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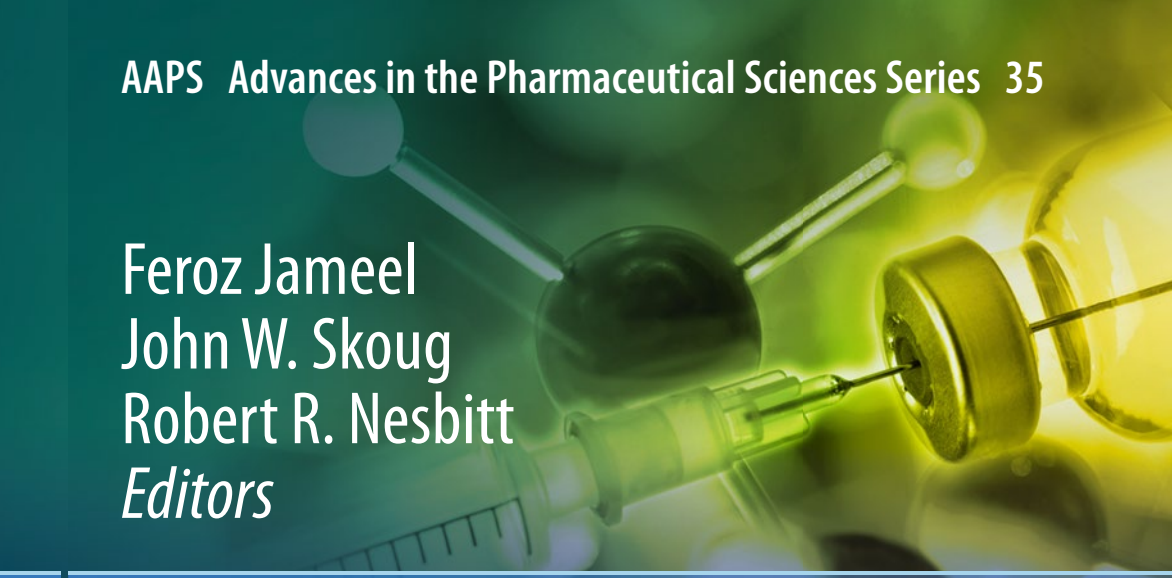

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Development of Biopharmaceutical Drug-Device Products

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Preface

The first monoclonal antibody (mAb) for therapeutic use was approved more than 30 years ago and ushered in the age of biologic-based drug discovery and development for pharma companies, and a new era of highly effective treatment options for patients. These therapies have had a major impact in cancer and autoimmune diseases and increasingly in neurologic, anti-infective and cardiovascular indications. With more than 60 biologics for therapeutic use having been approved worldwide as of 2018,[1] they have advanced from therapeutic novelty for pharma companies and regulatory agencies to mainstream molecular entities that comprise a significant fraction of the NME pipelines in most pharma companies. While the majority of approved biologic therapeutics are monoclonal antibodies, there is an increasing trend toward novel modalities such as bispecifics, antibody-drug conjugates (ADCs), fusion proteins and nanobodies. These newer modalities expand the applicability of biologic therapeutics by continuing to utilise the exquisite binding specificity and affinity of antibodies to bind two targets (bispecifics), to deliver toxic payloads to the target site (ADCs) and to enhance tissue penetration (nanobodies), thus opening up potential applications for intracellular and CNS-based targets.

Over the years, pharmaceutical development scientists have made many advances in the discovery and development of conventional mAbs. For example, most companies have implemented predictive (micro)analytical tools during discovery to influence candidate selection towards molecules that not only maximise binding affinity and minimise immunogenicity but also have physicochemical properties that enable the development of stable, high concentration formulations with manageable viscosity. Such an upfront investment has proven critical in avoiding costly downstream delays in clinical development due to challenges from a chemistry, manufacturing and control (CMC) standpoint. Among other advances are the development of platform formulation and analytical approaches to minimise

[1] K. Tsumoto, Y. Isozaki, H. Yagami, M. Tomita. Future perspectives of therapeutic monoclonal antibodies. *Immunotherapy* (2019) 11(2), 119–127.

timelines from candidate nomination to initiation of clinical development; understanding formulation excipient effects, primary container and materials effects, and manufacturing process parameters on product stability; the design of analytical comparability protocols to justify changes in drug substance or drug product processes that inevitably occur during the course of development; and patient-centric device delivery system design, especially for those products intended for at-home administration.

This book represents a comprehensive overview of the state of the art in biopharmaceutical drug/device product development. The scope is intentionally broad, and so we have divided the book into two parts: Part I focuses on preclinical and development considerations of antibody drug products, while Part II focuses on the development of the integrated drug/device combination product.

Chapters 1–11 provide a background on the structure and function of engineered antibodies, including novel molecular formats, high throughput preformulation characterisation to influence candidate selection and methodologies for their analytical and biophysical characterisation. Chapters 12–19 deal with formulation development topics, such as platform formulation strategies, high throughput formulation screening techniques, methods for assessing product robustness and clinical in-use studies to ensure that handling procedures do not compromise product quality prior to patient administration. Chapters 20–25 describe specific topics in process development, including lyophilisation, freeze drying, and mixing and filling considerations. Part I concludes with a comprehensive overview of peptide development challenges, including formulation, stability and delivery strategies. Information presented includes general overviews with comprehensive reference lists and case studies illustrating how particular methodologies are applied for both conventional mAbs and novel modalities.

In Part II, the focus is on combination product development. This combination of drug and medical devices enable at-home delivery of biopharmaceuticals, making therapy adherence easier for patients. However, this combination of drug and device brings together two distinct technologies and their associated development approaches, posing unique challenges to the organisations developing them. The specification-driven pharma development processes (GMP/ICH) and requirement-driven medical device processes (Design Control, ISO/IEC standards) have to be combined into an integrated approach to successfully develop combination products and manage them through their respective life cycles. It is not just about bringing these two development approaches together; success requires deep collaboration between drug product scientists and device development engineers. Because the medical device requirements, integrated development process and regulatory requirements for drug/device combination products are relatively new to many in the pharmaceutical field and are in fact rapidly evolving, a series of introductory chapters are included that cover the evolving regulatory framework with perspectives from both the US and EU industry experts. These chapters also cover the role of international standards, design control/risk management, human factors and their importance in the product development and regulatory approval process, and include a general chapter on a science and risk-based approach to

bridging between devices used in clinical trials and the to-be-marketed device. Part II concludes with several case studies highlighting both pharma and technology partner perspectives on patient-centric design and the development of autoinjectors as key injectable delivery devices for self-administration, challenges in on-market support and on-body delivery system development considerations.

It is critical to note that the underlying technology that makes up medical devices changes substantially faster than that of the drug product. The expectation of medical device technology to reflect the aesthetics and ease of use associated with consumer technology adds further pressure on the combination product life cycle. Hence, the device component of the combination product may have to be redesigned multiple times, also challenging the organisational approach, governance process and funding mechanisms to operationally sustain such products in pharmaceutical businesses.

In addition, different therapies in a company portfolio may require distinctly different types of delivery technology that span the range from pre-filled syringes, auto-injectors, on-body wearable injectors (larger volumes) and durable pumps with infusion-set type accessories. It is unlikely that one organisation can master the development and manufacture of all these delivery technologies – this predisposes biopharmaceutical companies to a partnership model for sourcing the delivery technology and combining that with the drug product to create the combination product. The partnership approach that is used to create and grow a drug portfolio has to be adapted to create and sustain a combination product throughout its life cycle.

The objective of this book is to cover this breadth of topics in order to provide both the novice and the experienced scientist a reference to all aspects of biopharmaceutical drug/device product development. It also provides insight into how organisations are handling the coming together of two development approaches (drug product and device), each multidisciplinary in their own right, with distinct technologies and associated challenges.

Finally, we would be remiss without acknowledging the time and effort of the contributing authors who represent leading experts in the biopharmaceutical industry. We are most grateful to them for sharing their expertise in this book and in so doing their passion for the science and engineering involved in biopharmaceutical drug/device product development. Enjoy the reading – we hope that it not only informs but will also spark new thinking that advances the field of combination products.

North Chicago, IL, USA

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Part I
Drug Product Development

Chapter 1
Monoclonal Antibodies: Structure, Physicochemical Stability, and Protein Engineering

Brittney J. Mills, Ehab M. Moussa, and Feroz Jameel

1 Structure of Monoclonal Antibodies

1.1 General Structure

A schematic diagram of the general structural features of mAbs is outlined in Fig. 1. The structure of a mAb molecule involves one pair of heavy weight polypeptides (heavy chains, HC) of 50 kDa each and one pair of lightweight polypeptides (light chains, LC) of 25 kDa each. The four chains are held together by hydrophobic interactions and interchain disulfide bonds to form a Y-shaped quaternary structure. According to the chemical structure, mAbs are classified into five classes or isotypes: IgA, IgG, IgD, IgE, and IgM. Since the vast majority of therapeutic mAbs are IgGs, the focus of this section is on the structural and functional properties of this class.

With regard to the amino acid sequence of IgGs, each of the two HC consists of one variable domain (VH) that is unique for each mAb and three domains that are constant across IgGs (CH1, CH2, and CH3). Similarly, each of the two light chains is comprised of one variable domain (VL), but only one constant domain (CL). Unlike the constant heavy domains, the chemical structure of the CL domain varies across IgG mAbs and results in two functionally similar isotypes: kappa and lambda.

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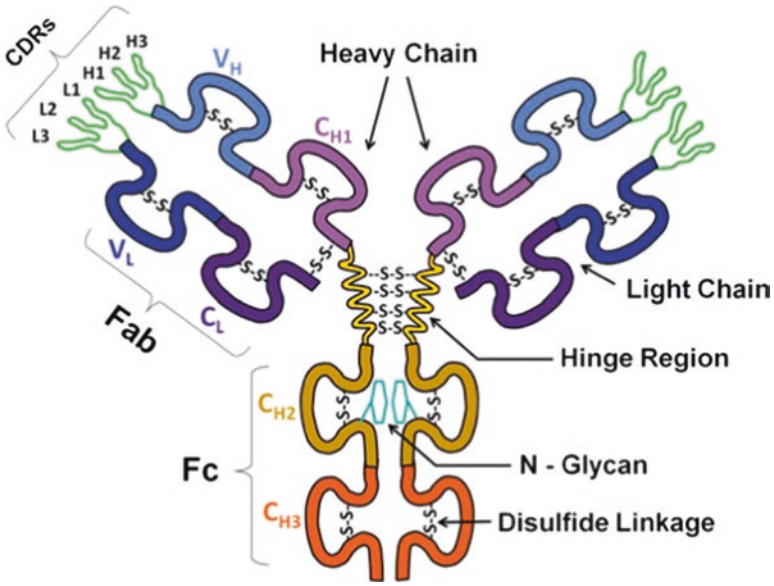

Fig. 1 Schematic diagram of the general structural features of monoclonal antibodies. Reprinted with permission from Moorthy et al. [3] Fab: antigen-binding fragment. Fc: crystallizable fragment. CDR: complementarity-determining region. L1-L3: CDR loops in the light chain. H1-H3: CDR loops in the heavy chain

Each set of the VH, CH1, VL, and CL domains forms one of two identical arms of antigen-binding fragments (Fabs), which are responsible for the specific function of the mAb. The two Fab fragments form the V-shaped head of the mAb structure. The neck of the Y-shaped mAb structure is termed the hinge region and is formed by a random coil structure that connects the CH1 and CH2 domains and hence connects the Fab arms with the tail of the mAb. IgG molecules are classified into five subclasses numbered 1–5 according to the length of the hinge region and the number of interchain disulfide bonds holding it together. The tail of the mAb structure, termed the crystallizable fragment (Fc), is formed by the CH2 and CH3 domains of the two HCs and is responsible for the effector functions and the pharmacokinetic properties thereof.

Each of the different domains constituting the polypeptide chains of the mAb is comprised of approximately 70–110 amino acids, which form 9–11 beta-sheets that are structurally organized into a beta-barrel structure. Each two adjacent domains interface through their hydrophobic regions resulting in one CH2-CH2, one CH3-CH3, two VH-VL, and two CH1-CL pairs. The hydrophobic interactions between the two CH2 domains are limited compared to the other pairs due to the coverage of the hydrophobic regions by glycan groups. The following subsections describe the features of the three main functional components of IgG mAbs: Fc, Fab, and the hinge region.

1.2 Crystallizable Fragment (Fc)

The Fc fragment constitutes the tail region of the Y-shaped mAb structure and contains the regions that bind to receptors and proteins that mediate several physiological functions. The interface of the hinge region and the CH2 domain contains the binding regions to complement proteins, as well as the Fc receptors (FcRs) typically present on the surface of the innate immune cells. The region connecting the CH2 and CH3 domains contain the binding sites for the neonatal Fc receptors (FcRn), protein A, and protein G.

Binding to Fc receptors stimulates the release of several inflammatory mediators and activates antibody-dependent cellular cytotoxicity (ADCC), whereas binding to the neonatal Fc receptors decreases elimination and increases plasma half-life by recycling the mAb molecules pinocytosed into the epithelial cells back into the blood stream.

In addition to the binding sites, the CH2 domain also contains a conserved Asn-X-Ser/Thr consensus sequence wherein the Asn residue at position 297 in each heavy chain is linked to a N-glycan group. This posttranslational modification plays a role in the binding to FcRs and is known to affect the pharmacokinetic profile of mAbs. It can also affect the solubility, stability, and immunogenicity of the molecule.

In general, the glycan group in mAbs is composed of an N-acetylglucosamine core structure connected to a bisecting mannose structure that is extended by additional N-acetylglucosamine molecules. This core structure can be further modified by the addition of fucose to the core N-acetylglucosamine and/or by the addition of terminal galactose molecules to the mannose arms. Typically, 1–3 major variants of the glycan group are formed during the expression of mAbs in cell culture.

1.3 Hinge Region

The hinge region is a random coil structure comprised of 12–62 amino acids depending on the IgG class [4]. It contains several proline residues that render the structure flexible and solvent accessible. Such flexibility allows the two arms of the Fab fragment to move relative to each other, a process that facilitates antigen binding. Solvent accessibility of this region, however, renders it more vulnerable than the rest of the mAb structure to clipping [5] and disulfide scrambling [6]. The former results in separation of the Fab and Fc fragments, whereas the latter may impact the biological activity and/or the effector functions of the IgG. In addition to proline residues, the hinge region also contains one or more cysteine residues that form interchain disulfide bonds, the position and the number of which vary in the different IgG subclasses. For example, in IgG1, the most commonly used IgG subclass in therapeutic mAbs, the hinge region consists of 15 amino acids and contains 2 interchain disulfide bonds that hold the two heavy chains together.

The interface of the hinge region with the CH2 domain of the Fc fragment has been shown to be involved in the binding to the Fc receptor [7] and hence plays a role in mediating the effector functions of the mAb.

1.4 Antigen-Binding Fragment

Each of the two variable domains (VH and VL) in each Fab arm contains several beta strands connected by loop structures. Each domain contains three loop structures that are spatially adjacent, although not sequentially consecutive. Together, the six loops in the variable light and heavy chains of each arm form the complementarity-determining region (CDR) of the mAb, which constitute the antigen-binding site of the Fab fragment [8]. The length and the amino acid sequence in these loops are highly variable across the different mAbs and hence determine the specific function.

Glycosylation in the CDR region has been shown to decrease antigen binding in some cases [9, 10] but increases binding affinity in another case [11]. Also, glycosylation in the variable chain close to the CDR region was found to affect antigen binding and specificity of a model mAb [12].

2 Chemical and Physical Degradation of Monoclonal Antibodies

In the development of an antibody-based therapeutic, a comprehensive assessment of the physicochemical properties of the molecule is completed to ensure that it exhibits the necessary attributes required for a successful clinical candidate. The route and rate of degradation are evaluated to determine the robustness of the molecule to external stressors that may be encountered during the product life cycle including manufacturing, shelf life, and administration. The type of degradation observed can be classified into two main categories: physical or chemical degradation. Although extrinsic factors such as solution conditions and temperature can modulate the observed degradation, intrinsic properties can provide insights into the susceptibility of a certain mAb for undergoing different types of degradation.

2.1 Physical Degradation

Aggregation

The primary mechanism of physical degradation occurs through the self-association of protein species or aggregation. As aggregation can be triggered by protein unfolding, preserving the overall fold (secondary and tertiary structure) is a key component

in achieving desirable drug-like properties. Under normal conditions, monomeric species exist in their folded state due to the energy barrier that prevents the protein from occupying an aggregation-prone state [13]. But, stressed conditions alter these energy barriers and shift the balance to population of the unfolded and aggregation-prone species [14]. Because antibodies contain multiple domains and regions susceptible to self-association, aggregation proceeds through the following intermediate stages [15, 16]:

(i) Protein unfolding: disruption of the energy barrier equilibrium to favor partially unfolded states [17]
(ii) Protein association: interaction between aggregation-prone regions (modulated by hydrophobicity or charge) of the unfolded monomers [18–20]
(iii) Nucleation (rate-limiting step): structural rearrangements to promote additional aggregation including alterations in surface charge, exposure of hydrophobic regions, and/or secondary structure changes to the more energetically favorable β-sheet orientation [14, 21, 22]

As the majority of aggregation is unfolding-driven, understanding the factors that contribute to the conformational stability will aid in limiting the aggregation propensity of the molecule. The domain unfolding which is responsible for aggregation initiation can vary as the aggregation-prone regions can be different from one mAb to another. In some instances, the CH2 domain unfolds first and triggers the aggregation process [23, 24], whereas it has also been shown that the unfolded Fab domain is more aggregation prone than the CH2 domain [25]. The susceptibility to aggregation is defined by the intrinsic properties of the molecule (primary and tertiary sequence) [16], but external factors (pH, excipients, temperature, sheer stress, and antibody concentration) also lead to structural fluctuations, which in turn affect unfolding and aggregation [16, 21, 26–28]. Because pH modulates the thermodynamics of unfolding of the domains, changes in solution conditions can alter which domain unfolding leads to aggregation initiation [25]. Stabilization of the CH2 and Fab domains is achieved by increasing the pH from acidic to near-neutral conditions [25, 29], with destabilization occurring at pH values below the pI of the protein [30]. As protein unfolding is also temperature dependent, incubation at temperatures above or below the T_m of unfolding will significantly impact aggregation. Incubation at temperatures above the T_m of unfolding can lead to loss in secondary and tertiary structural elements, which cause a higher degree of aggregation due to exposure of the hydrophobic core [31]. The aggregation nucleation rate also increases as the T_m of unfolding is approached [32], whereas the unfolding rate significantly decreases at temperatures closer to those used for long-term storage [30]. Finally, exposure of the protein to the air-liquid interface occurs during shear stress, which facilitates aggregation due to the loss of secondary and tertiary structure [33–35].

In addition to unfolding-induced aggregation, self-association can also occur between fully folded, monomeric species. Although monomeric aggregation can be caused by chemical cross-linking of free sulfhydryl groups or unpaired disulfide bonds [36, 37], colloidal association primarily occurs due to large regions of surface hydrophobicity, which can be present in both the Fc region [38, 39] and the CDRs

[40, 41]. Oftentimes, the CDRs contribute more significantly to colloidal instability due to the high occurrence of hydrophobic residues in these regions necessary for antigen binding [40–42]. In mAbs with global net charges, though, increases in colloidal stability are observed at pH values below the pI due to intermolecular repulsion [16, 30]. While weak interactions correlate with aggregation rates at low concentration [43, 44], it is much more difficult to make such association at high protein concentration due to non-idealities that exist only under high-concentration conditions. Along with molecular crowding leading to a decrease in free space, the higher viscosity that accompanies high-concentration solutions further limits molecule mobility [45, 46]. Even though these effects render higher concentration solutions more prone to aggregation under most types of stress [47], high-concentration solutions of mAbs are self-stabilizing to shear stresses [31, 48–51].

Opalescence and LLPS

Apart from aggregation, physical instability can be present in the form of reversible self-association (RSA), which leads to opalescence and/or liquid-liquid phase separation (LLPS). Opalescence is an optical property caused by Rayleigh light scattering of polarizable particles resulting in a solution state that appears turbid under white fluorescent light. It is due to enhanced light scattering caused by concentration fluctuations that occur near the critical concentration [52]. Per the European Pharmacopoeia, a solution is labeled as opalescent at 3 NTU and above [53]. Even though it may appear that particles have formed, opalescence is caused by soluble proteins and/or non-proteinaceous particles, as filtration leads to no differences in the extent of opalescence [54]. Although opalescence can occur without any aggregation or phase separation [55–58], lower temperatures often result in LLPS after the solution becomes opalescent. Fluctuations in thermodynamic properties (entropy and enthalpy) favor LLPS [59], which results in the formation of two phases with different concentrations, but the same chemical potential [60, 61]. Although LLPS itself is not caused by aggregation, the formation of the protein-rich phase can result in irreversible aggregation due to the high concentration in that phase [61]. The presence of two phases with differing concentrations will also lead to salt partitioning according to the concentration gradient and pH/ionic strength differences between the two phases [62].

Antibody concentration and storage temperature are two key factors that affect opalescence [55, 63–65]. The critical concentration is the concentration at which concentration fluctuations and opalescence are at a maximum. As it is primarily determined by the size of the molecule, the value should be similar for IgG1s. Most studies report the critical concentration as approximately 90 mg/mL [60, 64, 66], but some studies reported a lower range of opalescence (50–75 mg/mL) [67], and others have reported higher concentrations of solutions that have exhibited opalescence (100 mg/mL) [54]. On the other hand, the critical temperature is an intrinsic property that is influenced by the properties of the molecule. The critical tempera-

ture is defined as the temperature at critical concentration. As the temperature approaches the critical temperature, the extent of opalescence increases, but it is reversible upon temperature increase [55]. Another temperature-based parameter, T_{cloud}, is used to define the temperature at which LLPS begins, regardless of concentration. T_{cloud}, instead of B_{22},is a better measure of high-concentration physical instability as it can be measured under high-concentration conditions, whereas B_{22} is measured at low concentration [59].

RSA and opalescence are the result of intermolecular attractions between antibody molecules [55, 56, 68]. Even though antibodies exhibit high sequence similarity, the binding interface responsible for RSA is distinct for each antibody [42, 69, 70]. Most often, it is due to Fab/Fab or Fab/Fc interactions [38, 71], as the CDR region is the main site of sequence heterogeneity and has been widely implicated in intermolecular attractions [42, 70–74]. Because the surface properties of the antibody dictate the type of interactions that occur, pH strongly influences the propensity for RSA and opalescence. At pH values far away from the pI, electrostatic repulsive forces dominate due to the high charge associated with the antibody. At pH values near the pI, though, the net charge reaches its minimum leading to weakened global electrostatic repulsion between molecules. In this instance, short-range interactions such as H-bonding, van der Waals forces, and dipole interactions make significant contributions to the associations that occur between protein molecules. This higher propensity for RSA leads to increased opalescence [54, 56, 60, 67, 71, 75], as well as decreased solubility [54], at pH values near the pI. The type of species that form under conditions favorable for RSA and opalescence vary from antibody to antibody as both monomer-dimer-tetramer species [71] and monomer-trimer species have been reported [54].

As solution conditions affect the types of interactions that exist among molecules, opalescence is strongly influenced by the presence of excipients. Modulation of ionic strength is a commonly employed method to mitigate opalescence, but its effects cannot be generalized as it is dependent on the identity of the salt ion [76–80]. Most reports illustrate the effectiveness of increasing ionic strength in decreasing opalescence. Salt addition aids in masking nonuniform charge distribution on the surface of the protein, thus disrupting protein-protein interactions [54, 60] and causing the high-concentration antibody solution to behave similarly as observed under dilute conditions [71]. In some cases, though, opalescence increases as ionic strength increases [56, 57] due to the dominance of hydrophobic interactions under these conditions [67]. For this reason, arginine is commonly used due to its dual effect as an excipient to both modulate surface charge and weaken intermolecular hydrophobic interactions [81, 82], without altering the structural stability of the molecule [81, 83, 84]. Addition of arginine has also been shown to result in fully monomeric species under conditions favorable for RSA, whereas NaCl addition still led to monomer-dimer-tetramer species [71]. Therefore, elimination of all interactions responsible for RSA through arginine addition is currently the best route for mitigating opalescence.

2.2 Chemical Degradation

Chemical degradation involves the irreversible modification of residues within the protein sequence. Although it is not fully understood, the formation of species containing specific chemical modifications may play a role in the immunogenicity observed upon dosing with antibody-based therapeutics [85–89]. This could be due to both the increased likelihood of aggregation among chemically modified species and the formation of neo-epitopes, which would elicit an immunogenic response [90]. Chemical degradation can occur in any region of the mAb that contains residues prone to modification, but in a study of 37 antibodies, all sites of impactful degradation were located in the CDR [91]. Residues in the CDR are particularly prone to chemical degradation due to the flexibility and high solvent accessibility of this region as discussed earlier. Among the possible chemical degradation pathways, deamidation, isomerization, and oxidation are the primary ones observed in mAbs that can impact both the stability and function of the therapeutic entity.

Deamidation

Deamidation is the most common form of chemical degradation observed in mAbs. It results in the formation of more acidic species through the hydrolysis of the amide side chain of Gln or Asn residues (Fig. 2). The rate of deamidation is dependent on the solvent accessibility and structural flexibility of the region. The presence of

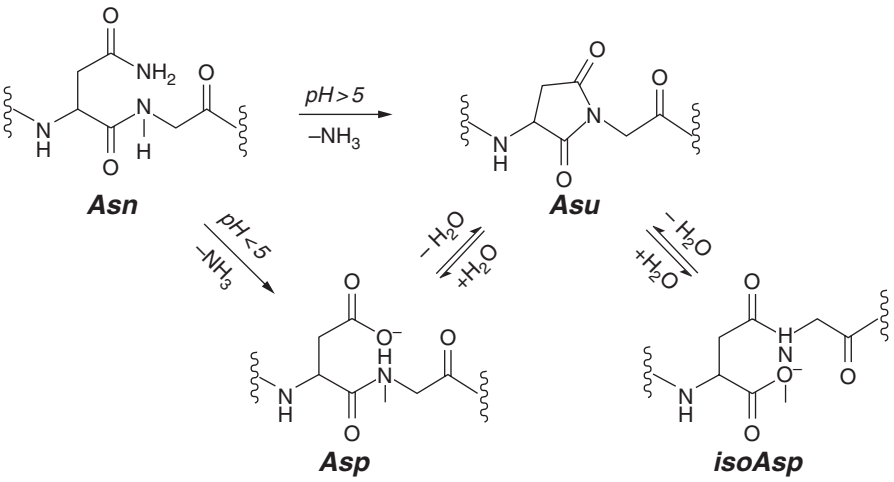

Fig. 2 Asparagine (Asn) deamidation and aspartate (Asp) isomerization. Deamidation of Asn proceeds through a cyclic imide (aspartyl succinimide, Asu) intermediate with loss of ammonia at pH > 5 to produce the Asp and isoaspartate (isoAsp). At acidic pH, the Asn side chain undergoes direct hydrolysis producing Asp product exclusively. Isomerization of Asp to isoAsp also occurs through Asu intermediate

small residues such as Gly or Ser next to an Asn or Gln will increase the likelihood of deamidation in comparison to bulkier residues [26, 92–98]. Extrinsic conditions such as pH, temperature, and buffer also affect the rate and degradation products [99]. At acidic pH (pH < 4), the Gln or Asn residues are converted to their carboxylic acid counterparts (Glu or Asp) [26], whereas at formulation-relevant pH values, deamidation is much slower and proceeds through a cyclic imide intermediate to form either Glu or Asp or their isomers. The succinimide intermediate formed by deamidation of Asn is much more stable than the Gln cyclic intermediate making Asn deamidation much more common [37]. The isoAsp degradant does not only result from the deamidation of Asn, but can also be caused directly by isomerization of Asp [36]. Asp isomerization follows a similar succinimide intermediate as Asn deamidation, and the resulting succinimide intermediate hydrolyzes to either isoAsp or Asp at a 3:1 ratio when at equilibrium [92].

The introduction of a structurally different species or alteration in charge can lead to changes in stability, as well as binding affinity of the resulting molecule. The effect of deamidation needs to be evaluated on an case-by-case basis as it has been reported to lead to decreases in binding affinity, potency, and stability [9, 98–103], whereas others have reported that the resulting succinimide led to no effect on binding affinity [104]. Effects on protein conformation and possibly binding affinity would be expected following Asp isomerization as an additional CH_2 is introduced into the peptide backbone and the side chain is altered [9, 18, 43, 101, 105–110]. The modification lengthens the peptide backbone and imparts additional flexibility [100], which causes substantial conformational changes at both the site of modification and nearby residues [111].

Oxidation

Oxidation is another critical chemical degradation pathway that is monitored during the development of biological modalities. The residues prone to oxidation include Met, Cys, His, Tyr, and Trp [26]. Although Met oxidation is almost always pH-independent, oxidation is generally influenced by both intrinsic and extrinsic properties [26, 112, 113]. Met is the most common residue to undergo oxidation to either Met sulfoxide [114] or sulfone [115]. A set of highly conserved Met residues within the Fc region (Met252, Met248) are especially susceptible to oxidation to Met sulfoxide [116–123]. Oxidation in this region has been shown to decrease stability and Fc receptor binding [116, 124, 125], but both heavy chains must be oxidized at Met252 to significantly affect the clearance [126]. While FcRn binding may be affected by high levels of Met252 oxidation, this modification does not impact the FcγRIIIa binding and subsequent ADCC activity [116]. Modifications in stability and FcRn binding suggest that structural alterations occur upon Met oxidation. The backbone amide hydrogen-bonding network is disrupted due to the presence of Met sulfoxide [124], which is more polar and larger in size than Met. These local conformational changes may affect CDC activity as many interactions occur at the CH2-CH3 interface to modulate CDC activity [127]. Even through the structure may

be altered by Met oxidation, no effects on antigen binding would be expected since the Fc region is not typically involved in antigen interactions. When this modification occurs within the CDR, however, the conformation is stabilized presumably due to the additional interactions present resulting in a slight increase in binding activity [128]. In addition to Met, Trp residues are also prone to undergo oxidation. Trp oxidation is induced by light exposure; photo-ionization can also occur if the residue is in close proximity to disulfide bonds [90]. The effects of Trp oxidation on biological activity are mixed [90, 122, 129]. Wei et al. showed that oxidation of a Trp residue in one of the CDR loops affected antigen binding and the biological function of a model mAb [129]. In a study by Dashivets et al., 94% oxidation of one Trp didn't affect binding in one mAb, whereas 43% oxidation of that same Trp in another mAb significantly affected binding [128]. Even if binding affinity was not affected, higher Trp oxidation led to lower thermal stability and increased aggregation.

Although chemical degradation may seem to only influence a small region within the mAb, this modification can have far-reaching effects that stabilize or destabilize the global structure. A study comparing Met oxidation and Asp isomerization in the CDR showed that although similar regions distinct from the modification are affected by the chemical alteration, Asp isomerization lead to increased flexibility, whereas Met oxidation had the opposite effect [100]. The extent of the structural changes caused by Asp isomerization was also greater in comparison to those elicited by Met oxidation. These results further confirm the necessity in evaluating the effect of individual chemical modifications on mAb structure and function.

N-Terminal Pyroglutamate

Presence of a glutamine on the N-terminus of the light or heavy chain can result in the cyclization of the N-terminal amine to form pyroglutamate (pGlu). In most instances, this posttranslational modification occurs during antibody expression and purification [130–133], most specifically during the bioreactor process. Cheng et al. reported over 90% conversion to pGlu after 15 days in the bioreactor [131]. In cases where pGlu formation does not occur during antibody processing, it has also been shown to occur during storage at accelerated conditions [134]. The conditions under which the antibody was stored strongly influence the rate of pGlu formation. The highest rate was observed at pH 4 and 8, with a minimum at pH 6, and preparation in succinate resulted in a higher percentage of conversion compared to histidine or acetate buffer. pGlu formation can occur on either the HC or LC if there is an N-terminal Gln, but the likelihood of it occurring on one chain preferentially can vary from antibody to antibody. In one case, 99% pGlu conversion occurred in the HC with only 2% in the LC [132]. On the other hand, under accelerated storage conditions, the pGlu conversion rate was slower in the HC vs. the LC [134].

Because pGlu formation results in loss of the N-terminal amine, the isoelectric point of the resulting molecule is altered in comparison to the native molecule. Analysis by near-UV indicated that the tertiary structure was not altered by pGlu formation, but it was hard to determine if structural differences observed by FTIR

were due to pGlu formation or caused by thermal stress [134]. Also, even though pGlu formation occurs within the CDRs, potency is not impacted if this region is not directly involved in the binding epitope [135]. Because pGlu has been observed in human endogenous IgGs, it is not likely a safety concern [136]. But, the presence of both N-terminal pGlu and non-cyclized Gln results in batch-to-batch variations in species, which makes it difficult to analyze and meet the specifications required of an FDA-approved product.

Fragmentation

Although not as widely reported as aggregation, mAbs may also undergo fragmentation through enzymatic or nonenzymatic hydrolysis of the peptide backbone at the hinge region or at a sequence containing either Asp or Trp [26]. Hinge region hydrolysis does not require specific residues. Rather, the rate is dependent on the flexibility and peptide sequence, as well as pH, with higher rates occurring either above or below pH 6 [26, 137, 138]. Asp-associated hydrolysis is also modulated by pH, and the rate is increased if the Asp residue is adjacent to a Ser, Val, or Tyr [26]. In general, the degree of fragmentation is insignificant and results in minimal effects on efficacy.

3 Advances in Protein Engineering to Improve Stability and Efficacy

The specificity of mAbs for their targets makes them highly suitable for use as therapeutic modalities. Their application was originally met with challenges due to inherent instabilities surrounding the primary sequence and the tertiary structure of the molecule, as well as immunological responses to the non-humanized versions [139, 140]. Improvements in hybridoma and recombinant expression technology have led to the generation of fully human forms, but protein engineering has taken it a step further to capitalize on the interactions of the therapeutic with FcRn and FcγRs to maximize half-life and activation of the immune system pathways. These advances in clonal technology have also increased the ease with which mutations can be made to achieve a product with desirable physicochemical properties.

3.1 Modification to Improve Stability

Physicochemical instabilities can arise both from the primary sequence and/or the tertiary structure of the mAb. Evaluation of the primary sequence allows for identification of amino acid segments that may be prone to chemical modification such as deamidation or oxidation. But, prediction of aggregation propensity is much more

difficult as it is reliant upon both the conformational and colloidal stability. Conformational stabilization by disulfide bond addition increased the T_m of the CH2 and CH3 domains by 20 °C and 35 °C, respectively [141, 142]. Removal of free sulhydryls has also been shown to decrease the aggregation propensity as this highly reactive residue is no longer present to cause protein misfolding or covalent cross-linking across monomeric species [143].

Aside from conformational stabilization, aggregation propensity is decreased by removal of aggregation-prone regions (APRs), as well. To successfully predict APRs, both the overall fold of the molecule and the dynamic structural fluctuations that may expose hydrophobic patches must be considered. Early predictive modeling systems had difficulty accounting for both variables, but the recently developed spatial-aggregation-propensity (SAP) model has successfully identified APRs that have been experimentally identified to contribute to self-association [144]. The SAP model gives the effective dynamically exposed hydrophobicity of a certain region on the surface of the molecule normalized to glycine [144]. The high- and low-resolution capabilities allow for it to successfully identify large APRs, as well as identify which residues should be mutated to decrease the hydrophobicity of the region.

Mutation of hydrophobic residues that significantly contribute to the aggregation propensity to a residue more hydrophilic in nature results in an increase in stability [16, 144]. As multiple mutations may lead to the complete removal of the self-association interface, a cumulative effect is observed upon the introduction of three mutations [144]. Greater increases in stability are observed when the hydrophobic residue is replaced with lysine instead of serine due to the larger size of the lysine side chain for shielding the hydrophobic region [144].

More APRs are located in the Fc fragment instead of the Fab fragment, with a large number of them located within the loops in the hinge region and CH2-CH3 interface [145]. Because these regions are very similar across Ig subclasses, most APRs are the same across IgG subclasses (IgG1, IgG2, IgG3, IgG4), and if they are different, the hydrophobic nature is at least conserved. But in the different classes (IgA, IgD, IgE, IgM), different APRs are present due to the structural diversity among the classes [145]. Because this interface contains a consensus motif that is highly hydrophobic in character, mutation in this region increases the solubility and stability of the antibody [144]. In addition to modifying colloidal stability, mutations within the CH2 domain (L234K/L235K) also have been shown to increase the conformational stability of the molecule [144].

While most APRs are located in the Fc domain, removal of APRs in the Fab domain will also decrease aggregation propensity, and some antibodies have also been shown to have larger APRs in the CDR over the Fc [146]. It is more difficult to identify mutations that will be successful at eliminating aggregation and maintain antigen binding within the CDR. In an anti-IL-13 mAb, a triple mutant that removed a hydrophobic patch (Phe-His-Trp to Ala-Ala-Ala) also resulted in decreased binding affinity [40]. For that reason, regions bordering the CDRs are usually targeted for mutation [16], and more success is observed when hydrophobic patches are replaced with negatively charged residues as opposed to positively charged or

neutral residues [16, 143, 147, 148]. Substituting Asp and Glu into HCDR1 and LCDR2 reduced aggregation propensity, but did not impair function as these residues do not directly contribute to antigen binding [148].

As opposed to eliminating the hydrophobic region through mutation, shielding of the hydrophobic region has also been shown to be a successful mechanism for reducing aggregation propensity. The glycan at Asp297 greatly improves the colloidal stability by shielding APRs as aglycosylated mAbs are less stable and more prone to aggregation [149]. Moreover, Voynov et al. demonstrated that disruption of the glycan group interaction with the mAb promotes aggregation [150]. The addition of a hydrophilic glycan near a region of hydrophobicity within the CDR also provides a shielding effect to decrease aggregation, and in this instance, no effect on antigen binding was observed [40].

3.2 Improving Efficacy and Half-Life Through Engineering

The success of an antibody-based therapeutic ultimately relies upon efficacy. Even if the drug-like properties are perfect, a molecule will not be successful if it does not achieve the desired therapeutic effect. For this reason, efforts have been made to engineer antibodies to modulate the effector functions and circulation half-life. The effector functions ADCC, complement-dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP) are immune responses that result in targeted cell death after interaction between the therapeutic mAb and the appropriate cellular receptor (i.e., FcγR) [140]. Enhancing the effector functions is pursued in antibody therapeutics that target cell surface proteins in certain cancers as it allows for a multiplicative effect of both the therapeutic and an internal cell-killing response. Decreasing effector functions through protein engineering has also been pursued. For example, CDC is linked to injection site reactions [151], and it may interfere with the induction of ADCC [152], so in these instances, it would be desirable to decrease the immune response.

Improved efficacy of mAbs used in the treatment of cancer can be achieved by increasing the affinity of the molecule toward specific FcγRs [153]. Enhancing the affinity to FcγR can be achieved by modulation of either the amino acid sequence or altering the glycosylation pattern [154, 155]. Mutagenesis resulting in the introduction of multiple antibody-FcγR interactions within the lower hinge and proximal CH2 regions will affect the observed response as these regions are critical for FcγR binding [153]. Multiple studies have been published showing the cumulative effect amino acid mutation can have on the affinity of therapeutic mAbs for FcγR due to additional hydrogen bonds, hydrophobic interactions, and electrostatic interactions [156, 157]. In turn, this increased affinity led to substantial increases in ADCC and ADCP [158–160]. Differentiation can also be achieved where mutation results in improved binding between the therapeutic mAb and the activating receptor FcγRIIIa, but reduced binding to the inhibitory receptor FcγRIIIb [159–161]. Finally, the residue chosen for substitution may be critical in modulating the interactions and

observed effects on effector functions. In the K326W variant of rituximab, use of Trp has been shown to be essential for the observed increases in CDC [162].

Although residue mutation can modulate effector functions, the glycosylation pattern of the therapeutic antibody has a far greater effect. For example, Scallon et al. showed that increased sialylation of the glycan group decreases binding to FcRs [163]. Removal of the core fucose of the glycan at position Asn297 results in a 50-fold increase in binding between the therapeutic mAb and FcγR, which leads to increases in both ADCC and ADCP [164]. Low fucosylation is a commonly employed method to increase ADCC and ADCP [165–168], and it has also been shown to increase the antitumor activity of a therapeutic mAb in comparison to its counterpart that had a normal glycan [169, 170]. Complete removal of the glycan, however, has the opposite effect and leads to decreased interaction between the therapeutic mAb and FcγR [171], thus limiting ADCC, CDC, and phagocytosis [172–176]. Effector functions can also be eliminated by using a cross-subclass approach in which the antibody is composed of parts from both the IgG1 and IgG4 subclasses. Elimination of effector functions is not suitable for mAbs used as cancer therapeutics but may be suitable for mAbs whose function does not rely upon ADCC and CDC such as for neutralizing, agonistic, or antagonistic antibodies.

In addition to effector functions, the half-life can be modulated through mutation as it also depends on interactions between the therapeutic mAb and a cellular protein (FcRn). FcRn is the cellular recycling machinery responsible for determining whether the mAb is processed into the lysosome for degradation or released outside of the cell, and this process is highly pH dependent [177–179]. At pH 6–6.5, binding between the therapeutic mAb and FcRn occurs, which leads to recycling outside of the cell. Elimination of these interactions must also occur at pH 7–7.5 to allow for successful release of the mAb from FcRn. For this reason, conserved histidine residues in the CH2 and CH3 domains are essential for this interaction as they become protonated at the acidic pH in the endosome and thus serve as suitable hydrogen bonding sites with FcRn. Mutation of H310 leads to complete loss of the interaction and undetectable binding between the mAb and the FcRn [153].

The addition of new interaction sites along with the conserved histidine residues will lead to increased affinity for FcRn and thus increase the half-life of the therapeutic. In the M428L/N434S mutant, an additional hydrogen bond results in an 11-fold increase in the affinity of the antibody for FcRn at pH 6 [180, 181]. The single mutant N434A also showed increased binding affinity at pH 6, but not pH 7.4, which resulted in increased half-life in cynomolgus monkeys [182]. Mutation of N434 to Trp, though, did not affect half-life as the binding affinity was increased at both pH 6 and 7.4 [182]. Another molecule known as the YTE mutant has been extensively studied due to the effects this mutation has on the in vivo properties of the molecule. This mutant exhibits a fourfold higher half-life in monkeys, tenfold higher affinity for FcRn, and fourfold higher tissue bioavailability in nonhuman primates [183]. The increased affinity is due to the stabilization of the complex by an additional salt bridge between Glu26 of the mutant and Gln2 of FcRn [180]. Although this mutation leads to optimal in vivo properties, the drug-like properties are compromised. Disruption of packing interactions leads to unfolding of a hydrophobic

segment and increased flexibility in the CH2 domain. This results in lower conformational stability and an increase in aggregation in comparison to the parental antibody [184]. Therefore, a balance needs to be achieved when trying to optimize in vivo properties while maintaining adequate physical stability.

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Chapter 2
Challenges and Considerations in the Design of Antibody-Drug Conjugates

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1 Introduction

Chemotherapy is currently the most common form of treatment for cancer. Chemotherapeutic agents kill cancerous cells or stop them from growing and/or spreading to other parts of the body. They can be grouped into different classes depending on their mechanism of action and composition (Table 1).

Despite the wide use of chemotherapeutics in cancer treatment, they possess narrow therapeutic windows due to the limited specificity between cancerous and healthy cells [1] and the increased rates of drug resistance [2]. These limitations prompted researchers to devise new ways by which the therapies can be made more selective and specific for treating cancer. Cancerous cells have differentiating features known as the "hallmarks of cancer [3, 4], which govern the abnormal ability of these cells to multiply and metastasize. In addition, several human cancers express unique tumor-specific or tumor-associated cell surface antigens [5], which can be of great value as targets. Monoclonal antibodies (mAbs) have been developed as therapeutic entities to target and bind the tumor-specific cellular antigens [6]. Tumor suppression occurs through one of the following pathways:

(i) Halting the signaling pathways of the cell, which eventually leads to cellular apoptosis

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Table 1 Classes of chemotherapeutic agents

	Mechanism of action	Examples	Types of cancer treated
Alkylating agents	Damage DNA to inhibit replication	Cyclophosphamide, melphalan, and temozolomide	Leukemia, lymphoma, Hodgkin's disease, multiple myeloma and sarcoma, as well as breast, lung, and ovarian cancers
Antimetabolites	Interfere with cellular metabolism	5-fluorouracil, 6-mercaptopurine, cytarabine, gemcitabine, and methotrexate	Leukemia and breast, ovarian, and intestinal cancer
Antitumor antibiotics	Cell cycle specific; multiple MOAs including DNA intercalation, DNA breakage via free radicals, and enzymatic inhibition	Actinomycin-D, bleomycin, daunorubicin, doxorubicin, and enediynes	Breast, lymphoma, leukemia
Mitotic inhibitors	Cell cycle specific; inhibition of cellular division via disruption of microtubules	Docetaxel, estramustine, paclitaxel, and vinblastine	Myeloma, leukemia, lymphoma, and breast and lung cancer
Topoisomerase inhibitors	Disrupts enzymes essential for DNA replication and RNA transcription	Etoposide, irinotecan, teniposide, and topotecan	Leukemia and lung, ovarian, and intestinal cancer

(ii) Activation of cell-mediated defense pathways including antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cellular cytotoxicity (CDCC), or complement-dependent cytotoxicity (CDC)
(iii) Affect tumor vasculature and stroma through inhibition of various pathways [7, 8]

Despite these various cell-killing mechanisms and advances in antibody engineering [9, 10], most mAbs display insufficient cytotoxic activity [11]. To overcome this, cytotoxic small molecules were combined with highly specific/selective mAbs to form a new class of anti-cancer drugs known as antibody-drug conjugates (ADCs) [12]. ADCs are composed of an antigen-specific mAb that is linked to a cytotoxic small molecule drug (Fig. 1). The site specificity associated with the mAb molecule leads to a reduction in off-target side effects due to the targeted delivery of the conjugated drug to the specific cell of interest [12]. Thus, in principle, ADCs increase the therapeutic index (the ratio of the toxic dose to the efficacious dose) of potent cytotoxins. Hence, an ADC aims to combine the specificity of a mAb with the potency of an indiscriminate toxin.

Early development of ADCs faced significant setbacks as they failed to undergo internalization and demonstrate increased drug specificity and decreased toxicity [14]. These ADCs were mainly designed to release the toxin either via intracellular

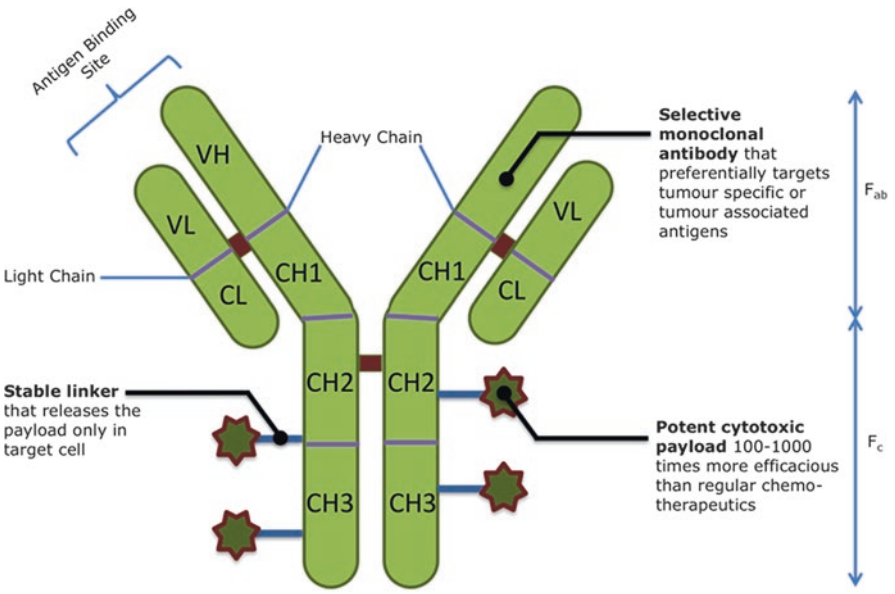

Fig. 1 Structure and various components of an ADC. (Reprinted with permission from Ref. [13])

lysosomal degradation or by extracellular degradation of the linker by tumor-specific enzymes and/or the low pH environment of the tumor [15].

More recently, development efforts have shifted toward independently developing each component of the ADC (antibody, linker, and warhead) to optimize pharmacologic properties of the ADC and improve the therapeutic index of these compounds. While ADCs have been in development for almost 20 years, there is still a significant gap in knowledge regarding how various ADC components impact PK/PD, toxin delivery to tumors, efficacy, and off-target toxicity [16]. Although the concept of ADCs is theoretically simple, it still remains a challenge to combine the various components into an optimized and functional therapeutic agent.

To date, only six ADCs are currently on the market: brentuximab vedotin (Adcetris® by Seattle Genetics), trastuzumab emtansine (TDM1) (Kadcyla® by Genentech), inotuzumab ozogamicin (Besponsa® by Pfizer), gemtuzumab ozogamicin (Mylotarg® by Pfizer), polatuzumab vedotin (Polivy™ by Genentech), and Lumoxiti™ (moxetumomab pasudotox-tdfk by AstraZeneca Pharmaceuticals). Adcetris® is used for the treatment of relapsed or refractory Hodgkin's lymphoma or by those with relapsed or refractory systemic anaplastic large cell lymphoma [17], Kadcyla® is used for the treatment of breast cancer [18], Besponsa® is used for the treatment of relapsed or refractory B-cell precursor acute lymphoblastic leukemia, Mylotarg® is used for the treatment of acute myeloid leukemia, and Polivy™ is used for the treatment of large B-cell lymphoma. Lumoxiti™ is used for third-line treatment of hairy cell leukemia and is an ADC utilizing a toxic peptide as opposed to a small molecule warhead. ADCs remain a key focus in the biopharmaceutical indus-

try even though the success rate thus far has been low. The chance of successfully bringing an ADC to market will hopefully increase due to the numerous advances made in the design of ADCs [19, 20], as well as in the preclinical and clinical space [21–23]. ADCs have also recently been considered for non-oncology indications [24]. Additionally, drug conjugates using scaffolds other than antibodies are being investigated. Of particular interest are small-format and small molecule drug conjugates, which theoretically offer better tumor penetration properties, as well as provide opportunities to further modulate and optimize PK properties [25]. While out of scope of this chapter, non-oncology ADCs and non-antibody-based conjugates are still subject to many of the design and stability considerations as a traditional ADC.

The primary objective of this chapter is to provide the biopharmaceutical scientist an updated, comprehensive review of the current knowledge surrounding drug product development of oncology ADCs based upon the developments in the last few years. Additionally, challenges and considerations, specifically in the formulation development of ADCs, will be discussed.

2 Mechanism of Action (MOA)

A general mechanism of action of ADCs is depicted in Fig. 2. The mAb component of the ADC enables it to circulate in the bloodstream until it finds and binds to tumor-specific cell surface antigens. mAb-antigen interaction leads to endocytosis of the entire complex into the cell through clathrin-coated vesicles [26]. In the interest of preventing unwarranted release of the cytotoxin prior to cellular internalization and maximizing drug delivery to cancer cells, the linker has to be stable in the bloodstream and release the active form of the cytotoxic drug when required [27].

After encapsulation into the endosome, the ADC either will undergo FcRn-mediated recycling out of the cell or will be degraded by the lysosome depending on the pH of the environment. As the intracellular environment of cancer cells is generally more alkaline than healthy cells, the ADC remains in the endosome in cancer cells [28], whereas in healthy tissue, it is transported out of the cells limiting off-target cell death due to non-specific delivery of the ADC [29].

Within cancerous cellular environments, the ADCs should not be transported out of the cell. Therefore, the ADC will proceed through the intracellular degradation pathway through the lysosome, resulting in subsequent release of the drug into the cellular environment. Depending on the type of toxin used, the released toxin can cause cellular death by one of several mechanisms of action (e.g., DNA alkylation, DNA breaks, microtubule disruption, topoisomerase inhibition, RNA polymerase inhibition).

Considering the MOA described above, there are several considerations for optimization of an ADC to produce a suitable therapeutic construct: target antigen selection, selection/engineering of the monoclonal antibody, selection of the linker, selection of the warhead, type of conjugation chemistry, and drug-to-antibody ratio (DAR). These items may have a profound impact on the efficacy, pharmacokinetics,

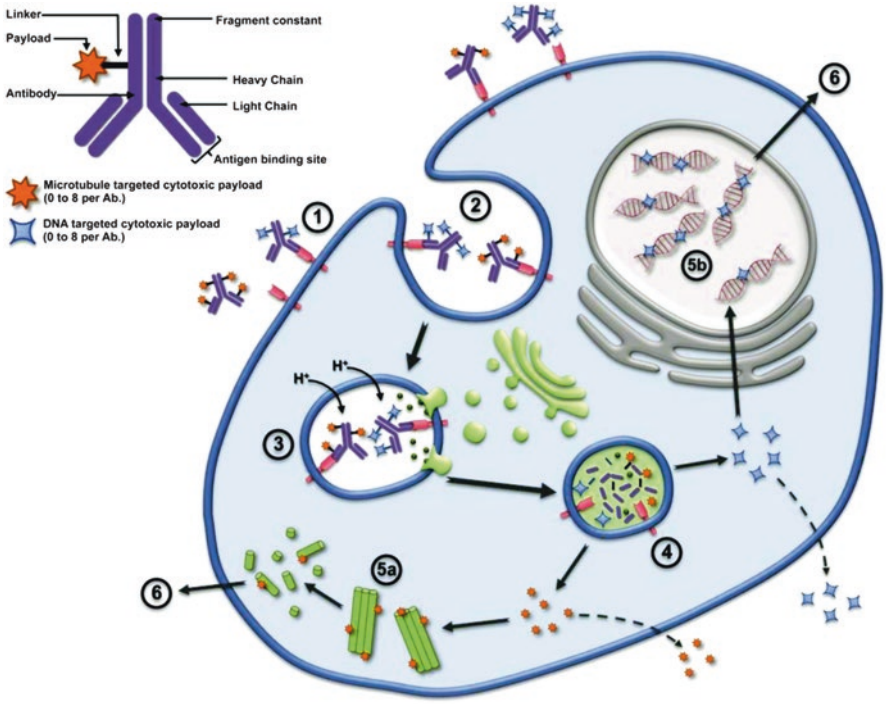

Fig. 2 The ADC first enters the cell upon binding to the tumor target cell's antigen (1). The ADC-antigen complex then undergoes internalization into the endosome (2) followed by fusion of the endosome and lysosome, inducing acidification and enzymatic reactions (3). The acidic environment and enzymes within the lysosome mediate cleavage of linkers, thus releasing the drug into target cell cytosol (4), whereby the cytotoxin disrupts the microtubule network (5a) or binds to the minor groove of the DNA thereby blocking replication (5b). Ultimately, the damage caused to the target cells results in cellular apoptosis (6). Some cytotoxins are released from the cell and may cross the membrane of neighboring cells causing bystander effects, while others do not (e.g., DM1). (Reprinted with permission from Ref. [30])

and safety of an ADC and can also impact the manufacturability and pharmacological stability of the ADC. Oftentimes, the efficacy and safety of a therapeutic construct are established prior to (or in in spite of) manufacturability considerations. However, successful organizations will pursue these considerations in parallel throughout development of the therapeutic.

3 Target Antigen Selection

The first priority in the development of an ADC is the identification of the appropriate antigen for the desired therapeutic target. Different types of cancer require different minimum values of tumor-antigen density for ADC efficacy. Therefore, the

chosen antigen can vary depending on the type of indication being treated. The primary antigens of choice are proteins located on the surface of the cell due to their accessibility and large influence on cellular processes, but other molecules located on the surface such as gangliosides and various glycoproteins are also suitable targets [31, 32].

In addition to being specific for the tumor type of interest, cell surface antigens should also exhibit the following characteristics [33]:

1. Successfully internalized into the cell, but not altered during the internalization process
2. Specific and high levels of expression on the target cells with minimal expression on normal cells (although recent evidence suggests antigen density may not be a critical factor governing ADC efficacy) [34]
3. High occurrence within the desired patient population (antigen present within tumors of proposed patients)
4. Low levels of shedding of the antigen to minimize binding of the ADC to antigens not localized on the cell surface

In addition to fulfilling these requirements, understanding the factors, which influence the internalization process, is critical. For example, different rates of internalization and degradation have been observed for the different epitopes of the HER-2 receptor. Other factors also include high interstitial tumor pressure, the regulation state of the antigen, and the presence of other barriers to the uptake of the cytotoxin [35, 36].

Targeting antigens present in the stroma and vasculature that support the growth and spread of tumor cells have also been investigated because of the less propensity of somatic mutations and hence the low risk of drug resistance. These targets include the vascular endothelial growth factor (VEGF) and its receptors or extracellular stromal tissue [37–41]. Another target for ADCs that is under investigation are the tumor-initiating cells (TICs) or cancer stem cells that are responsible for tumor growth [42].

4 Selection of the Monoclonal Antibody

mAbs have been used as the targeting moiety in the generation of ADCs due to their high degree of specificity and affinity for the antigen of interest. The high affinity of the mAb for its target antigen is necessary for successful binding and internalization into the cell of interest [41]. While the specificity and affinity may be the same across various forms of the antibody (murine vs. fully human/humanized), use of fully human or humanized mAbs is preferred to limit immunogenicity concerns. Immunogenicity resulting from a lack of specificity or the formation of human anti-drug antibodies (ADAs) may limit the observed therapeutic effect due to faster than desired elimination of the drug from the bloodstream [43, 44].

It would be an added benefit if the antibody possessed intrinsic antitumor activity and is capable of interfering with the biological activity of the target antigen, in addition to the cytotoxic activity arising from the conjugated drug, as is observed in Kadcyla® [45]. This can be achieved by modifying the residues within the Fc sequence of the mAb to promote interaction with the Fc Gamma receptors (FcγRs), thus initiating the immune effector functions [46]. There are three main types of immune effector functions: antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cellular cytotoxicity (CDCC), and complement-dependent cytotoxicity (CDC) [47]. ADCC facilitates tumor cell death via direct interaction between the Fc region of the antibody and effector cells, which leads to macrophage-induced phagocytosis or the release of toxic granules by natural killer (NK) cells [48]. CDCC also relies upon the macrophages and NK cells to cause cell lysis, but it differs in that the interaction is between the C3b protein and the effector cells. Finally, CDC results in cell death due to the activation of a proteolytic cascade caused by the interaction between the CH2 domain of the antibody and C1q. The proteolytic cascade leads to the formation pore-forming structures that release cellular contents [49]. Because the effector functions are highly dependent on interactions between the mAb and various signaling proteins, the presence and extent of effector activity observed will vary depending on the specific mAb isotype used. IgG1 isotypes exhibit the highest degree of ADCC and CDC in comparison to the other isotypes [50, 51], which may be due to the sequence differences within the Fc domain. The IgG4 version of gemtuzumab ozogamicin exhibits minimal effector activity [52], whereas the IgG1 isotype of the same molecule demonstrates much better ADCC effector function [53]. On the other hand, the IgG3 antibodies exhibit high CDCC, but sub-optimal half-lives. While the presence of FcγR-binding functionality of the antibody may benefit an ADCC or CDC mechanism of action, the presence of this binding functionality presents a theoretical toxicology liability for the ADC due to a potentially unnecessary valency, as ADCs main MOA is cytotoxicity and not ADCC or CDC. Mutation of the antibody to remove this functionality may be an option to suppress certain types of off-target toxicities. Removal of this functionality through de-glycosylation or other mutations may alter the drug-like properties of the antibody or ADC and require careful PK, manufacturability, and stability consideration.

5 Selection of Linkers

The linker is responsible for covalently attaching the drug (toxin) to the antibody, and its chemistry is critical to the safety and efficacy of the ADC [54]. The design of the linker can have a profound impact on PK/PD, specificity, and stability of the ADC, thus being a critical factor in safety, efficacy, and, ultimately, the therapeutic index of the molecule. One of the most important considerations during linker selection and ADC construction surrounds the mechanism of linker cleavage and resulting plasma

stability. The linker must exhibit suitable stability within the circulation to achieve delivery of the toxin to the site of interest, yet be sufficiently "unstable" to allow for release of the toxin once inside the cell of interest [55]. In the process of identifying linkers capable of achieving both requirements, two classes of linkers have emerged: cleavable and non-cleavable.

5.1 Cleavable Linkers

Cleavable linkers are sensitive to different intracellular and extracellular conditions (e.g., pH, proteases, and reducing environment) and may be designed to exploit specific conditions outside of or within the target cell [56]. Currently, there are three types of cleavable linkers that are commonly employed: hydrazone, disulfide, and peptide linkers [54].

Hydrazone linkers are acid-labile and are sensitive to acidic environments. They undergo hydrolysis in the acidic environment of the lysosome to release the cytotoxic drug [57]. However, when tested with Mylotarg® in clinical studies, hydrazone linkers failed to demonstrate the necessary stability and safety profiles [58]. The second category, disulfide linkers, is based on the reducing environment of the intracellular environment in comparison to the extracellular environment. The high concentration of glutathione within tumor cells leads to cleavage of the disulfide linker via its sulfhydryl moiety and promotes anti-neoplastic therapy [59]. In comparison to hydrazone linkers, disulfide linkers exhibit reduced off-target toxicity due their enhanced stability in the bloodstream. The third type of cleavable linker is the enzymatically labile peptide linker where a dipeptide is used to link the cytotoxic drug to the mAb. These ADCs rely upon the presence of proteases within the lysosomal compartment to cause peptide bond cleavage and subsequent release of the toxin [60]. Breakage of the peptide bond within the bloodstream is not a large concern as these proteases require an acidic environment for activation. The dipeptide is usually composed of valine and citrulline along with a spacer molecule that separates the large cytotoxic drug from the mAb. Among the three cleavable linkers, enzymatically labile linkers demonstrated lower in vivo toxicity as a result of greater specificity, increased stability, and a longer half-life [61].

5.2 Non-cleavable Linkers

Non-cleavable linkers do not contain a specific site for cleavage to occur. Rather, they rely upon the normal protein degradation process within the lysosome for release of the drug. In ADCs generated using these linkers, lysosomal degradation of the mAb into its amino acid components results in the release of the linker and toxin, usually attached to the conjugated amino acid. Although T-DM1 generated

with the non-cleavable thioether linker is stable in the bloodstream and is associated with less toxicity compared to trastuzumab conjugates generated using reducible disulfide linkers, its use is limited only to ADCs targeting antigens that undergo efficient intracellular internalization [62].

5.3 Bystander Effect

In addition to the linker properties affecting the ADC degradation mechanism, they also influence the effect of the cytotoxin on neighboring cells [54]. This effect is known as the bystander effect, and it can lead to death of the healthy cells located next to the cancerous cells. Efflux of the cytotoxin and subsequent action on neighboring cells is possible only if cleavable linkers are used, as the cleaved entity is uncharged, rendering it capable of crossing the lipid bilayer. In the case of the non-cleavable linkers, the linker-drug entity that remains after cleavage is charged and therefore not capable of cellular efflux [63]. This difference leads to the observation of wider efficacy in the case of the cleavable linkers, but their ability to cause the bystander effect depends upon the type of conjugation chemistry used [64]. This ability to infiltrate neighboring cells may be beneficial in the case of solid tumors that do not exhibit high and homogenous target expression, even though the concept surrounding the bystander effect seems opposite of the "targeting" mechanism that is desired for an ADC [65].

6 Selection of Cytotoxin

Generally, cytotoxins must be stable in the liquid state over 2–3 days at room temperature to allow for successful conjugation and ADC production. They must be "attachable" to a linker to allow coupling to the antibody. The design of cytotoxins (including any potential prodrugs and toxin-related catabolites) is critical to ensure the cytotoxin, prodrug, or cytotoxin catabolite possesses the required properties to remain active within the acidic lysosomal environment and successfully enter the cytosol and/or nucleus after exiting the lysosome (via diffusion or active lysosomal transport) [66]. Another key consideration is the susceptibility of the warhead to multidrug resistance pumps, which can be upregulated in certain types of cancer [67]. Consideration should also be made regarding the ability of free warhead (or warhead-related catabolites) to non-selectively permeate non-target cells. The ability of free warhead (or warhead-related catabolites) to permeate back into cells should be considered either for increasing the bystander effect (described above) or for reducing off-target toxicity, if so desired.

To meet all the above requirements, early ADCs utilized small molecule drugs that were previously used as therapeutic entities themselves. Because some of the

early small molecule toxins used in ADC generation such as anthracycline and methotrexate did not elicit the necessary therapeutic effect due to the low doses required [68, 69], more potent cytotoxins were employed in future ADCs. Although most cytotoxins are used to target mitotic cells, the selection of the correct toxin depends on the mechanism of action and the nature of the cancer.

Of the six approved ADCs, five use a small molecule toxin from one of the following categories, which make them highly desirable for future ADCs: calicheamicins, maytansinoids, or auristatins. The calicheamicins, which are used in Mylotarg® and Besponsa®, induce DNA damage [70], which eventually leads to cell death by apoptosis [71]. The DNA damage occurs due to direct binding of the cytotoxin to the minor groove within the DNA double helix. Reactive species are then formed, leading to cleavage of the strand at multiple locations, eventually resulting in cell death by apoptosis due to degradation of the DNA double helix. Two additional classes of cytotoxins known as the duocarmycins and pyrrolobenzodiazepine (PBD) exhibit a similar mechanism of action. Both also exert their effect on the minor groove within the DNA double helix with duocarmycins acting as a DNA alkylating agent and the PBDs acting as a minor groove cross-linking agent [72, 73].

The cytotoxin used within Kadcyla® [74], which falls under the category of the maytansinoids, causes cellular apoptosis by binding to the microtubules and interfering with the mitotic pathway [75–77]. They limit microtubule formation by stalling the cell in metaphase by binding to the "plus" end of the microtubule and blocking the formation of tubulin dimers. In addition, the GTP molecule on the β-subunit becomes hydrolyzed leading to the disassembly of existing microtubules, further preventing mitosis [78].

Similar to the maytansinoids, the auristatins also interfere with the cell cycle [75, 76], but in a completely opposite manner. Whereas the maytansinoids cause shrinking of the microtubule, the auristatins cause excessive growth of the microtubule. Binding of the auristatins to the β-subunit prevents the hydrolysis of GTP molecules, thus limiting the ability of the microtubules to separate into sister chromatids. Therefore, the cell is stalled in the metaphase stage of the cell cycle. The maytansinoids have also exhibited success in the clinic with the approval of Adcetris® and Polivy™. Depending on the properties of the linker used, the auristatins may exhibit the bystander effect. If used with a more hydrophobic linker, the bystander effect may be observed; but, if used with a charged linker, the effect may not be as prominent. Depending on the indication, the bystander effect may or may not be desirable [79].

While ADCs themselves are directed prodrugs (requiring target specificity and catabolic conversion to release the active payload cytotoxin), cytotoxins can also be designed as prodrugs themselves in an attempt to improve the therapeutic index. One example of this includes ADCs with disulfide containing toxin prodrugs [80]. Generally, careful consideration of any liabilities introduced by these functional groups is important for assessing in vivo and formulation/in-use stability.

7 Conjugation Process

ADCs are can be formed using a variety of chemical and enzymatic conjugation techniques, which can have a significant impact on the conjugation site(s), DAR, and/or DAR heterogeneity. Generally, chemical conjugation methods utilize solvent-accessible native or engineered amino acids which, through a controlled chemical reaction, are covalently bonded to the linker to form the ADC. On the other hand, enzymatic conjugation utilizes certain posttranslational modifications and results in more homogeneous DAR. As such, the conjugation process should be carefully selected based on the target site specificity of the conjugation site(s) and the required DAR distribution [81, 82].

Lysine and cysteine are the two main native amino acids that have been widely utilized as non-specific conjugation sites for producing ADCs. The non-specific nature of the conjugation and the large number of surface-accessible lysine residues result in broad DAR distributions that pose several challenges as it pertains to PK and toxicity profiles, manufacturing processes, and analytical control strategies. Relative to the lysine residues, native cysteine can be better utilized to generate less heterogeneous DAR. To this end, interchain disulfides are selectively reduced to provide 2–8 free thiol groups that act as conjugation sites to form ADC with less heterogeneous DAR.

Heterogeneity of the DAR distribution and conjugation sites play a critical role in the efficacy and safety of ADCs. In order to generate highly homogenous DAR, site-specific conjugation approaches must be employed. These approaches include (1) engineering cysteines or non-canonical amino acids into mAbs in order to enable selective conjugation reactions to the payload and (2) insertion of a "tag" to facilitate enzymatic-catalyzed conjugation.

Engineering two non-native cysteines into the heavy chains of a mAb has been shown to form a highly homogenous ADC with a DAR of 2 [83]. Another similar approach involves engineering out of one of the two cysteine residues involved in the interchain disulfide bonds to make the other cysteine residue available for site-specific conjugation. While successful in achieving control over DAR distribution and site specificity, these approaches may result in unwanted disulfide bonds within the mAb or between neighboring mAb molecules, leading to disrupted binding capacity [84] or aggregation, respectively. To mitigate these challenges, specific reduction processes have to be developed and optimized.

Similar to the naturally abundant cysteine residues, non-natural amino acids such as selenocysteine or acetylphenylalanine can been engineered into mAbs [85–87] to enable site-specific conjugation through specific chemistry that is unique to them compared to the natural amino acids. For example, selenocysteine resembles cysteine in structure, but has a selenium atom instead of sulfur, whereas acetylphenylalanine resembles phenylalanine, but carries a ketone group [88]. Additionally, cell-free expression systems have also been employed to design, screen, and produce ADCs containing non-natural amino acid-based conjugation handles [89].

In addition to chemical conjugation, certain enzymes have been employed to catalyze the conjugation of both native and genetically engineered antibodies or to incorporate unique reaction handles to enable specific chemical reactions with counterpart functional groups. One enzymatic conjugation approach utilizes transglutaminases to catalyze the conjugation of a primary amine-containing linker to the primary amide side chain of a specific glutamine to form ADCs with a homogenous DAR of 2 [89, 90]. Other enzymes used for this purpose include glycotransferases and formylglycine-generating enzymes [81]. β-1,4-Galactosyltransferase (GalT) and α-2,6-sialyltransferase have also been used to add an aldehyde group on the N-glycan terminus of the asparagine residue present on the Fc region to serve as a conjugation site for aminooxyfunctionalized drugs [70].

8 Drug-to-Antibody Ratio (DAR)

The selection of the optimal DAR for a given ADC is critical and has to be carefully considered. Since only a very small percentage of the administered drug actually enters the tumor cells (<0.01% injected dose per gram of tumor) [91], it would seem that higher DAR and/or more highly potent warheads would be necessary to achieve enough efficacy of the ADC. Nevertheless, high DAR may lead to increased clearance and/or unwanted immunogenicity [92], and highly potent toxins can cause off-target toxicity. In general, ADCs with a DAR 2–4 have been shown to provide good a balance between slow clearance and high potency. In addition to DAR, the hydrophobic nature of the drug-linker also affects clearance [93]. Accordingly, modulating the hydrophobicity of the linker-drug can enable the use of higher DAR ADCs that have desirable clearance profiles [94] and would have higher therapeutic indices if used with low potency toxins. In this vein, the therapeutic index can also be improved using conjugates composed of two different toxins that have complementary therapeutic effects [95].

9 Pharmaceutical Stability Considerations

An antibody-drug conjugate (ADC) includes a native mAb with a small molecule drug attached through a linker. The position of the linker connection with the antibody is important for stability [96, 97]. Covalent linkage to solvent-accessible lysine residues alters the overall surface charge of the molecule, whereas cysteine conjugation may significantly disrupt the integrity of molecule conformation and change the overall thermodynamics of unfolding [98]. The hydrophobicity of the drug and the inherent heterogeneity of the number of linker-drugs per antibody molecule are additional factors that can contribute to molecule instability [99]. Therefore, the site of conjugation, linker-drug properties, and overall DAR are important factors to consider when designing a formulation as the solution conditions must facilitate suitable stability of the biologic in addition to the hydrophobic linker-drug entity.

9.1 Stability Implications Due to Lysine Conjugation

During the formation of lysine conjugates, the lysine residue is activated to form an intermediate species capable of forming the covalent attachment with the linker-drug. This activated species has been shown to have decreased physical stability in comparison to the naked mAb, as well as the fully conjugated ADC [100]. This is due to the presence of exposed maleimido groups in the activated form, which are prone to aggregation. The aggregates formed are primarily non-cysteine related, suggesting that cross-linking is mediated through other interactions such as between the activated lysine residues. Lower degree of covalent aggregation in the fully conjugated ADC implicates the activated lysine residues as the primary drivers of the instability associated with the ADC intermediate.

Standard lysine conjugation involves the use of surface-accessible lysine residues as the site of linker-drug attachment. This form of conjugation alters the overall surface charge of the molecule as positively charged amine side chains are now converted into neutral amide bonds. Alterations in overall surface charge have been shown to impact physical stability in the case of proteins [101] so understanding the effects of lysine conjugation on the stability of an ADC is an important parameter to consider. With lysine conjugation, though, it is difficult to predict the impact of conjugation on stability because the heterogeneity associated with this method can lead to drastically different sites of conjugation from lot-to-lot. Even though the typical mAb can contain greater than 80 lysine residues [100], they are not all accessible for conjugation. Various reports have shown that anywhere from ~40% to 80% of the total lysine residues are actually conjugated [101, 102]. A large majority of the conjugation occurs within the CH2 domain as this is a more flexible region, which makes these residues more accessible for conjugation. Therefore, it is not unexpected that the largest differences in stability are observed within the CH2 domain of the ADC. Although the conformational stability is affected less so than observed with cysteine-based conjugation [63, 103], the T_m of the CH2 domain within lysine conjugates is decreased in comparison to mAb alone [100, 104]. In addition to conformational destabilization, lysine conjugation also leads to decreased colloidal stability, as in increase in aggregate formation was observed in the ADC in comparison to the native mAb during storage at elevated temperature [100, 105]. This change could be caused by both the elimination of a charged residue on the protein surface and the introduction of a hydrophobic drug onto the surface of the molecule.

9.2 Effect of Disulfide-Bond Elimination During Cysteine Conjugation

Because of the large degree of heterogeneity associated with lysine conjugation, other conjugation methods have received more interest recently. One of these involves conjugation using the native cysteine residues, which is achieved through

selective reduction of the interchain disulfide bonds. Even though heterogeneity is still present in these conjugates, the site of conjugation is limited to eight sites, instead of the 80+ sites available using lysine conjugation. Of the ten potential isoforms for DAR 2–8 species, there is a preference for certain isoforms. In the lower DAR species (DAR 2), conjugation primarily occurs in the Fab domain, with conjugation occurring at the hinge region only as higher DAR is required [106]. Heterogeneity can be reduced by chromatographic purification of unwanted DAR species.

Conjugation through native cysteine residues involves breaking the interchain disulfide bonds. As these bonds are one of the main linkages preserving the antibody framework, alterations in conformational stability are expected. But the conformational perturbations observed in conjugates are greater than those caused by simply eliminating the disulfide bonds [97]. Therefore, the introduction of the linker-drug into these locations has steric implications that influence the folding stability of the molecule. As DAR increases, the distance between the CH2 domains also increases [106]. These results provide further confirmation that the primary conjugation site in lower DAR species is in the Fab domain, whereas higher DAR species have conjugation in both the Fab and CH2 domains. Regardless of the method used (DSC, far-UV CD, intrinsic fluorescence), the thermal stability of cysteine-conjugated ADCs is lower than their unconjugated mAb counterparts. The CH2 domain unfolding is also less reversible in the conjugate [97]. The difference in thermal stability, though, is not accompanied by changes in overall secondary or tertiary structure [97, 105, 106].

Although cysteine-based conjugation would be expected to only impact the conformational stability, differences in colloidal stability are also observed that are not solely due to the presence of the hydrophobic linker-drug. Upon conjugation, the solvent exposure of the cysteine residues is increased [97]. If the cysteine residues are not fully conjugated or capped, they could remain available to form disulfide bridges between ADC molecules. The decreased conformational stability can also lead to colloidal destabilization. After unfolding, the ADC is more prone to aggregation, which was not observed in the parental mAb [97]. Hence, elevated temperatures may lead to CH2-initiated unfolding, which directly impacts the aggregation propensity of the molecule [97, 105, 107]. In addition to aggregation, increases in fragmentation have also been observed due to the elimination of the interchain disulfide bonds [98].

9.3 Linker-Drug Hydrophobicity and DAR

One important property that needs to be considered for pharmaceutical stability during the selection of a linker-drug during ADC design is the inherent hydrophobicity of the small molecule components. Hydrophobic linkers coupled with hydrophobic toxins often imbalance the surface properties of the ADC, potentially causing poor

solubility and stability (aggregation) and provoking undesired immune responses during circulation in the bloodstream. At every DAR load, the linker-drug leads to a 4–6× increase in nonpolar surface area in comparison to polar surface area [97]. The degree of linker-drug hydrophobicity is also important and can be directly correlated to the rate of aggregation during thermal stress [107].

Self-interaction between molecules becomes more likely when the surface properties of the molecule are changed, thus decreasing the colloidal stability of the molecule. Colloidal stability is not the only parameter influenced by the extent of linker-drug hydrophobicity as T_m of the CH2 domain decreases as linker-drug hydrophobicity increases [107]. To combat the hydrophobicity inherent of commonly used drugs, hydrophilic linkers containing negatively charged moieties such as sulfonate groups [108, 109], polyethylene glycol (PEG) groups [109, 110], or pyrophosphate diester groups [111] can employed. Peptide linkers are often hydrophobic and can induce the aggregation of ADCs. Doxorubicin conjugates composed of the hydrophobic peptide linkers Phe-Lys or Val-Cit linkers were found to form noncovalent dimers [112], whereas the addition of a hydrophilic methoxytriethylene glycol chain onto doxorubicin inhibited its aggregation [113]. Although not always feasible, modulation of the linker is the easiest way to address the stability concerns associated with the hydrophobicity of the linker-drug.

As increased hydrophobicity accompanies the higher DAR species, it is not surprising the aggregation propensity increases with increasing DAR [98, 105, 106]. In samples with mixed DAR species (various percentages of DAR 2, 4, 6, 8), the aggregates generated after thermal stress were primarily composed of the higher DAR species (DAR 6 and 8) [106]. The aggregates were fully intact, suggesting that the DAR-induced colloidal destabilization in this instance was driven by the alterations in surface hydrophobicity and not conformational destabilization. In purified DAR samples, DAR 6 species also exhibited a lower aggregation onset temperature than DAR 2 and 4 species [96]. With the cysteine-based conjugates, higher DAR is also accompanied by decreased conformational stability. In these instances, the stability is not affected solely by the hydrophobicity of the linker-drug. Rather, the introduction of a bulky substituent into the hinge region and the elimination of hinge disulfide bonds that accompany the higher DAR species lead to lower $T_{m,onset}$ as DAR increases [98, 101, 105, 106]. The broadening of the peak corresponding to the CH2 domain in the DSC thermogram is indicative of a less cooperative structural transition and less compact structure overall. Loss of key structural elements such as the hinge disulfide bonds, which is more likely as DAR increases, would be expected to lead to changes in the conformational stability of the ADC.

10 Formulation Considerations

Designing the appropriate formulation for an ADC can be a difficult task as one has to consider the stability of all the three components of the ADC. Among the three components, the stability of the drug is relatively of less concern, but the stability of

the mAb and linker-drug is of major concern when it comes to the stability in the liquid state. Although ADCs are often lyophilized to enhance the shelf-life, a minimum of few weeks of liquid stability is required for flexibility in manufacturing operations. Disaccharides such as sucrose or trehalose are often added to the composition of formulations as cryoprotectants and lyoprotectants to protect the mAb during freezing, drying, and storage. Since ADCs are potent, the relative solid content of the mAb in the formulation will be small; hence, addition of bulking agents such as sucrose, mannitol, or glycine is desirable. Compared to sucrose, mannitol and glycine are preferred as they form a crystalline matrix, which enhances the elegance and mechanical strength of the cake, in addition to providing efficiency to an otherwise lengthy lyophilization process [109].

In addition to stabilizing the solid form of the drug, solution stability must also be maintained. Preservation of the overall fold of the molecule is achieved through the use of conformational stabilizers such as sucrose, whereas colloidal stability is achieved through modulation of ionic strength and protein-protein interactions. Conjugation of a largely hydrophobic linker-drug to the surface of the protein negatively impacts the aggregation propensity of the molecule. High ionic strength formulations will lead to decreased electrostatic repulsion between ADC molecules, thus favoring the hydrophobic interactions and aggregation [98]. The counterion used in the salt has also been shown to impact aggregation. Studies utilizing both NaCl and Arg-HCl illustrate the stabilizing effect of the guanidinium group as higher aggregation was observed in NaCl formulations in comparison to Arg-HCl [98]. Along with affecting colloidal stability, ionic strength may also lead to differences in conformational stability. Although worse at higher NaCl concentration, addition of even a small amount of NaCl may lead to a decrease in conformational stability as measured by DSC [98]. This could be due to the disruption of noncovalent attractive forces as increased fragmentation was also observed. Excipients can also exert their stabilizing effect through direct interactions with the cytotoxin. Certain excipients may be more likely to interact with a specified structural class of toxin due to both the hydrophobicity and the size of the small molecule. Accordingly, toxin-based “platform” formulations may be a suitable approach for formulation development as the cytotoxin is usually the main driver of instability within the ADC. Finally, addition of surfactants is also required to limit aggregation induced by interfacial stressors as is the case for mAbs.

Addition of excipients to stabilize the linker-drug entity in particular has not been widely discussed in the literature. Aside from the hydrophobicity-driven destabilization of the ADC as a whole, degradation of the small molecule component itself can occur. Investigating the stability of the linker-drug during ADC stability studies can be inherently complex as it is difficult to discern if the observed changes are due to the small molecule component or the mAb. Therefore, completing stability studies on the linker-drug itself may uncover any liabilities of the small molecule that would limit its successful use in an ADC.

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Chapter 3
Enabling Biologics Combination Products: Device Ability in Protein Therapeutics

Bernardo Perez-Ramirez

1 Introduction

Combination products are defined as therapeutics combining two or more products (drug/device, biologics/device, biologics/drugs, or drug/device/biologics) regulated and sold as a single unit [21, 80, 94]. As these pharmaceutical and biological therapies and treatments have evolved, so has the need to develop appropriate delivery mechanisms for these applications [18, 19, 23, 26]. When developing a combination product, there are many aspects to be considered – relationships between device development and the pharmaceutical or biologic, early establishment of regulatory and clinical strategies, understanding user needs, determining product requirements, as well as device manufacturing variation [94]. All the above requirements are directed to improve the patient experience where patient insight drives the system development [62]. This integrated development is based on five principles: (1) All teams must always start with a common end in mind which is the patient experience and the factors and attributes of the product that make a best in class/preferred treatment experience. (2) All teams involved must share a common understanding of drug-device combination system architecture and components including all the subsystems and their respective constituents and how they interact between each other, namely, active component, formulation, and primary container. (3) Technical design decisions related to one sub-system must be considered within the perspective on its potential impact on other components and with the understanding on how these could impact the overall drug-device combination system and in particular the factors/attributes of the product that make the best in class/preferred treatment experience [62, 94]. (4) Successful drug-device combination system design does

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not reside in selecting the best individual components, but in selecting the best combination of individual elements to create a best in class/preferred treatment. (5) Drug-device combination product robustness is directly linked to the ability to identify and to put under tight control (in close collaboration with suppliers) any variable across the system architecture for which a variation could impact the treatment experience.

Therefore, a quality target product profile (QTPP) is required for successful development and defined as: "a prospective summary of the quality characteristics of the drug product that ideally will be achieved to ensure the desired quality, taking in account safety, and efficacy of the drug product" [31]. The remaining of the chapter describes the essential elements of device integration and early drug ability/device ability potential of biologic constructs as well as required analytical tools. Equilibrium binding thermodynamics for optimization of a protein-collagen sponge drug delivery is discussed in detail together with the utilization of silk protein for sustained delivery of monoclonal antibodies through the creation of lyogels.

2 The Patient-Centric Approach and Drug-Device Integration

In recent years, it has been a shift of the pharmaceutical landscape toward device-mediated biologics-based injectable therapies to treat chronicle diseases, moving from a situation where drug-device combination (DDC) development was an insulin-centric niche activity [36] to a situation where DDC development is now a strategic imperative representing about half of research and development pharma portfolio including monoclonal antibodies, fusion proteins, enzyme replacement therapies, hormones, and also gene therapy [26, 62].

To deliver this portfolio, several core device delivery technology platforms are required:

1. Disposable single-use standard and safety prefilled syringe (PFS) of 1 and 2 ml to be used as stand-alone product combination or as primary container for auto-injectors
2. Reusable and disposable pen injectors [12] based on 1.5 and 3.0 ml cartridges for daily administration of peptides in particular within the diabetes field
3. Disposable single-use auto-injectors based on 1 and 2 ml syringes as primary container for the weekly or less frequent delivery of mAbs
4. Disposable single-use large-volume device/wearable injectors [18] based on 3 and 10 ml primary containers for bolus injection (2 ml and above) for the weekly or monthly delivery of mAbs

The patient treatment experience is the key endpoint that drives DDC market success. Patient preferences and human-centered designs are more and more a critical differentiator [80, 94]. Consequently, improving patient outcomes must be

Table 1 Patient outcomes

Patient's response	Output
Physiological	Efficacy, safety
Physical	Pain
Cognitive	Simplicity, ease to use
Emotional	Aesthetics

the ultimate goal of DDC development [62] and could be analyzed in four main dimensions (see Table 1).

The patient insight drives the drug combination system development that in turn is composed of several individual units/pieces, highly integrated. The fundamental components of the drug-device integration are (i) the drug substance, (ii) the chemical formulation, (iii) the primary container, and (iv) the delivery device [62].

Each component technical design decision must be considered within the perspective on the impact on the other components, namely, drug substance, formulation, drug product, and delivery device. All sub-systems are closed linked. The first seen by the patient is the delivery device, but the first made and the first locked is the drug substance followed by the formulations. Hence, the design of the drug substance and the formulation drives the design requirements of the other subsystems [62].

3 Device Ability Begins in Discovery Research

The "biologic" is one important component of the final drug product. As such, proper selection of the new molecular constructs incorporating physicochemical and biopharmaceutical characterization [24, 60, 65, 80, 100] should be performed early in discovery research to identify suitable candidates to move into clinical development and eventually to the market [33, 60]. The challenges been faced by an early evaluation of suitable candidates are several: (i) usually minimal amounts of protein are available, (ii) process not completed defined yet, (iii) assays need to be high-throughput, and (iv) global probes of structure and function should be employed [60] using single-value deconvolution analysis [70] or similar approaches. As discussed in the preceding section, there is a necessity to incorporate convenience to patients in drug product development; this has prompted the development of high protein concentration dosage forms for subcutaneous administration which are typically limited to 1.5 ml [26, 42]. High protein concentration drug products have presented several challenges associated with stability and solubility that is insufficient to meet expected dosing [68]. Hence, the need to identify device ability factors as part of the overall drug ability potential of the biologic constructs is imperative. What is currently missing is a formal selection criteria, similar to the one employed for organic synthetic modalities to improve bioavailability by the oral route of administration, namely, the Lipinski's rule of five [41]. Due to the

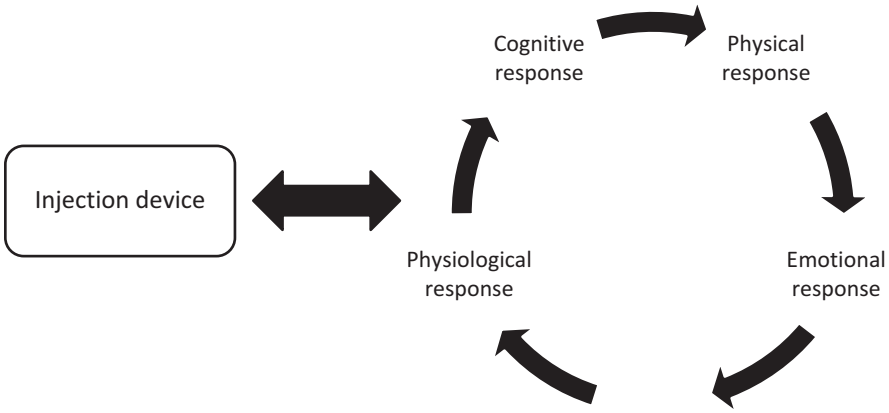

Fig. 1 Patient response to an injection device

complexity of protein biologicals compared to small molecules, the device ability criteria for the former are still elusive. How to design biologic protein sequences and structure for the benefit of the patient injection experience? How to design the formulation for the benefit of the patient injection experience with no pain and ease of use? Earliest choices are critical; no world-class development could compensate for poor early design (Fig. 1).

3.1 Device Ability Tools for Predicting Solution Behavior

Managing high protein concentration viscosity behavior has been the main challenge when monoclonal antibodies are concentrated beyond 100 mg/ml [40, 68, 69, 77, 102]. Highly concentrated or crowded antibody solutions can be defined as non-ideal solutions where individual antibody molecules are in close proximity between them and the distance could be of the same order of magnitude as the size of the molecule [38]. This in turn increases the frequency of encounters and the duration of interactions among the molecules. This could translate into poor recovery during ultrafiltration/diafiltration process, clogging of filters, poor injectability, denaturation, aggregation, liquid-liquid phase separation, particulation, and precipitation. Those are common consequences of highly viscous solutions in addition to potential loss of potency and changes in pharmacokinetic profile and/or product safety. Nevertheless, all monoclonals do not have the same increase in viscosity as function of protein concentration [17, 40, 68]. In modulating the viscosity properties, sequence and structural properties of variable regions in monoclonal constructs are key in determining the solution behavior. For example, experimental evaluation of net charge, Ɛ-potential (zeta potential), and pI of Fv (variable fragments) regions were found to correlate with viscosities of highly concentrated antibody solutions [40], and highly viscous solutions contain negatively charged patches on their Fv

regions. A combination of experimental and computational approaches has been also used to probe the link between charged residues (and type of charge in the CDRs) and viscosity and self-associating behavior [98, 99]. A spatial charge map (SCM) tool has been developed for screening antibody solutions using information on charge distribution of the Fv portions of the mAbs. Thus, rational structure-based reengineering of the Fv portion of mAbs can help mitigate viscosity and tendency to form reversible self-associations [27]. Hydrophobic patches could be natively exposed and/or exposed due to dynamic fluctuations or as the result of ligand binding-induced conformational changes. Solvent-exposed hydrophobic residues could lead to self-association and increased viscosity. Targeted mutations to these self-association-prone regions, identified by molecular dynamic simulations, could allow designing better device-able mAb [16]. A different approach has been designed to analyze viscosity of monoclonal antibodies based on the observation that globular proteins lacking the flexibility of antibodies have much lower viscosity than antibodies at comparable volume fraction [66]. The model shows that the solution viscosity of monoclonal antibodies is consistent with molecular entanglements that are exacerbated when the molecules bind together to form large complexes due to their elongated shape and intrinsic flexibility [66].

Measurements of diluted solutions of monoclonal antibodies by static or dynamic light scattering have been used to probe solution behavior with the expectation that those results could be applicable at high protein concentrations [13, 17, 28]. Protein aggregation is expected to be the predominant degradation profile at high protein concentration since chemical stability is independent of protein concentration. Thus, Rayleigh (classical) scattering can allow the calculation of the virial coefficient (A22), a thermodynamic measure of the solution's deviation from ideality [103]. In addition, light scattering is a convenient way to determine the diffusion interaction coefficient (kD), that is, a function of B22 and protein hydrodynamics. B22 is the designation given to the second virial coefficient when obtained by other methods different from static light scattering such as analytical ultracentrifugation [96], SEC [6], and hydrophobic interaction chromatography [76]. Those parameters could be good indicators of solution conditions where a protein could remain soluble or lead to aggregation and precipitation (Fig. 2). It has been shown that for more concentrated solutions, the value of B22 could be over- or underestimated with the conventional analysis for significantly repulsive and attractive interactions and the application of the Kirkwood-Buff analysis could be more appropriate in those cases [10] and valid at low and some high protein concentrations when one considers strong repulsions/attractions. Ideally, the techniques outlined above could be employed in a universal/platform buffer. The approach could be beneficial for formulation screening in conjunction with methodologies and models to predict domain-domain preferential contacts based on sequence information [5, 39, 56, 78, 84, 97]. Those contacts include electrostatic interactions (charged residues), non-electrostatic interactions (solvation, London dispersion forces), and steric effects (atomic radii and molecular geometry) [14]. Nevertheless, universal methods that could predict the behavior of protein solution (in particular aggregation and oligomerization) in diluted buffers and extrapolate to high protein concentrations should

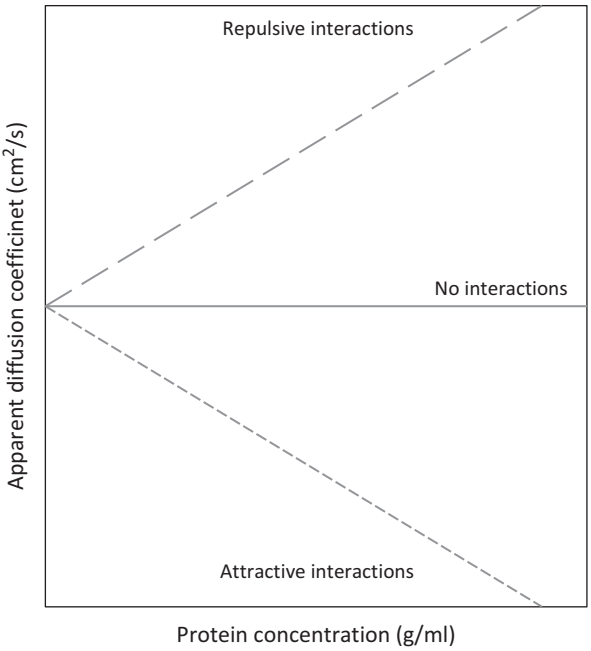

Fig. 2 Model for diffusion interaction parameter kD for three different monoclonal antibodies as determined by light scattering. The sign and magnitude of kD represents the sum of all the protein-protein interactions

be evaluated carefully for potential pitfalls and artifacts. The final formulation buffer could have a tremendous impact on the solution behavior of the protein, through synergistic effects among excipients employed as stabilizers, cryoprotectants, or tonic modulators, as well as interactions with the primary container or the drug delivery matrix in a combination product. In a typical drug product formulation, excipients could be excluded or preferentially bound to particular domains in the protein. When an excipient (ligand) upon binding to a protein induces self-association or dissociation [67], analysis by Scatchard plot shows that those isotherms are curvilinear [52]. The linkage between ligand binding and protein self-association could be dissected by careful analysis of the resulting Scatchard plots, where we can distinguish four situations, namely, (i) ligand binding that induces self-association, (ii) ligand binding that facilitates self-association, (iii) ligand binding that both induces and facilitates self-association, and (iv) ligand binding that cross-links protein [75].

The energetic of the solution,[1] dictated by the interaction of excipients with charged and/or polar residues in proteins, has a profound impact on the ability of a

[1] The phenomenon has been described in the literature as *colloidal stability* in reference to a particle remaining suspended in solution at equilibrium and the ability to resist self-association, sedimentation, particulation, flocculation, and coagulation. However, proteins, due to the nonuniformity

folded protein to self-associate [57], precipitate, and/or crystalize [7, 85]. In a protein solution, different pathways could lead to precipitation/crystallization including aggregation, self-association, gelation, and liquid-liquid phase separation (LLPS) [15, 35, 45, 53, 79, 91, 93]. The kinetics of aggregation, self-association, and gelation are usually slow and vary from protein to protein due to the different mechanisms involved in protein oligomerization. Thus, measuring properties of the solution that could lead to gelation or LLPS could provide an orthogonal evaluation of the so-called colloidal[2] stability [46, 93]. LLPS is a spontaneous segregation of a homogenous protein solution, below a certain temperature (LLPS temperature), into coexisting protein dilute and protein-rich liquid phases [20, 25, 73, 74] (Fig. 3). Thus, the ability of a protein to undergo LLPS is an indication of the strength of the averaged overall inter-protein attractive interactions that could also drive self-association resulting in aggregation and potential higher viscosity. Typically, proteins that undergo LLPS have the diluted phase in perfect thermodynamic equilibrium (equal chemical potential) with the condensed phase. A lower temperature favors the condensed phase, leading to LLPS. Therefore, LLPS temperature can also serve as an indicator of the energetic of the solution (stability) of monoclonal antibodies over long-term storage [93]. However, in most antibody solutions, the inter-protein interactions are too weak to cause LLPS above the freezing point of the solution [92]. Nevertheless, LLPS could be induced in the laboratory at temperatures above freezing by using poly(ethylene glycol) (PEG) that is preferentially excluded from proteins [4, 8] favoring conformational stability [58, 59] without affecting inter-protein interactions [3, 87]. When two protein molecules are close enough so that PEG molecules cannot fit in the space between them, the unbalanced local osmotic pressure adds an additional attraction between the two protein molecules. Native interactions are not affected. The dense phase (precipitate) mimics a highly concentrated drug product (Fig. 3). The method has been employed to evaluate the so-called colloidal stability (referred in this chapter as the energetic of the solution) of five different monoclonal antibodies under several solution conditions [93]. In addition, Wang and collaborators have been able to provide a quantitative assessment of solubility through the PEG-induced LLPS approach by evaluating the "binding energy" (ε_b) in the condensed phase, deduced from the solubility measurements, representing the strength of attractive interactions between antibodies. The ε_b parameter depends on both the physical-chemical properties of the monoclonal construct and the solution conditions (excipients, pH, ionic strength, visco-reducers, etc). The great advantage of the procedure is the use of minimal amounts of protein to simulate the highly protein-concentrated environment through the PEG-induced LLPS.

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of their charge, dynamic conformations, and the ability of different domains to establish unique interactions with the solvent and cosolvent, may not behave as particles in solution. Hence, the *energetic of the solution* (increasing or decreasing) could be a better descriptor than *colloidal stability.*

[2] See footnote 1.

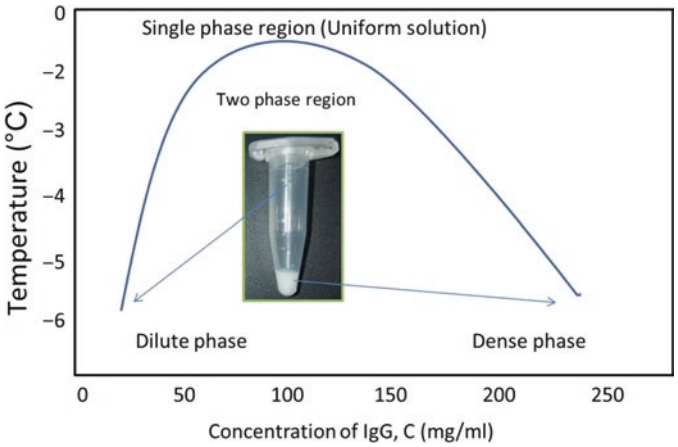

Fig. 3 Modeled coexistence curve for PEG-induced liquid-liquid phase separation for a monoclonal antibody in 10 mM sodium phosphate, pH 7.2, 150 mM NaCl. (Data obtained according to the procedure described by Wang et al. [93])

4 Combination Products: Matrices and Scaffolds

4.1 Protein "Aggregation" and Association upon Interaction with Drug Delivery Matrices

There are several clinical indications where it is often necessary to retain a therapeutic protein at the delivery site for optimal efficacy. The success of BMP-2, for example, in eliciting bone regeneration in vivo, depends on extended residence time at the surgical site [81, 82, 95]. To accomplish this, BMP-2 is required to be administered in combination with a biomaterial matrix. The ideal matrices for BMP-2 delivery are characterized by adequate porosity to allow cell blood infiltration, appropriate mechanical stability against compression and tension, biocompatibility, and biodegradability among other key characteristics. In addition to the properties of the matrix or biomaterial employed in drug delivery, it is important to evaluate early on, during development, the impact that the drug delivery system could have on the chemical and physical stability of the active protein. Thus, interaction of the protein with the intended delivery system or the process for preparing the combination product could adversely damage the protein, reflected as denaturation, leading to "aggregation," cleavages, or induction of other physical and/or chemical instabilities. To that effect, the quality of the protein being eluted from the delivery system during in vitro release should be analyzed for oligomerization, fragmentation, oxidation, thermal stability, and bioactivity among other key parameters. Ideally a 100% recovery of the protein with no loss in biological activity or chemical and physical properties should be the goal of the initial in vitro release studies.

Ionic strength and pH are experimental variables utilized to modulate the interactions between a protein and a delivery system that could impact the in vitro release profile. The remainder of this section will outline an example of the use of equilibrium binding measurements to extract relevant thermodynamic parameters to optimize protein release from a delivery matrix.

Delivery System Saturation: The rhBMP-2 Example

Theoretical approximations can be obtained for the saturation of an implantable matrix with the protein intended to be delivered. In the case of rhBMP-2 and the collagen matrix used as the delivery system, two simplest scenarios can be contemplated to determine the occupancy of the collagen matrix [50]. A theoretical low limit of binding can be calculated as the mass of rhBMP-2 that binds in a saturated monolayer to the total surface area of the collagen as determined by Brunauer-Emmett-Teller (BET) analysis [49]. A theoretical high limit of occupancy can be calculated as the mass of rhBMP-2 that occupies the free volume in the collagen matrix [50]. Therefore, on the basis of those calculations, an approximation of 5 and 11 μg rhBMP-2/mg of collagen matrix incorporates the range of dimensional orientations for a monolayer of rhBMP-2 molecules occupying the collagen matrix surface [49]. By contrast, the high limit of binding of rhBMP-2 to a collagen matrix could be approximated as saturation in the free volume inside the collagen matrix (Fig. 4). In this case, the high volume saturation accounts for 67.3 mg rhBMP-2 per milligram of collagen cylinders [50]. This theoretical approach gives an initial identification of the limits of saturation of a delivery matrix and guides further experi-

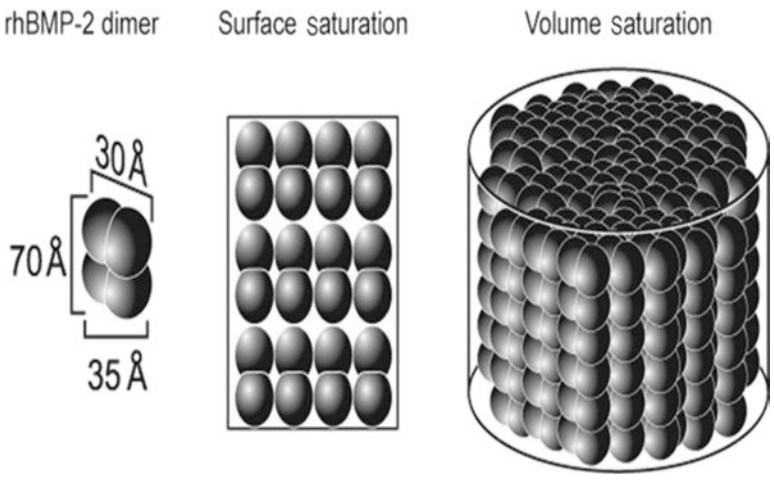

Fig. 4 Schematic of rhBMP-2 and collagen interactions as surface saturation and volume saturation. (Reprinted from Morin et al. [50], with permission of the copyright holder, The American Chemical Society)

ments to control the release profile of a given protein and optimize the desired pharmacokinetic profile. Those experiments involve understanding the strength of binding of the protein to its delivery matrix, identification of potential linkages to self-association or "aggregation," and how solution variables modify those interactions.

Extracting Thermodynamic Data from Equilibrium Binding Analysis

To experimentally evaluate the type of interaction that a protein undergoes with a delivery matrix (binding strength) and type of binding (monolayer versus multilayers), we can use the binding of rhBMP-2 to a collagen delivery matrix as described previously [50] as a model as well as the example of the binding of rhBMP-2 to precipitated hydroxyapatite [11]. Table 2 shows the binding strength of rhBMP-2 to collagen as determined from Scatchard analysis of the equilibrium data. In this particular example, it is clear that the binding strength is modulated mainly by pH rather than ionic strength. As the pH is increased from 4.4 to 5.4, the amount of protein bound to the collagen is increased significantly. The contribution of protons to the linkage free energy of binding ($\Delta\Delta G^{\circ}_{\text{binding}} = \Delta G^{\circ}_{\text{pH 5.4}} - \Delta G^{\circ}_{\text{pH 4.4}}$) could be estimated from the data in Table 2 as ~1.35 kcal mol^{-1}. Ionic strength appears to

Table 2 Binding strength of unfractionated rhBMP-2 to collagen

pH	NaCl (mM)	ν or ν_T[a] (μg/mg)	K_b or K_{b1}[b] (M^{-1})	App $\Delta G_1{}^{\circ c}$ (kcal mol^{-1})	K_{b2}[d] (M^{-1})	App $\Delta G_2{}^{\circ c}$ (kcal mol^{-1})
4.4	None added	80	6.6×10^3	−5.19	ND[e]	–
4.4	12	170	5.4×10^3	−5.08	ND	–
4.4	25	140	6.3×10^3	−5.18	ND	–
4.9	None added	110	2.4×10^4	−5.94	ND	–
4.9	12	260	2.6×10^4	−6.02	8.4×10^3	−5.35
4.9	25	260	2.3×10^4	−5.95	1.0×10^4	−5.45
5.4	None added	150	6.3×10^4	−6.54	1.2×10^4	−5.56
5.4	12	260	4.3×10^4	−6.32	8.1×10^3	−5.33
5.4	25	260	7.8×10^4	−6.67	9.0×10^3	−5.39

[a]ν_T is the total number of binding sites per unit of substrate (collagen) for biphasic binding (where two or more modes of binding are observed by a nonlinear Scatchard plot). Comparable to ν for simple binding (determined from the *x*-intercept of Scatchard plots where one mode of binding is observed as a linear Scatchard plot)

[b]K_b or K_{b1} is the equilibrium binding constant of rhBMP-2 to collagen matrix determined from slope of linear trend line of Scatchard plots

[c]$\Delta G_1{}^{\circ}$ and $\Delta G_2{}^{\circ}$ are the apparent standard free energies for observed monophasic and biphasic observed interaction between rhBMP-2 and collagen matrix at 25 °C, where $\Delta G^{\circ} = -RT \ln K_b$

[d]K_{b2}, equilibrium binding constant determined from trend line of final portion of data in a nonlinear Scatchard plot

[e]ND represents those Scatchard plots that were linear, and therefore biphasic interaction was not detected. Reprinted from Morin et al. [50], with permission of the copyright holder, The American Chemical Society

have only a minor effect on the linkage free energy of interaction, accounting for approximately 0.3 kcal mol^{-1}. The thermodynamic approach in this analysis could be applicable to assess formulation parameters for drug products delivered by vehicles such as a collagen matrix or other biomaterials. Modulation of the binding interactions could help in finding the conditions that provide the optimal release profile for a given indication.

4.2 Silk Protein Scaffolds for Biomedical Applications: Sustain Release of Monoclonal Antibodies

The successful development of therapeutic drug products needs a multidisciplinary approach in order to provide the best options to patients and caregivers. One key factor is the development of versatile functional materials that can interact with and within biological systems for stabilization, improved pharmacokinetic, or sustained release. Those materials can be found in nature or synthesized in the laboratory in the form of synthetic polymers, ceramics, and metals [37]. Within the many naturally available materials, we can find the silk produced by silkworms and spiders. Because of the characteristics of silk protein, several reports have been published recently using silk in a variety of biomedical applications including tissue and organ regeneration and cell adhesion [2, 86]. As members of the fibrous protein family, silks are strong materials with good mechanical strength that have been used as suture in wounds [2]. The silk proteins have excellent biocompatibility and minimal or no immunogenicity [44, 47] and controllable biodegradability [89, 90]. Fibroin silk protein could be converted into a variety of physical assemblies including fibrous scaffolds, films, membranes, hydrogels, sponges, nanofibers, and microfibers [1, 22, 83, 88]. Because of the highly desirable properties of the silk material, there are already products in the market based on silk materials such as Seri® Surgical Scaffold, from Allergan, MA. A resorbable matrix of fibroin yarns functionalized with RGD cell-binding domains is approved for anterior cruciate ligament repair. In the area of gene therapy, recombinant silk analogs have found applications to deliver plasmid DNA offering advantages over liposomes and synthetic polymers due to the ability of the silk protein to be modified [54].

The 2 main proteins that comprise silkworm silk, namely, fibroin and sericin, consist of 18 different amino acids with abundance of glycine, alanine, and serine; spider silk primarily contains glycine and alanine-enriched fibroin protein [34, 48, 101]. Silk fibroin consists of disulfide bound heavy and light chains of ~370 kDa and ~25 kDa, respectively [32]. Chemical modifications can be done to the silk protein to improve the existing properties or tailor to specific interactions with a given protein. For example, covalent decoration of silk films with integrin recognition sequences (RGD) as well as parathyroid hormone (PTH, 1–34 amino acids) and a modified PTH 1–34 (mPTH) involved in the induction of bone formation has been obtained [72]. Osteoblast-like cell adhesion was significantly increased on RGD

and PTH compared to plastic, mPTH, and the control peptide RAD [72]. The section below describes the development of silk-based lyogels as a potential scaffold for sustained delivery of monoclonal antibodies.

Silk Lyogels

Maintaining stability of proteins during encapsulation in a drug delivery scaffold has presented some challenges due to use of high shear stress procedures (sonication) or the use of organic solvents [63, 64]. In addition, the potential unfavorable contacts of the protein/polymers (hydrophobicity) could lead to faster degradation of the encapsulated protein [9, 71]. A lack of protein polymer compatibility leads to stability problems of the protein during storage or under in vivo release conditions. For example, hydration and degradation of microspheres made by poly D,L-lactide-co-glycolide (PLGA) are prerequisites for the release of protein during bio-erosion phase; this results in an acidic microenvironment (due to formation of lactic and glycolic acids) resulting in sufficiently low pH, which can lead to denaturation of proteins [71]. Other important aspect to consider in selecting the right delivery system is the ability to maximize the encapsulation efficiency of the desired protein and enable scale-up for large-scale manufacturing. Also, the method of encapsulation should be such that by manipulating the formulation conditions, different types of release profiles of the encapsulated material can be produced. Taking in consideration the above requirements, a procedure has been developed to prepare freeze/dry silk gels (silk lyogels) with the ability to be loaded with proteins for local delivery [29, 30].

Silk is a material that is naturally capable of creating a physically cross-linked hydrogels [29]. Those hydrogels could be created by simply sonicating the silk fibroin protein to facilitate cross-linking and then leaving the material to form a gel in the presence of the desired protein that is added after the sonication procedure, minimizing in that way potential degradation of the loaded protein (Fig. 5). Briefly, the silk hydrogels and lyogels were prepared by sonication of different concentrations of silk fibroin solutions according to the procedure of Guziewicz et al. [29]. Sonication was performed in 8 ml of silk fibroin solution. Sonication power was adjusted for each concentration from 20% to 65% amplitude to achieve a sol-gel transition within 2 hours. The sonicated solutions were cooled in a room temperature water bath for 1 minute after each sonication step, 30 and 10 seconds, sequentially. Single hydrogel pellets were prepared by placing 0.2 ml sonicated fibroin solution into a 96-well plate. The plate was allowed to sit at room temperature uncovered until successful sol-gel transition, in which the solution turned opaque and water droplets appeared on the surface. For hydrogels and lyogels containing antibody, lyophilized antibody was added to the sonicated solutions to a target concentration of 5 mg/ml. The solution was gently inverted to promote homogeneity and dissolution of lyophilized antibody in the solution. After sufficient mixing, the solution was transferred to 96-well plates and allowed to gel. The lyogels were prepared by lyophilizing

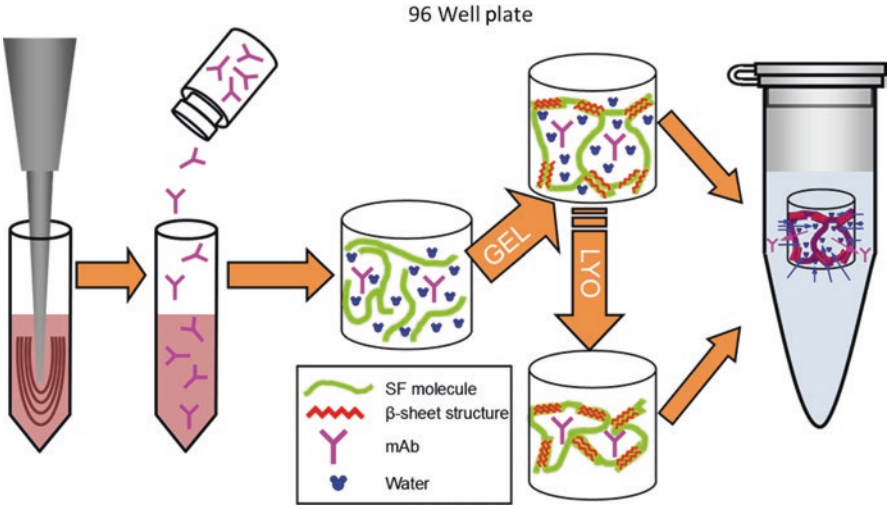

Fig. 5 Schematic for the preparation of silk lyogels from hydrogels

the hydrogels in the 96-well plates as described previously [29]. The process of lyophilization did not significantly impact mechanical properties of the construct, as comparable values were observed in rehydrated lyogels and the parent hydrogel constructs [29]. The mechanical properties of silk lyogels also compared favorably to those of other sponge-like materials typically used for tissue engineering and drug delivery [29]. The rigid nature of the lyogels is highly desirable as it would facilitate potential handling and implantation.

Silk lyogels and hydrogels ranging from 3.2% (w/v) to 12.4% (w/v), loaded with 1 mg antibody at 5 mg/ml, were incubated at 37 °C in 10 mM sodium phosphate buffer, 150 mM NaCl, and pH 7.4 (PBS). Collected release medium was assay for antibody concentration [29]. As shown in Fig. 6a, antibody release from hydrogels reached a plateau within 11 days at all silk concentrations. Approximately 80% of the antibody was released from the lowest silk concentration (3.2% w/v) hydrogels. Cumulative antibody release decreased to 67.5%, 52.5%, and 46.6% for 6.2% (w/v), 9.2% (w/v), and 12.4% (w/v) hydrogels, respectively. The antibody release data was normalized using cumulative release at day 26 as a reference (Fig. 6b). Decreased rates were observed as function of increasing silk concentration. Antibody release was also evaluated from lyogels, and a significant decrease in the rate of release was observed when compared with hydrogels. Over 38 days, only 14.8% of the antibody was released from 6.2% (w/v) silk lyogels during the same period (Fig. 7a). The normalized release data (Fig. 7b) show that a release plateau was not achieved in 6.2% (w/v), 9.2% (w/v), and 12.4% (w/v) silk lyogels. Antibody release profiles from the silk lyogels demonstrated the role of hydration resistance in altering silk-antibody interactions, leading to a significantly altered sustained release profile when compared to hydrogels [29].

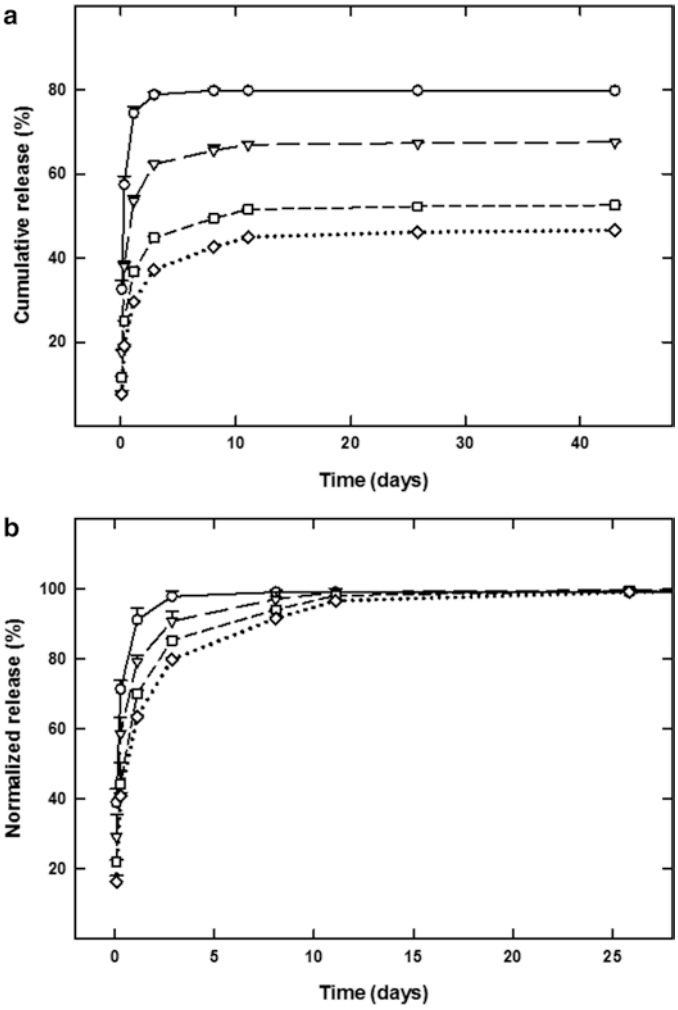

Fig. 6 Antibody release from sonication-induced hydrogels at varying silk concentrations: 3.2% (circles, solid line), 6.2% (triangles, long dashed line), 9.2% (square, short dashed line), and 12.4% (diamond, dotted line). (**a**) Cumulative release as a function of time. (**b**) Cumulative release normalized to 100% at the release plateau from day 25 to day 43. Where error bars are not visible, they fall within the symbols. Lines were added as a visual aid. (Reprinted from Guziewicz et al. [29], with permission of the copyright holder, Elsevier)

Water is primarily excluded from the hydrophobic silk II (β-sheet) secondary structure [43]. Removal of water during lyophilization increased the interactions between silk and antibody molecules [29]. These enhanced interactions are expected to decrease release rates for untrapped antibody and/or increase trapped antibody. Hence, based on these observations, silk lyogels could be prepared in two forms:

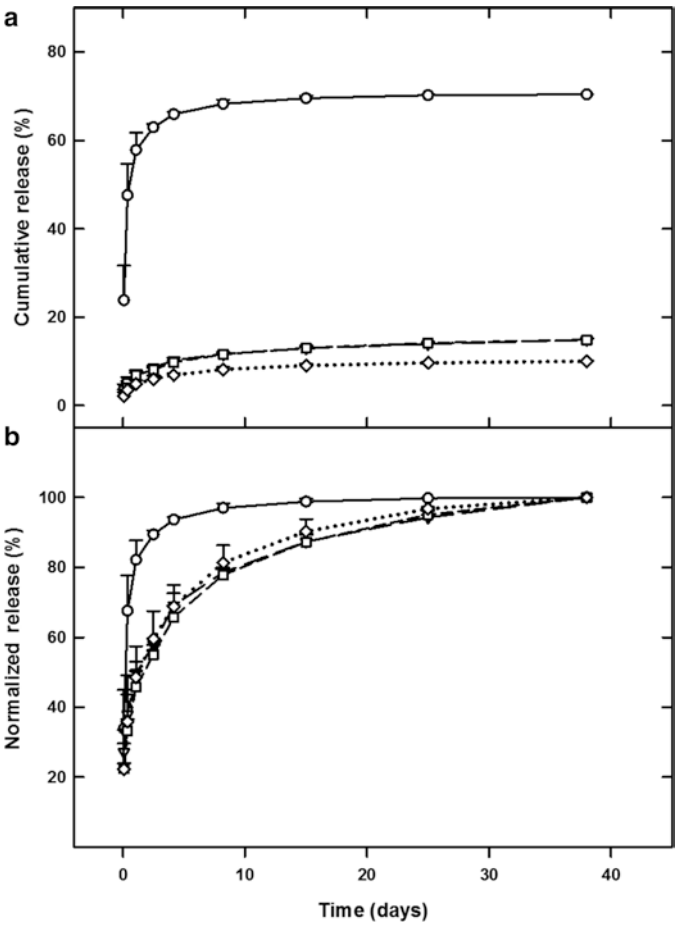

Fig. 7 Antibody release from lyogels at varying silk concentrations, 3.2% (circles, solid line), 6.2% (triangles, long dashed line), 9.2% (squares, short dashed line), and 12.4% (diamonds, dotted line). (**a**) Cumulative release as function of time. (**b**) Cumulative release normalized to 100 at day 38. Where error bars are not visible, they fall within the symbols. Lines were added as a visual aid. (Reprinted from Guziewicz et al. [29], with permission of the copyright holder, Elsevier)

(1) low density β-sheet networks (LDBN) and (2) high density β-sheet networks (HDBN). This could be one way to control the release profile of antibodies from lyogels. Limited solvent penetration and availability for the disruption of silk-antibody hydrophobic interactions could allow creating better lyogels for diverse applications in protein sustain release by introduction of excipients that could modulate the critical antibody-silk interactions. Also a combination of formulation and chemical modification and/or engineering of the silk protein [54] could produce suitable materials that could allow improving sustain release by modulating hydrophobic and hydrophilic interactions of lyogels with the protein of interest.

5 Summary

The quality target product profile (QTPP) is a key element that needs to be defined early in the development cycle in order to have a successful drug-device combination product [61]. All the elements need to be evaluated in a holistic way, meaning the active molecule (and its physical and chemical limitations), the primary container, and the formulation. The design space for the QTPP could be impacted by the properties of materials (extractable and leachable from drug delivery device), where product contacts are made. This potential interaction over time (stability) can possibly alter the efficacy of the drug and sterility, reflected in physical and/or chemical instabilities (aggregation, precipitation, deamidation, cleavages, etc), which in turn lowers the efficacy of the drug product for therapeutic effect.

Deep understanding of the root cause of oligomerization can permit the design of mitigation strategies. Proteins are dynamic entities, constantly sampling different partially folded states as a function of temperature and other solution variables. These variables dictate the standard free energy between the native, unfolded, and partially folded "aggregation"-prone states leading to oligomerization. Because of this, not all oligomerization events in proteins are similar. Altering the variables, such as temperature, pH, salt, and ligands, could induce a protein to "aggregate" as a consequence of "unnatural folding" to balance the thermodynamically unfavorable interactions between solvent and exposed hydrophobic residues in proteins. By the same token, these same variables may induce a protein to self-associate, in mostly the native state, to counteract the unfavorable interactions with the solvent. Hence, "aggregation" and reversible self-association could both lead to turbidity, opalescence, phase separation, and precipitation. However, by proper analysis of thermodynamic binding data, the formulation scientist can distinguish between "aggregation" and self-association and if those events are rather induced, mediated, or facilitated by ligands.

Depending on the system chosen to deliver a therapeutic protein, an understanding of the interactions and impact of the delivery system on the physical and chemical properties of the protein is condition sine qua non. Thus, equilibrium binding measurements to obtain relevant thermodynamic parameters, under different solution conditions, could provide insights into the nature of the protein delivery system interaction and its modulation.

Using a monoclonal antibody as a pharmaceutically relevant model protein, sustained release from silk hydrogels was evaluated in comparison with a novel sustained delivery matrix prepared by lyophilization of hydrogels, namely, lyogel. Antibody release rates were substantially decreased in the lyogel compared to the parent hydrogel material. Based on a combination of type of excipients employed during the preparation of the lyogels and engineering of the silk protein [51, 55], it could be possible to prepare better biomaterials for optimal sustain release.

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Chapter 4
Production Strategies and Challenges with IgG-Based Bispecific Ab Formats

Chen Gu and Diego Ellerman

1 Introduction

Unlike regular monoclonal antibodies which are directed to a single protein, bispecific antibodies are, as the name reveals, directed against to different antigens or epitopes on the same protein. This difference allows combining two therapeutic agents into one molecule which results in a simplified development path in some cases. More importantly, the physical connection of two binding domains with different specificities enable a wide array of mechanisms of actions not accessible to monoclonal antibodies [1]. Among the most widely used applications of bispecific antibodies are redirecting the cytotoxic activity of T cells for oncology and infectious diseases, the crossing of the blood-brain barrier for neurological indications, and a more tissue-specific antibody delivery for agents that may have systemic toxicity [2]. While some of these applications are still in preclinical or clinical development, two bispecific Abs have been approved for commercialization and are showing positive results in patients. Blinatumomab (Blincyto®) is a T-cell engager that targets CD3 on T cells and CD19 on B cells for the treatment of B-cell malignancies [3, 4], and Hemlibra® is a molecule that mimics the coagulation factor FVIII by bridging together FIX and FXa [5, 6]. Blincyto® has shown overall high response rates in different phase III clinical trials [7]. In turn, Hemlibra® has been recently reported to dramatically reduce the number of bleeding in patients with hemophilia [8]. Because of the good performance of the marketed bispecific antibodies as well as the new therapeutic approaches they offer, bispecific Abs are becoming a major component in the biotherapeutic arsenal for a wide array of indications. A critical hurdle that had to be solved before bispecific Abs could transition from research

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tools to become drug candidates was the development of robust production processes that enable the industrial production to support clinical trials [9]. Different strategies relying on distinct antibody engineering solutions have been devised to generate bispecific antibodies in a scalable fashion. The most common Ab formats are based on antibody fragments that are connected by linkers (like Blincyto®) or use two sets of engineered Fc fragments that drive the formation of heterodimers (like in the case of Hemlibra®) [1, 10, 11]. Each of these two main groups of formats are highly diverse, containing a number of different variants [10]. Although the small size of fragment-based Ab formats confers them advantages in some applications that require fast clearance from circulation, the long half-life of antibody formats containing the Fc fragments is preferred for numerous applications. In addition, the Fc fragment can be engineered to possess or not effector functions depending on the requirements of the specific application. A variety of Ab formats based on the structure of an IgG have been developed with different mindsets: some approaches prioritize keeping the architecture of the bispecific as closely as possible to a natural IgG, while others put more emphasis on a simplified production process. In this chapter we discuss how the design of some of the most commonly used IgG-based antibody formats impacts the production process and anticipates potential challenges associated with them.

2 Overview of General Strategies to Produce IgG-Like Bispecific Antibodies

Initial attempts to make bispecific Abs used the fusion of two different hybridomas to generate a quadroma [12–14]. Because the resulting quadroma expressed two different heavy chains (HCs) and two different light chains (LCs) that could associate with different combinations, an important challenge was to purify the correctly assembled bispecific Ab from the collection of other non-cognate species. For many years this was the main hurdle preventing the advancement of bispecific Abs into clinical trials [9]. As noted above, different solutions to overcome the initial limitation have been developed [1, 10, 11], and bispecific antibodies are progressing into the clinic. However, evolution of the different fundamental technologies continues, in part fueled by the goal of enabling a simplified, more robust production process. Three main strategies for designing IgG-based antibody formats can be distinguished: (a) antibody formats where the two different specificities are encoded by one single heavy chain (HC) and one single light chain (LC), (b) antibody formats consisting of three chains (either two HCs and one common LC, one HC and two LCs, or one HC, one LC, and one hybrid chain) but containing engineered features that either reduce the formation of mispaired species or simplify their removal, and (c) antibody formats composed of four different chains also containing engineered mutations for the reduced formation or simplified removal of mispaired species. Representative Ab formats for each case and the implications on the production process are indicated in Fig. 1. Bispecific Abs composed of one HC and one LC have a

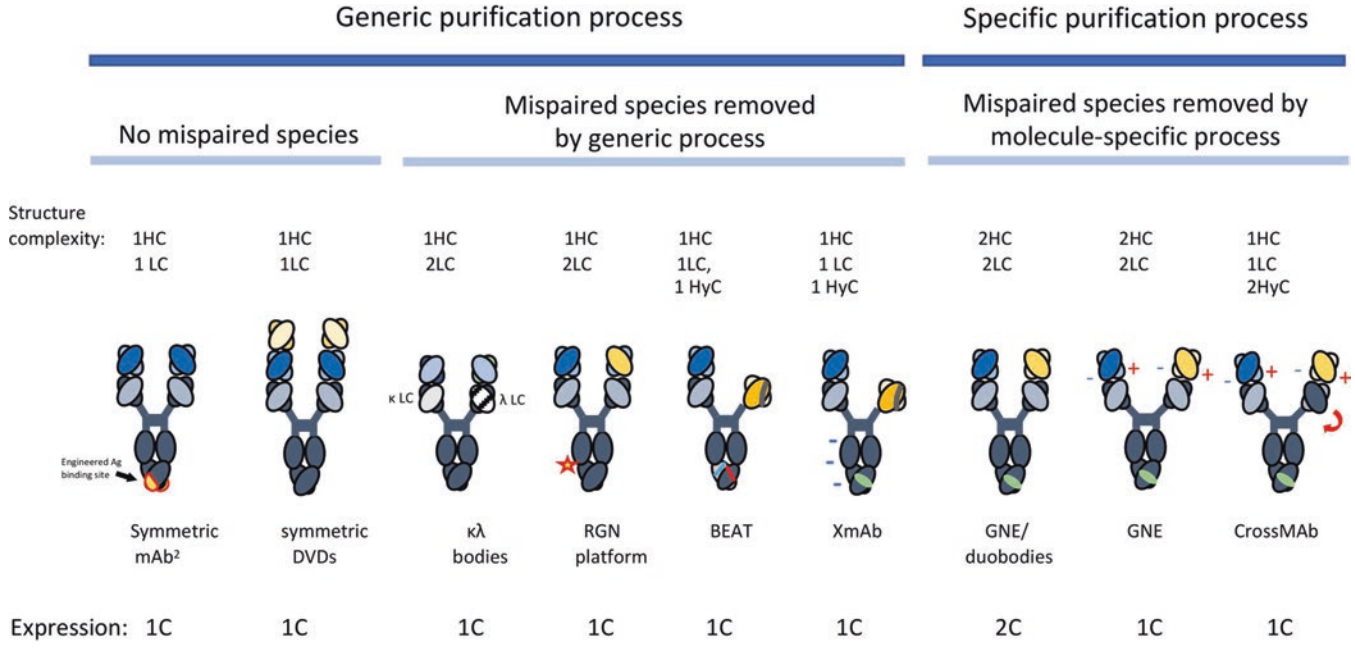

Fig. 1 Selected bispecific Ab formats grouped according to the complexity of the purification process. The complexity of the structure of each Ab format is indicated: HC, heavy chain; LC, light chain; HyC, HC/LC hybrid chain. Exemplary Ab formats consisting of one HC and one LC that can be purified with standard mAb process include mAb^{2TM} (Fcab indicated by the yellow cap) and symmetric dual-variable-domain Abs (DVDs). Representing the group of Ab formats that may form mispaired species but that can be removed by generic purification processes are the κλ body, BEAT®, XmAb®, and the platform developed by Regeneron. The different LC used in the κλ body are indicated (κLC or λLC). The star represents the "star substitution" in Regeneron's platform. The red and blue stripes represent the grafted interface from the α and β chains of the TCR in the BEAT® platform, whereas the pink oval represents the IgG3 C_H3 domain. The green oval represents the engineered C_H3 interface in XmAb® and other platforms. The "−" symbolizes charge mutations introduced to enable purification by cation exchange chromatography. The group of Ab formats that may form mispaired species that require molecule-specific processes for removal of unwanted products is represented by formats used by DuoBody Abs format, CrossMAb™, and the formats used by Genentech (GNE) and Chugai. The green oval represents an engineered C_H3 interface, the "+" and "−" charge mutations, and the arrow of the swapping of domains creating two hybrid chains in the CrossMAb™ format. The row at the bottom indicated where each format enables expression in a two-cell system (2C) or single-cell system (1C)

reduced likelihood of chain mispairing and thus can be produced, in general terms, as regular antibodies. On the contrary, the presence of either two LCs or two HCs in the other Ab formats raises the possibility of mispaired species which results in a range of increased complexity in the production processes. A detailed discussion of the structural and engineering aspects of the different Ab formats can be found in excellent reviews on the subject [1, 11]. Although we have grouped the different Ab formats according to their convenience for manufacturing, that does not imply that it is the only factor relevant for selecting an Ab format for a given application. Besides the obvious freedom to operate without infringing intellectual property, other considerations like the requirement of symmetric or asymmetric Ab formats for specific applications and potential immunogenicity risks must be taken into account [2]. In the sections below, we describe some of the experiences and challenges that have been reported in the development of different IgG-based Ab formats.

3 Bispecific Ab Formats Composed of One HC and One LC

3.1 Mab²™

This Ab format was developed by F-star, and unlike most of the other Ab formats, it adds the second specificity by recruiting it onto the C_H3 domain of the Fc fragment. Loop regions at the C-terminal tip of the molecule can be diversified to bestow binding to the desired target. The resulting modified Fc has been dubbed Fcab for *Fc* with *a*ntigen *b*inding properties [15]. When a specific Fcab is grafted onto a regular Ab, it results on a bispecific symmetric molecule, bivalent for each specificity. A number of Fcabs with specificities for HER2 [15], integrin $\alpha_5\beta_3$ [16], and VEGF [17] have been described, but the most advanced in terms of clinical development is a bispecific Ab called FS118, consisting of a Fcab specific to LAG3 and Fabs binding PDL1, currently in phase I clinical trials. What makes this Ab format attractive is the potentially simplified manufacturing process enabled by the lack of HC or LC mispaired species. Absent other complications intrinsic to the format, a simplified process not only reduces costs and development time but also streamlines transferring the production process to a contract manufacturing organization (CMO). This is a critical aspect for companies without an integrated manufacturing facility and a convenient attribute for bigger companies that outsource part of their production. In fact, FS118 is being produced by CMC Biologics, a CMO. Although no publications are yet available describing the manufacturing scale production of Mab²™, challenges may potentially arise derived from the modified C_H3 domains. The process of diversifying loops in the C_H3 domain may lead to a destabilized protein structure with reduced thermal stability [16, 18]. An example where the reduced thermal stability of a Fcab against HER2 was improved by directed evolution has been reported by Traxlmayr et al. [19] However, in cases where only a partial recovery of the thermostability is possible, it could eventually lead to challenges for formulation groups.

In terms of how different applications may fit or affect the utilization of Mab^{2TM}, it is relevant to comment on the bivalent or monovalent binding ability of the Fcab. The Mab^{2TM} format is symmetric; therefore it has two binding sites per Fc. Because the two binding sites on a Fcab are closer to each other and are more rigid than the binding sites on two Fabs, it is possible that for Mab^{2TM} against large antigens, the position of the epitope could be critical for avoiding steric hindrance and enable bivalent binding. This could be a limitation of the format for some applications where avidity is important. On the other hand, some applications like crossing the blood-brain barrier through binding to the transferrin receptor (TfR) and T-cell engagers may require monovalent binding [2]. For those applications the Mab^{2TM} format would not be the preferred one unless used in an asymmetric version that ensures monovalent binding to either TfR or CD3. Although that is certainly possible, the asymmetric version would not carry the advantages of a simplified manufacturing process of the symmetric form described here.

4 Bispecific Ab Formats Composed of Three Chains

4.1 κλ Body

This Ab format was described by researchers in Novimmune in 2015 [20]. Earlier approaches to produce bispecific Abs resorted to the use of common LCs to solve the mispairing between LCs and HCs while using heterodimeric C_H3 domains to direct the preferential formation of HC heterodimers [21]. In a related approach, κλ body utilize a common heavy chain to prevent the formation of HC mispaired species. Unlike other approaches however, the κλ body platform does not utilize engineering to drive the association of the cognate HC-LC pairings but instead relies on purification to remove the undesired monospecific products. In addition to each parental Ab using the same HC, one of them is selected to contain a kappa LC (κLC) and the other Ab a lambda LC (λLC). The co-expression of all three chains leads to the formation of a mixture of species containing the bispecific molecule as well as IgGs with either two κLCs or two λLCs. Subsequent purification steps with resins that can specifically bind to κLC (Kappa select) or λLC (Lambda Fab select) remove the undesired products (Fig. 2). In a panel of 46 different κλ body that were analyzed by transient transfection, the percentage of bispecific antibody obtained ranged between 11% and 51% [20]. A subsequent report indicated that in over 300 κλ body analyzed at research scale, the bispecific species constituted the most abundant species, but occasionally antibodies could show low percentage of bispecific content [22]. In a case study of a κλ body with unbalanced expression of the LCs leading to a low (~20%) percentage of bispecific Ab formation, Magistrelli et al. first tried to improve the expression of the limiting chain (κLC). Codon optimization improved the expression of the κLC, but it remained several folds lower than the level of the λLC, and thus the λLC monospecific IgG remained the most abundant species produced. The opposite strategy of reducing the expression of the λLC

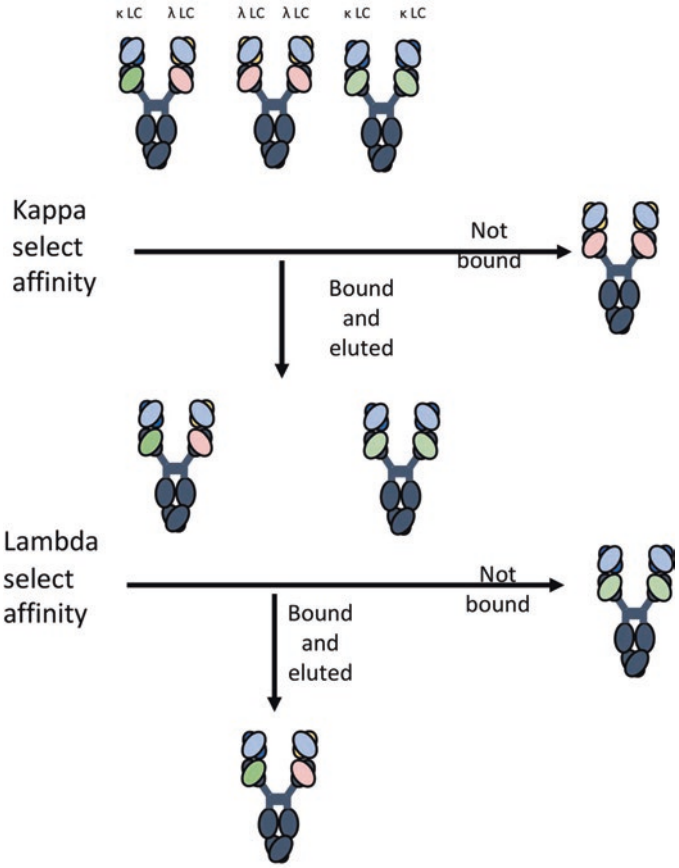

Fig. 2 Purification strategy of κλ body. The bispecific κλ body is represented along with the two monospecific IgGs (IgG κLC, IgG λLC) formed during co-expression of the common HC, the κ LC, and the λ LC. The first step, protein A chromatography, does not discriminate between the three forms. The following Kappa-Select step removes the λ LC monospecific IgGs, while the third step, Lambda Fab Select-based chromatography, removes the κ LC monospecific IgGs

proved instead more successful. Expression of the λLC was reduced to different levels by introducing codons less frequently used in CHO cells (codon deoptimization). The increasing deoptimization of codon usage led to a concomitant increase in the κ/λ ratio and the percentage of κλ body formed, reaching 42% for the best variant. The variant with the highest degree of codon deoptimization however led to an excess of κLC that impacted the formation of bispecific IgG. Importantly, the increase in bispecific Ab formation in transient transfection by the combined codon optimization of the κLC and deoptimization of λ LC was also observed in stable pools. Thus, κλ body with poor yields due to unbalanced expression of the LCs but showing unique biological activities could be advanced into development by fine-tuning codon use. With regard to developing this platform to a manufacturing process, Fischer et al. provided examples of the performance in cell line development

using two different κλ body. Five stable transfected clones for one κλ body and two clones for the second κλ body were grown in fed-batch cultures showing titers above 1.5 g/L in most of the cases. Although a substantial variation in the distribution of the monospecific IgGs between different clones was observed (~35–60%), the percentage of κλ body was less variable (~33–47%). For one of the two κλ body, the stability of cell lines was assessed over 50 generations, with no variations observed in the distribution of the bispecific/monospecific ratio or in the titer. Downstream process was evaluated at 100 L scale for one of the κλ body: 2 g/L titer was achieved at harvest with a 45% content of bispecific Ab, 42% of monospecific κLC, and 13% of monospecific λLC. Because in this case the monospecific κLC was the most abundant undesired product, a Lambda-select chromatographic step that bound the bispecific antibody and let the κLC monospecific Ab flow through was placed after the initial protein A capture, followed by the Kappa-select step. As reported by Fischer et al., each chromatographic step selective for the LC isotypes achieved complete removal of the corresponding monospecific species to render a final product with undetectable product-related contaminants [20]. The specific recovery of the κλ body in each step was ~90%; however the final yield of the process was 35% due to the fact that the κλ body were initially 45% of the total IgG. The purified κλ body was then used in stability studies. In regard to the biophysical properties of κλ body, thermostability studies indicated domain unfolding at temperatures comparable to a regular mAb, probably owing to the lack of engineering in the framework of κλ body. Also, colloidal stability studies showed a κλ body had good stability at 10 mg/mL in a standard buffer for 3 months. In summary, the κλ body platform utilizes a generalized, simple process for production and generates bispecific molecules free of non-native sequence that could increase the risk of immunogenicity. The simplified production process however comes at the price of potentially higher cost of goods than other processes due to the lower yields of bispecific Ab and the use of less cost-effective resins. Currently there are many κλ body in preclinical studies [23, 24], but none has advanced to clinical trials yet.

4.2 BEAT® and Regeneron's Platform

These two formats are composed of three chains and have in common that rely on differential binding to protein A to remove small amounts of HC homodimers formed during production.

BEAT® (*B*ispecific *E*ngagement by *A*ntibodies Based on the *T*-Cell Receptor)

BEAT® is a platform developed by Glenmark that is based on heterodimerization regions of the T-cell receptor. According to researchers that developed this format, the T-cell receptor has structural homology with an IgG; thus fragments of the α and β chains of the TCR can be exchanged with those from an IgG preserving the

domain structure. In particular, those sequences responsible for the heterodimerization of the TCR can be grafted onto the C_H3 domain to drive its heterodimerization. Testing different engineered constructs, Skegro et al. identified mutations that drove the formation of a heterodimeric Fc with ~94% efficiency [25]. As mentioned before for the Mab^{2TM} platform, modifications in the structure of the C_H3 domain may lead to a reduction of the thermostability of the molecule. In the case of BEAT®, the authors reported a melting temperature of 70 °C for the modified structure, which is below the melting temperature of the wild-type C_H3 (~82 °C) of human IgG1. Because unfolding of the C_H3 domain is the driving force for aggregation in acidic conditions [26, 27], this reduced thermal stability could be a liability for long-term storage stability. At present no specific study of this aspect of the BEAT® platform has been reported. To reduce the risk of LC-HC mispairing, the BEAT® format uses a Fab × scFv Ab format. Because the V_H and V_L domains are connected in a single chain (scFv), their association is greatly favored, preventing major LC-HC mispairing. In regard to the engineered feature that facilitates purification of the bispecific molecule from HC homodimers, BEAT® borrows the sequence of human IgG3 for one of the Fc domains. IgG3 is an isotype that does not bind to protein A; thus IgG3/IgG3 homodimers do not bind to protein A, whereas IgG1/IgG3 and IgG1/IgG1 bind to protein A but with different affinities. Using a pH step elution method, separation of the different component was achieved: a first step at pH 5 washed any unbound IgG3/IgG3 species, and a second step at pH 4.1 eluted mainly the IgG3/IgG1 heterodimer, while the IgG1/IgG1 homodimers eluted at pH 3 (Fig. 3). The efficiency of this process at industrial scale or any associated challenges has not been reported, but Glenmark has two BEAT® molecules in phase one clinical trials: GBR 1302 (CD3 × HER2) and GBR 1342 (CD3 × CD38). As discussed below for other formats, scFvs are often prone to dissociation and intermolecular association leading to the formation of aggregates. Different strategies are available to reduce this liability including the introduction of a stabilizing disulfide bond, grafting the CDRs onto a stable framework, CDR engineering [28], or by swapping kappa and lambda framework regions [29]. It is not clear however whether the BEAT® platform utilizes any of these strategies to mitigate a potential unstable scFv domain.

Regeneron's Platform

This Ab format is similar to BEAT® in the shared use of differential binding to protein A to remove HC homodimers. Unlike BEAT®, this Ab format relies on a common light chain to solve the HC-LC association problem. Different strategies are available to generate bispecific Abs with a common LC, for example, taking the LC of Ab A to generate a library with a diversified HC to screen for binders to antigen B. An alternative approach is to raise Abs against antigens A and B in transgenic mice expressing a fixed human LC but allowing recombination in the variable domain of the heavy chain. Another difference with BEAT® is that Regeneron's platform does not use any C_H3 interface engineering to drive the formation of heterodimers. The lack of enforcement of the association of the HCs into the bispecific

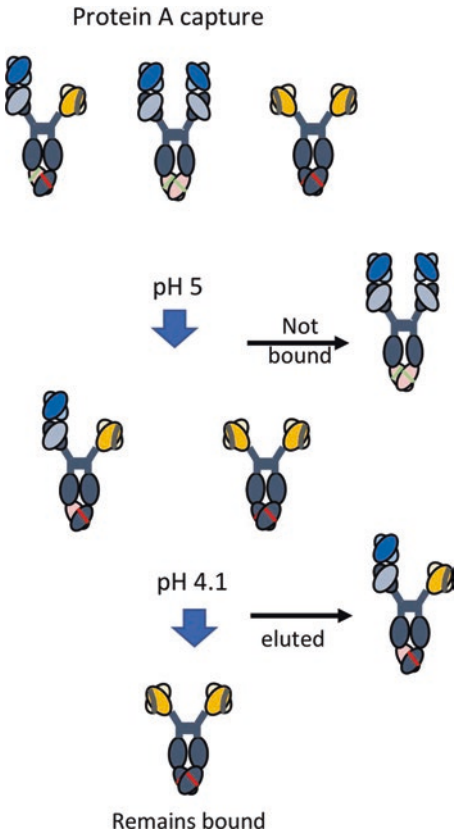

Fig. 3 Purification strategy of BEATs. The pink ovals in BEATs represent the C_H3 domains with IgG3 sequences. The red and green stripes represent the α and β chains of the TCR grafted in the CH3 domains. After loading, protein A is washed at pH 5 to remove any unbound IgG3/IgG3 homodimers. Step elution at pH 4.1 dissociates IgG1/IgG3 heterodimers from the column, while IgG1/IgG1 dimers remain bound

molecule results in the production of ~50% of undesired material. In this regard, this platform shares with κλ body the reduction in titers of the bispecific molecule with its potential impact on the cost of the final product. Unlike BEAT® which uses the entire IgG3 C_H3 domain, Regeneron's platform introduces only two amino acid substitutions from IgG3, $H_{435}R$ and $Y_{436}F$ [30], which were previously known to ablate IgG1 ability to bind protein A [31]. The process of purifying HC homodimers using a pH step elution after capture with protein A presented in this case some hurdles. Because resins based on recombinant staphylococcal protein A (SpA) are able to bind the Fab of some Abs (i.e., V_H3 framework), the process initially worked only on a subset of bispecific Abs and required additional purification steps for Abs containing V_H3 domains [63]. Because almost half of the germline V_H genes belong to the V_H3 family, the initial problem would have impacted potentially a large number of molecules. Out of the five domains in SpA, only two (domains D and E) can bind the V_H domain, whereas all domains bind the Fc domain [32–34]. Therefore, resins based on minimalized version of SpA lacking domains D and E, such as MabSelect SuRe, could enable separation of heterodimers from homodimers of IgGs containing the V_H3 framework. However, Tustian et al. reported that the original

version of that resin was made on a matrix with large particle size and pores of small diameter that did not enable efficient separation of the different species [35]. The subsequent development of an improved resin, named MabSelect SuRe pcc, allowed the separation of the bispecific Ab from homodimers in a single step [36]. The process was tested at a clinical manufacturing scale (GMP, 2000 L reactor) with purity of the final product >98% [36]. Because the BEAT® platform described above also utilizes differential binding to protein A, it is expected that it also requires a careful selection of protein A resin to maximize resolution of the different species. In the initial publication by Skegro et al. [25], 1 mL MabSelect Sure columns were used, but likely for manufacturing scale processes, the use of MabSelect SuRe pcc or with similar properties could be beneficial.

4.3 XmAb® Platform

This Ab platform is similar to BEAT® in the sense that they use a Fab × scFv format and a heterodimeric Fc to drive the formation of bispecific Ab. However, they differ in the nature of the mutations at the C_H3 interface and the strategy for removing the HC homodimers. The XmAb® platform was developed with the aim of achieving a structure with high thermal stability properties. Thus, only isovolumetric changes were introduced at the C_H3 interface to minimize perturbations in the structure. The selected mutations enabled the formation of heterodimeric Fc with ~95% efficiency, and the resulting Fc showed a melting temperature of ~77 °C, only ~6 °C below the melting temperature of the wild-type C_H3 of IgG1 [37]. In addition to mutations in the C_H3–C_H3 interface, the XmAb® platform also introduces charge mutations in positions that are solvent exposed to facilitate the purification of HC homodimers by ion exchange chromatography (IEX) (Fig. 4). One mutation in C_H1, one in C_H2, and three in C_H3 on the same HC introduce negative charges that led to differences in the pI between the homodimeric and the heterodimeric Fc (pI—6.0, 6.4, and 7.3). The platform was tested initially in the context of T-cell-engaging bispecific Abs where one arm targets CD3 on T cells and the other arms binds a tumor-associated antigen. For the T-cell-redirecting Abs, Xencor used a scFv anti-CD3 that was optimized for thermal stability to reduce the aggregation risk associated with this format. The anti-CD3 Ab was used in the high pI arm, and to further increase the pI difference with the other arm, it included a charged $(GKPGS)_4$ linker [37]. Six different constructs produced at a research scale were purified by CEX-rendering bispecific Abs with no homodimers detectable by analytical size exclusion (SEC) chromatography. The method was also tested at clinical production scale with a reported yield of ~60% and heterodimer content higher than 90% after the initial protein A purification step. A potential liability for this format is the immunogenicity risk derived from introducing solvent-exposed mutations to drive the differences in pI. Although the immunogenicity risk does not impact technical development, it could be a liability for clinical trials. In fact, Xencor has a number of XmAbs in

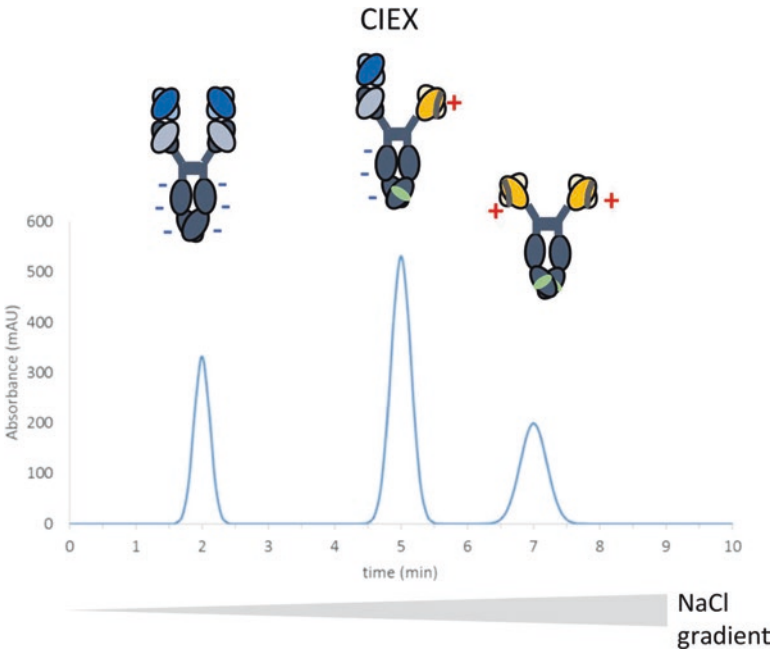

Fig. 4 Purification strategy of XmAbs. Mutations introducing negative charges in one of the Fc are indicated with the "−" symbols, while mutations introducing positive charges in the scFv linker is indicated with the "+" symbol. The bispecific antibody is separated from minor amounts of HC dimers using an IEX chromatography step with a salt gradient elution. Shown is a conceptual scheme of the strategy

phase I clinical trials, XmAb 13,676 (CD3 × CD20), XmAb 14,045 (CD3 × CD123), XmAb 18,087 (CD3 × Sstr2), and XmAb 20,717 (PD-1 × CTLA-4), so data on the potential incidence of anti-drug antibodies (ADAs) may be available in the near future.

4.4 ART-Ig

This is a bispecific Ab format developed by Chugai. It utilizes electrostatic steering to drive the heterodimerization of the Fc and a common LC, so the format enables expression of all three chains in a single cell. Importantly, Hemlibra®, the second bispecific Ab approved for commercialization of the two currently in the market, has been developed using this Ab format. The development of Hemlibra® is an interesting case of optimization of the lead candidate to solve different functional and developability challenges. First, the lead molecules showed faster clearance than expected. Analysis of the molecule indicated the presence of a positively charged

patch on the variable domain of the anti-FIXa arm [38]. Initial attempts to disrupt the patch by directly mutating the residues resulted in loss of activity, so an alternative strategy consisting in the introduction of negative charges in adjacent amino acids finally provided with a solution [38]. During development of the lead clone, it was also noticed that its solubility was poor, precipitating at concentrations between 4 and 40 mg/mL. To solve this problem, in addition to counterbalance the charge patch responsible for the poor pharmacokinetics (PK), additional hydrophobic residues in the variable domains were replaced by hydrophilic residues. Substitutions with positive effect were combined to render an antibody with good solubility at concentration >100 mg/mL. After the PK and solubility problems were solved, it was noticed that the pIs of the HCs of the lead antibodies targeting anti-FIX and anti-FX were so close that homodimeric species would not differ significantly to the bispecific molecules and therefore their removal by IEX would have been difficult. To overcome this challenge, Sampei et al. introduced mutations in the variable domains of each HC such that it resulted in differences of at least 0.5 units in pI [38]. The pI optimized version of the Ab could be resolved from minor HC mispaired species by cation exchange chromatography when expressed at a production scale (2500 L fermentation). It is unclear whether these mutations provide an ad hoc solution for the Hemlibra® Ab or, alternatively, they provide a generalized solution that can be adopted for a robust platform. A subsequent publication from Chugai on an anti-glypican3/anti-CD3 T-cell engager described a molecule-specific set of mutations to enhance the pI difference between the HCs [39], suggesting that mutations involved in the pI engineering are customized for each molecule. In the case of Hemlibra®, a number of mutations were introduced to overcome different problems related to activity and developability, thus increasing the risk of immunogenicity. However, Sampei et al. described that each mutation was analyzed using two different in silico software programs. Those mutations flagged as with increased immunogenicity risk in the in silico test were substituted by other options with lower scores in the analysis. Results recently disseminated from two phase III trials (HAVEN 3 (NCT02847637), HAVEN 4 (NCT03020160)) indicated that in addition to Hemlibra® being efficacious, no ADAs have been detected so far. Thus, a careful analysis of the immunogenicity of highly engineered molecules could be critical for the success in the clinic.

5 Bispecific Ab Formats Composed of Four Chains

Like every single Ab format, those composed by four chains have advantages and disadvantages. One advantage is the freedom to combine two different Abs originating from a wide variety of discovery platforms. On the other hand, both HC and LC can mispair increasing the number of potential unwanted products that need to be purified away or characterized. Two groups of formats can be distinguished based on whether they allow expression on a single cell or not.

5.1 Ab Formats Requiring Expression in Two Cells

With the exception of κλ body, all other Ab formats discussed so far contain solvent-exposed non-native sequences. One important advantage of Ab formats composed of four different chains that are expressed in two different cells is that the structure of the Ab does not contain any solvent-exposed nonnatural sequence that could be recognized by anti-drug antibodies (ADAs). These Ab formats do contain mutations in the C_H3–C_H3 interface that drive the heterodimerization of the Fc, but they are not solvent accessible and therefore not accessible to Abs either. While the non-native sequences contained in other Ab formats may or may not be immunogenic in humans, the absence or concealing of non-native sequences in the Ab formats described in this section minimizes the risk of immunogenicity. Genmab and Genentech have developed this type of Ab formats using different solutions to drive heterodimerization of the C_H3 domain. While Genentech uses the "knob into hole" mutations described by Ridgway [40], Genmab uses different mutations in the C_H3 domain to drive heterodimerization of the HCs as discussed below. An early approach taken by Genentech to produce this type of format was the co-culture of cells expressing the two distinct half antibodies. Initially this approach was developed in bacteria [41] and later on also implemented in mammalian cells [42]. As mentioned above in the section of κλ body, the balanced expression of all chains forming the bispecific antibody is important to maximize the yield while minimizing the complexity of product-related contaminants. Another advantage of the two-cell expression systems is that differences in the expression levels of the two antibodies can be easily compensated without the need to optimize codons or promoters. In the co-culture approach, the effect of differential expression levels of the two different half Abs could be countered by adjusting the ratio of the two different cultures such that it led to a balance protein levels of the two half antibodies [41]. The homogenization of the co-culture followed by protein A capture led to the efficient formation of the bispecific Ab with some excess of half Abs that could be removed by hydrophobic interaction chromatography (HIC). The generalized applicability of this procedure was shown on a panel of 27 different Ab combinations at different scales. An alternative approach to the *E. coli* co-culture is the independent expression and purification of each half Ab in either *E. coli* or mammalian hosts followed by an in vitro annealing step. This step consists basically in a reduction step to break the intra-chain disulfide bond between the two cysteines in the hinge region followed by annealing of the complementary half Abs and the reoxidation of the hinge disulfide (Fig. 5). A detailed study of annealing conditions identified temperatures, pH, and reduced glutathione concentrations that optimized the formation of the bispecific species minimizing the formation of aggregates [43]. Using the optimized reaction conditions, the process could be scaled up with an assembly efficiency of 90% in 6 hours [43]. The in vitro annealing of two half antibodies at large volumes may lead to the formation of a variety of impurities such as excess half antibodies, covalent and non-covalent knob/knob and hole/hole homodimers, and high molecular weight species. As described by Giese et al., the characterization

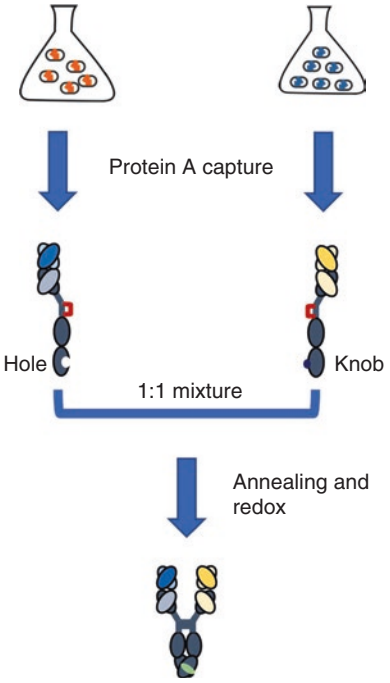

Fig. 5 In vitro annealing process. The "knob" and the "hole" half antibodies are expressed in independent cultures and purified through protein A chromatography. They are then incubated at a 1:1 molar ratio in vitro at pH ~8 in the presence of reduced glutathione. After >6H at 32 °C, annealing and reoxidation are complete

of chromatographic properties of the starting half Abs using a high-throughput screening allows for the rapid development of downstream processes for the removal of process-related impurities [44]. Using this approach on a test case, Giese et al. developed a three-column chromatography for a bispecifiic Ab with 64% yield and 99% purity [44]. The automated, high-throughput screening resin has been utilized subsequently to quickly develop a purification process for other bispecific Abs. As mentioned above, an advantage of the process using two cells and an in vitro annealing process is that it allows for a tight control of the equimolar amounts of each half antibody.

Similar to the Ab format developed by Genentech, DuoBody Abs is a class of bispecific antibody developed by researchers at Genmab. The production of this type of bispecific antibody utilizes a process called controlled Fab arm exchange (cFAE) to drive the correct chain pairing between the two antibodies [45]. This production process was developed based on a naturally occurring phenomenon of IgG4 subclass of antibodies, where IgG4 antibodies can exchange half molecules with other IgG4 antibodies generating antibodies with bispecificity in vivo [46–48]. In order to promote heterodimerization in the IgG1 backbone, corresponding point mutations must be made in the C_H3 domain of each antibody. These mutations, $K_{409}R$ and $F_{405}L$, are necessary to enable antibodies in the IgG1 domain to undergo cFAE in the presence of a reducing agent. These mutations have been shown to

promote the dissociation of the homodimers due to unfavorable C_H3–C_H3 interactions ($K_{409}R$–$K_{409}R$ or $F_{405}L$–$F_{405}L$) while favoring the interaction of the heterodimer ($K_{409}R$–$F_{405}L$) [45]. Similar to the "knob into hole" process, generating bispecific Abs using cFAE requires separate expression of the two antibodies followed by a two-step purification of MabSelect Sure and anion exchange chromatography to remove contaminants. By expressing the antibodies separately, the process eliminates the potential LC mispairing problem and enables the use of two different LCs. After purification, the two antibodies are mixed together in the presence of reducing agent in order to drive the formation of the hinge disulfide and proper heterodimerization formation [45, 49]. Purification of annealing mixture involves using diafiltration to remove excess reducing agent. Production with cFAE at the lab scale produced bispecific antibody with 95% bispecific and 5% homodimer, which was similar to the quality at a manufacturing level [45]. However, depending on the mechanism of action of the bispecific Ab, the presence of homodimers in the final product can result in non-specific binding and activation of receptors. Thus, to improve product quality, it is likely that subsequent purification steps could be required for specific applications. Genmab is advancing GEN3013 (CD3 × CD20, NCT03625037) as well as co-developing a number of other DuoBody Abs with Janssen Biotech (CD3 × BCMA, CD3 × CD123, CD3 × $GPRC_5D$, cMET × EGFR), so more details on the development of these molecules may be available in the near future. In summary, the Ab formats described in this section although they require a more involved production process provide versatility to assemble Abs from different discovery platforms, control of unbalanced expression of Abs, and low immunogenicity potential.

5.2 Ab Formats Composed of Four Chains Enabling Expression in a Single Cell

As is evident from Fig. 1, there is a growing trend for bispecific Ab formats to enable the expression in a single-cell system. This is mainly because it simplifies the process and operations for manufacturing with the consequent reduction in costs. Three approaches have been described to enable the co-expression of four different chains minimizing the formation of LC mispaired species when used in combination with a heterodimeric C_H3. One is the introduction of mutations in the HC-LC interface that drive the formation of the orthogonal HC-LC pairs. Another approach is the use of crossed-over domains in hybrid heavy/light chain as used in the CrossMAb™ technology [50]. The third approach is the engineering of disulfide bonds that only form and lock the orthogonal combinations of LC and HC pairs [51, 52]. Because more information is available on the different aspects of the production of Ab formats with engineered HC-LC interfaces beyond the research scale than for the other two approaches, it is discussed in more detail below.

Ab Formats with Engineered HC-LC Interfaces

Several independent engineered HC-LC interfaces have been reported for the orthogonal pairing of HCs and LCs [53, 54]. Although a description of a complete downstream process at a production scale has not been described yet for any of these designs, we describe below one Ab format that has been validated at the stable cell line stage. Dillon et al. described the development of several mutant HC-LC interfaces to drive the correct association between the chains [55]. Two of the best performing solutions, v10 and v11, drove the efficient association (>90%) of HC and LCs in a panel of different bispecific Abs tested. Importantly, the biophysical properties of the engineered Fabs were comparable to those of the parental antibodies. In particular, the melting temperature (T_m) and the binding affinity were comparable between engineered and parental Fabs. Bispecific Abs of either variants v10 or v11 produced by expression in a single-cell or in separate cells and then assembled in vitro were also comparable in their PK properties and biological activities in vitro. The content of bispecific Ab obtained using the engineered variants was also evaluated in stable cell lines. About 10% of the clones obtained showed near-quantitative assembly of the bispecific IgG, and the titers of the non-optimized top 5 clones ranged between 0.6 and 1.1 g/L [55]. Thus, this engineered Ab format for single-cell expression is very efficient at promoting the correct association of the LCs, and this property translates into stable cell lines. One potential challenge for this type of approach is that the number and complexity of product-related impurities are higher than using the two-cell system (Fig. 6). Because they differ only in one LC to the correctly assembled bispecific molecule, the LC mispaired species are likely to be very similar in their biochemical properties, making their detection and removal more challenging. The use of high-resolution Orbitrap-based mass spectrometry allowed the detection of mispaired LC species down to 0.3% and the precise quantitation of amounts down to 1% [56, 57]. Although a publication describing the complete production process at a scale compatible with use in clinical trials is yet not available, the high efficiency of these designs to drive the correct assembly of the bispecific antibody together with possibility of finding stable cell clones showing quantitative formation of bispecific molecule and availability of tools for characterizing and quantifying the presence of LC mispaired species provide a solid technical background for the successful implementation of the format at an industrial scale.

6 Conclusion

Different bispecific antibody formats are being developed and applied in different therapeutic areas. The lack of developability of the initial approaches utilized to produce bispecific antibodies was an insurmountable hurdle for many years. Although nowadays those initial limitations have been overcome, developability

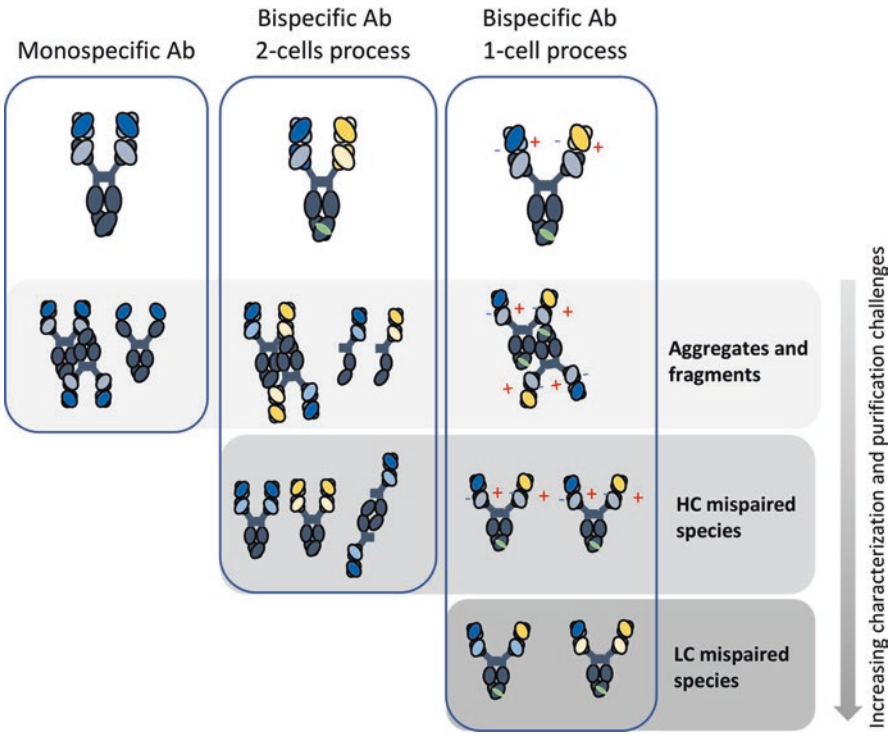

Fig. 6 Comparison between a monospecific Ab, a four-chain bispecific Ab format produced by the in vitro annealing process, and a single-cell process. The potential impurities arising from each process is shown below the corresponding Ab format. The impurities are arranged in an increasing order of biochemical similarities to the bispecific molecule. Impurities more similar to the bispecific molecule present increasing challenges for their detection and removal

continues to be an important force driving the evolution of bispecific Ab formats. The different Ab formats discussed in this chapter present advantages and disadvantages in terms of simplified production process, and in some cases, a very simple production route is engineered at the expense of creating potential disadvantages in other aspects of the molecule. However, the perceived liabilities discussed here for some Ab formats (high cost of goods, reduced thermostability, non-native IgG sequences) need to be further investigated before we can have a clear assessment of their impact on their success achieving a simple, developable process as well as in their clinical efficacy. As multispecific or multivalent Abs are providing with improved functionalities over regular bispecific Abs in some niche areas, the complexity of the molecules is also increasing. Understanding the advantages and challenges associated with the different formats and their production strategies will contribute to a solid foundation for developing the next generation of IgG-based multispecific therapeutics.

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Chapter 5
Lessons Learned in Understanding Dual Variable Domain-Ig (DVD-Ig) Structural Complexity to Select DVD-Ig Lead and Therapeutic Candidates

W. Blaine Stine, Dana I. Filoti, and Tariq Ghayur

1 Introduction

The DVD-Ig format is one of the many bispecific biologic formats described during the past 20 years. These formats fall into various structural classes [1], and structural differences may impact functional outcomes. About 50 bispecific biologics from a few structural classes are now in various stages of clinical development, with two bispecific drugs approved [1]. The emerging interest in DVD-Ig/bispecifics as a therapeutic modality stems from the many exciting opportunities such molecules offer as these formats increase target/target biology space for new therapeutic concepts and therapeutic modalities. These opportunities can be described into three categories, in terms of degree of novelty/innovation:

First, additive outcome (1 + 1 = 2), similar to the combination of two mAbs, but as a single therapeutic agent, e.g., neutralizing two soluble cytokines such as IL-1α/IL-1β and VEGF/ANG2 [2–4].

Second, the outcome is synergistic (1 + 1 = <2) and is not observed with the combination of two mAbs. In this instance, the outcome is "novel" and reveals an unexpected target(s) biology, e.g., simultaneous binding of two epitopes on a receptor [5] or binding two receptors on the same cell or different cells [6].

Third, the outcomes can be only achieved with a DVD-Ig or bispecific biologics. This category includes concepts such as (i) bring immune cells in close proximity

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to cancer cells [7]; (ii) deliver biologics across the blood-brain barrier [8]; (iii) bring two proteins together to catalyze an outcome [9]; (iv) deliver an antibody binding domain to an intracellular (endosomal) target [10]; etc.

In order to achieve novel synergistic and unique functional outcomes, the molecular architecture is critical. The molecular distance, position, and valency of the two target binding domains or variable domains (VD) likely influence the unique desired outcome. Figure 1 depicts some examples of bispecific formats where these structural features are highlighted.

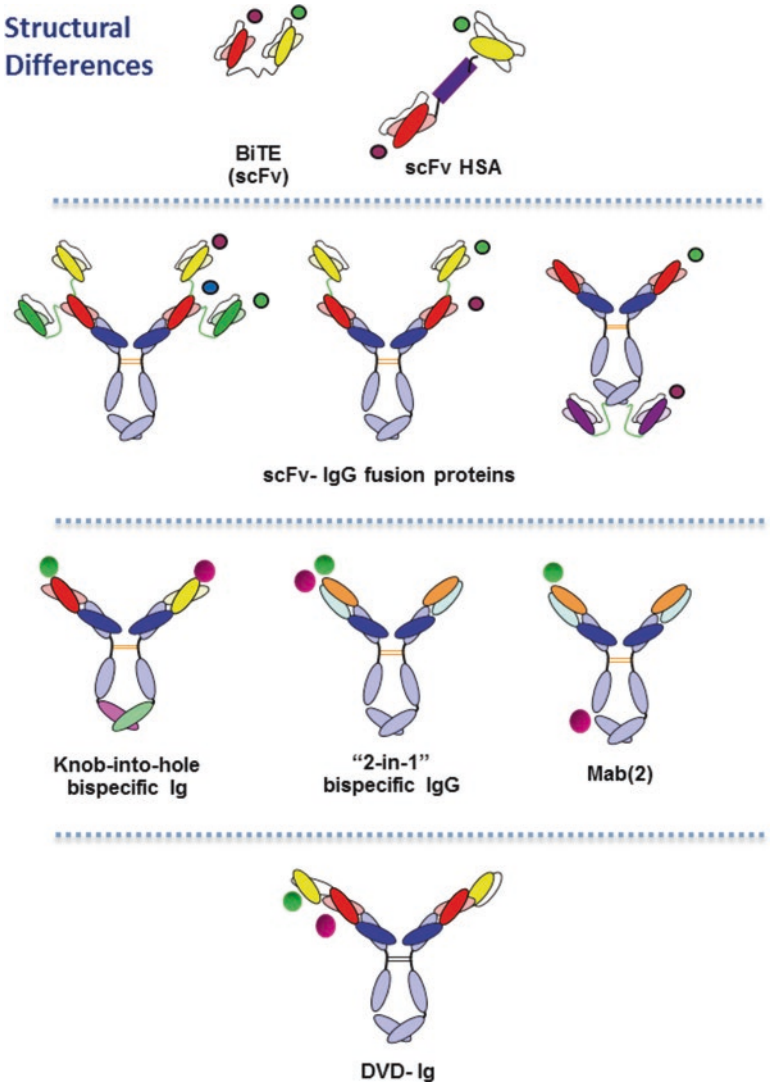

Fig. 1 Examples of structural differences among various bispecific formats. The main variable are (i) mAb symmetry, (ii) valency, (iii) VD distance and position, and (iv) linker length. These structural differences may have unique functional implications and development challenges

2 DVD-Ig Format

The dual variable domain (DVD)-Ig molecule retains the symmetry of an IgG1 molecule. It is bispecific and tetravalent where each Fab arm has two variable domains VD1 (inner) and VD2 (outer) linked in tandem via amino acid sequences derived mostly from "linkers" that connect C_H1/CL and VH/VL or hinge region in an IgG1 mAb (Fig. 2). In addition, the G4S linkers can be used to link mouse, humanized or fully human VDs (Fig. 2). The DVD-Ig molecule has a molecular mass of ~200 kDa compared with ~150 kDa for IgG1; the extra 50 kDa comes from the added VD2 (VH and VL) to the existing VD1 of an IgG1 (Fig. 2). In this architecture, the VD1 is supported by C_H1/CL, and VD2 is supported by VD1 (Fig. 2). As such, the DVD-Ig molecule can bind two targets simultaneously, contains mostly all natural sequences, is modular in architecture, and is amenable to high-throughput design and synthesis each with unique VD1, VD2 sequence and position or orientation (e.g., VD1 A (inner),VD2 B (outer) or VD1 B (inner),VD2 A (outer)), and different linker lengths, e.g., long-long (13–15a.a) or short-short (5–7a.a.) linkers connecting the VD1–VD2 heavy and light chains or linker combinations, e.g., long linkers connecting the VD1–VD2 heavy chains and short linkers connecting the VD1–VD2 light chains and vice versa. This structural flexibility of DVD-Ig molecule allows us to explore multiple structural variables simultaneously in in vitro and in vivo functional assays/models for desired functional outcomes and in biophysical/biochemical assays to select for stable molecules with good manufacturability, drug-like properties (DLP) and good pharmacokinetic (PK) profiles.

Three DVD-Ig molecules have entered clinical trials: (i) ABT-122 for RA and psoriasis, (ii) ABT-981 for osteoarthritis, and (iii) ABT-165 for solid tumors. ABT-122 and ABT-981 have completed Phase 2. In addition, several DVD-Ig molecules have been tested thoroughly for various structural, functional, and drug-like properties, including PK in cynomolgus monkeys. Overall, these data suggest that DVD-Ig molecules behave like IgG1 molecules and therapeutic grade molecules, with good manufacturability and biochemical and biophysical properties, and PK profiles (cyno and

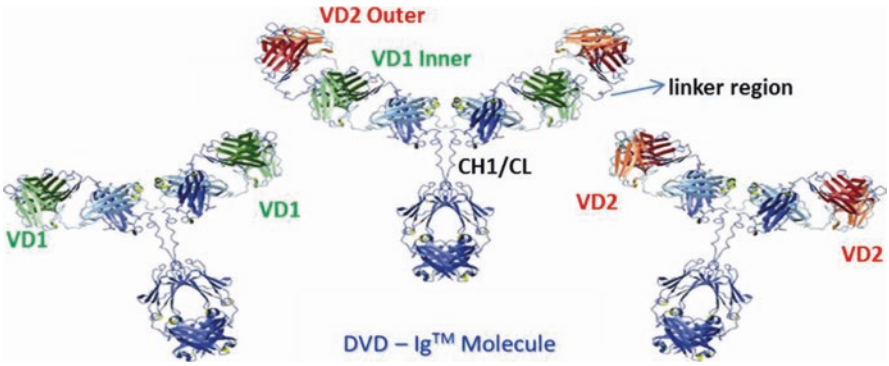

Fig. 2 Schematic representation of a DVD-Ig molecules. Each Fab arm of DVD-Ig has two variable domains (VD1, VD2)

humans) can be selected. More importantly, based on human, cyno and rodent data, it appears that the DVD-Ig format per se may not be immunogenic, but rather in some instances, immunogenicity to DVD-Ig may be dependent upon target biology [11]. In addition, several other laboratories have successfully made and tested DVD-Ig molecules in various preclinical animal models and demonstrated desired outcomes [12]. Thus, the DVD-Ig format is a robust platform for making bispecific biologics for basic preclinical research and as a therapeutic modality.

Based on our experience in development of mAb therapeutics and in evaluating 1000s of DVD-Ig molecules, in this chapter we will briefly discuss (i) critical aspects considered in developing the DVD-Ig platform and (ii) the lessons learned in selecting therapeutic grade DVD-Ig molecules, specifically with regard to how various structural components impact (a) structural-functional properties, (b) biophysical and biochemical properties, (c) PK profiles, and (d) manufacturability (i.e., expression, formulation, etc.).

2.1 Aspects Considered in Developing the DVD-Ig Format: Utilize Existing Platforms and Expertise

The successful delivery of any novel biotherapeutic to patients requires the translation of discovery concepts to clinical enablement. The vast majority of these biotherapeutics are full-length mAbs, typically using a well-defined IgG1 framework regarding the numbers of S-S bonds and glycosylation profile. The advantages of closely aligning the DVD-Ig architecture with naturally occurring IgG1 were the expectation that DVD-Ig will retain many of the favorable properties of human antibodies including long serum half-life, a high degree of intrinsic stability, unique specificity, and low immunogenicity [13]. With the large number of IgG biotherapeutics that have been developed, significant manufacturing experience has been gained which facilitates the development of the next generation of biotherapeutics.

Manufacturing Platforms

Production of drug substance and drug product can leverage learnings from one program to the next by minimizing cell culture and purification process development, analytical methods development, and formulation development. However, deviating from an IgG format typically comes at a cost. For example, formats lacking an Fc region not only have significantly shorter in vivo half-life (which may or may not be desirable) but also are not compatible with protein A-based capture methods. Formats such as knob-in-hole where two distinct heavy chain and light chain pairings are brought together through an asymmetric Fc interaction also require the removal of unpaired chains which can complicate purification process development and compromise overall process yields. Some formats also require the use of multiple expression vectors beyond the typical light chain and heavy chain vectors used for IgG expression. This may complicate the generation of a highly stable and

productive host expression system needed for robust manufacturing. These considerations were taken into account during the development of the DVD-Ig architecture enabling the successful application or modification of existing development platforms built around IgG therapeutics to DVD-Ig bispecifics.

Expression Platforms

Expression systems for DVD-Ig bispecifics can be based on widely used Chinese hamster ovary (CHO) expression platforms. Vector construction, transfection, selection, and cloning methods are all highly similar to those used for traditional monoclonal antibody development (Fig. 3). One difference that has been observed is that,

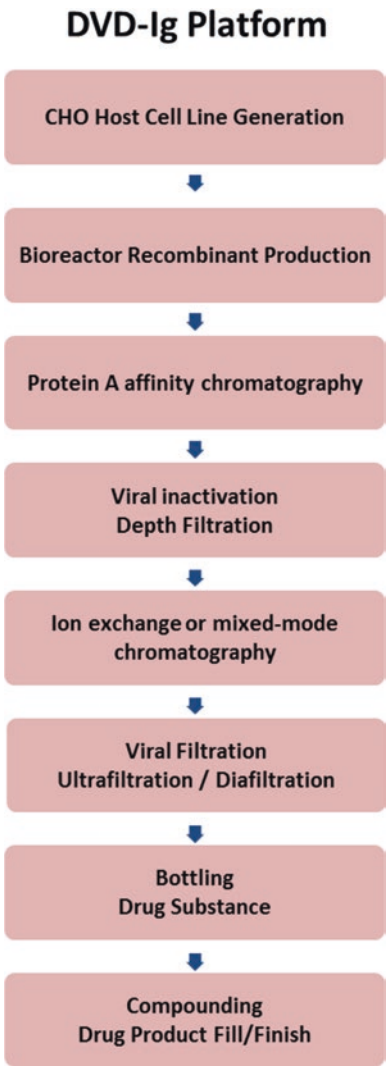

Fig. 3 Schematic of the DVD-Ig purification platform

in general, titers can be lower for DVD-Ig than typically observed for IgG. However through appropriate selection and screening, multi-gram per liter productivity has been achieved for DVD-Igs. In general DVD-Ig-based master cell banks have also proven to be very comparable to IgG-based master cell banks regarding stability and robustness. Since the host system is based on standard CHO expression, cell culture conditions can also leverage manufacturing platforms that take advantage of the benefits associated with chemically defined media, fermentation in modern bioreactors, and disposable manufacturing technologies.

Purification Platforms

Purification of DVD-Ig bispecifics can utilize protein A capture methods because of the presence of a standard Fc region in the DVD-Ig architecture. Protein A is a highly efficient capture method for clarified harvest containing high titer DVD-Ig. Additional purification steps for aggregate removal, host cell protein, and other process and product-related impurities are routinely used during the manufacturing of DVD-Igs. Aggregate levels in clarified harvest can be slightly higher for some but not all DVD-Ig bispecifics compared to IgGs. However conventional downstream aggregate removal steps such as hydrophobic interaction chromatography or mixed-mode chromatography have shown to be effective at lowering total aggregate levels into the same ranges typically encountered for IgGs.

One manufacturing step that can differ for DVD-Igs is the process of ultrafiltration and diafiltration. Here the DVD-Ig containing process intermediate is buffer exchanged and concentrated using ultrafiltration membranes. The increased molecular weight intrinsic to the DVD-Ig architecture which is typically around 200 kDa may require additional process optimization. Formulated drug substance and drug product can also be expected to follow platforms established for IgGs. Although as with any biotherapeutic, process optimization for DVD-Igs is necessary to increase efficiency and robustness and to achieve the desired quality attributes.

In summary, these benefits of aligning DVD-Ig architecture closely with an IgG format have enabled comparable development timelines and costs for DVD-Ig bispecifics.

2.2 Understanding the Biology of the DVD-Ig Architecture to Select DVD-Ig Lead Candidates

In an effort to build an understanding of how particular attributes of the parental antibody sequences used in the construction of DVD-Ig bispecifics influence the final construct, an initiative was undertaken in which a large panel DVD-Igs was constructed from existing monoclonal antibody sequences. Variable region pairings were made using the same common linkers and IgG1 isotypes so that the physicochemical differences could be directly attributed to the variable region sequences

and the relative orientation. By creating matched pairs of DVD-Igs containing identical variable region sequences, two orientations (VD1 inner, VD2 outer and VD1 outer, VD2 inner) enable comparisons between identical stretches of amino acid sequence. The amino acid composition to the matching pairs of DVD-Igs is identical, and the linear stretches of amino acids are identical except for the junctions between inner and outer domains. The creation of this panel of VD-matched DVD-Ig enables the comparison of distinct structural and physicochemical properties with their corresponding parental mAbs. Some of the key lessons learned from such studies are summarized briefly in Table 1 and the subsequent sections.

Table 1 Features to consider in selecting a therapeutic grade DVD-Ig

Key parameters	Features to consider	Lessons learned
Expression and manufacturability	1. Good transient (HEK293)and stable expression (CHO) profiles 2. Compatibility with platform processes	1. VD sequence combinations and VD positions impact expression levels more than linkers 2. HEK293 expression levels may not predict CHO cell expression/amplification levels
Biophysical/ biochemical properties (drug-like properties, DLP)	1. Aggregation propensity (solubility, viscosity, intrinsic stability) 2. High-concentration formulation	1. VD position impacts DLP. Intrinsically less stable VDs prefer C_H1/CL proximity, i.e., VD1 position 2. Linker design to limit overall VD2 flexibility (a) May impact aggregation
Functional properties (binding two targets simultaneously)	1. Simultaneous, sequential, or conditional binding of both targets and/or epitopes 2. Affinity, potency, and specificity of both VDs	1. The right VD sequence combinations, i.e., VD a.a. sequences from multiple mAbs/targets, VD positions, and linkers must be determined empirically 2. Other aspects: (a) Paratope position within VD: intrinsic VD flexibility and linker combinations are needed to maintain inner VD target binding (b) Target biology: dynamic size, oligomeric state, location (soluble, surface) important for inner VD positioning
PK/PD and ADA	1. PK: good PK profile, t1/2 $\geq$ 10–12 days 2. ADA: minimal or no ADA	1. PK: Mostly PK is similar to mAbs; but parental mAb PK does not predict DVD-Ig PK (a) >10% aggregation in a serum stability assay reduces T1/2 and increases clearance. This is mostly observed if VDs with less intrinsic stability are placed in VD2 position (see text below) 2. The DVD-Ig format is not immunogenic per se. Immunogenicity may be related to target biology

Structure-Function Relationship

In an IgG1 mAb, the variable domain (VD, target binding domain) is connected via linkers to and is supported by the C_H1/CL domain (Fig. 2). This C_H1/CL domain – VD linkage – also provides intrinsic structural "stability" to the VH/VL interactions of the VD domain. In most instances, the transient expression profiles of the full-length IgG1 or single-chain Fv (scFv) have been used as a tool to identify "stable" VH/VL combinations [13–15]. In the DVD-Ig architecture, the added second VD2 (the outer domain) is connected via a second set of linkers to and is supported by the VD1 (Fig. 2). As such, the VD2 is distal to and not supported by C_H1/CL. This arrangement increases the numbers of interactions that might occur between the VH/VL of the two VDs and the added linkers (Fig. 4). Thus, in the DVD-Ig architecture, the VD1–VD2 sequence combinations, their position (orientation), and the sequence and length of linkers may impact the expression levels, the functions of the two VDs, and the overall stability (drug-like properties) of the DVD-Ig molecule. Therefore, we took a systematic approach to understand how particular attributes of parental mAb VD sequences used to make DVD-Ig and the second set of linkers influence the various properties of the DVD-Ig molecule.

Initially, over a 1000 DVD-Ig molecules were constructed using VD sequences from existing mAbs. The creation of this panel of matched DVD-Ig enables the comparison of distinct structural and physicochemical properties with their corresponding partners in the same Fc framework and with the same VDs simply in opposite orientations. The VD pairings (VD1, VD2) were made using same linkers and IgG1 isotypes so that the physicochemical differences such as monomer stability could be directly attributed to the VD sequences and the relative VD orientation (e.g., VD1 inner,VD2 outer and VD1 outer,VD2 inner). By analyzing in comparison, coordinated pairs of DVD-Ig containing identical VD sequences, but in two orientations as shown in Fig. 5, allow understanding the structure-function crossing point. These DVD-Igs were also characterized for their ability to bind both target antigens with similar affinities to the parental monoclonal

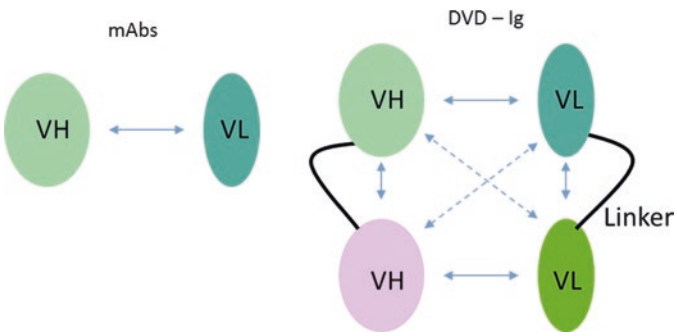

Fig. 4 Schematic representation of the potential interactions that occur between different components of a mAb and a DVD-Ig VD

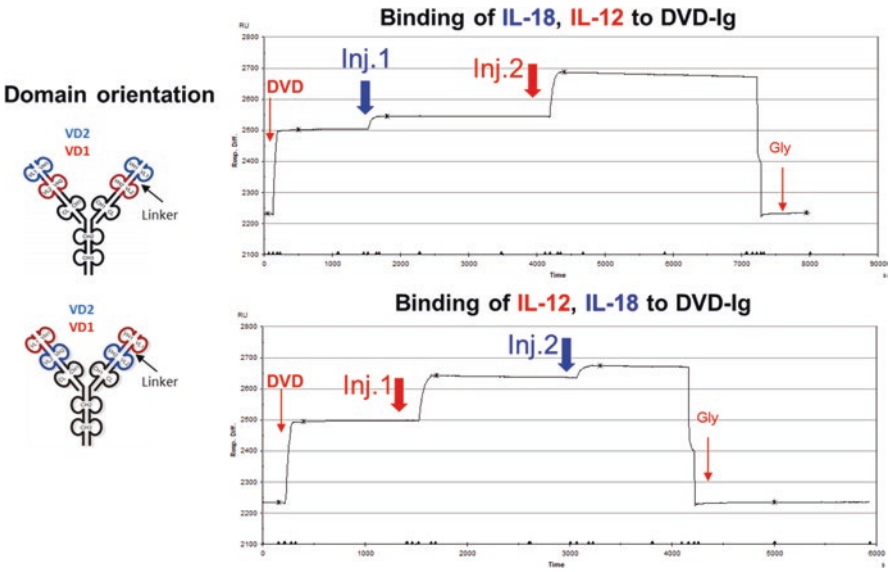

Fig. 5 DVD-Ig binds two targets simultaneously as assessed by surface plasmon resonance (SPR)

antibodies used in their construction. In addition, binding studies using surface plasmon resonance (SPR) revealed how the presence of a bound antigen in one variable region does not affect the ability of the second variable region to bind its target antigen (Fig. 5).

Expression Levels: The Impact of VD Combinations, Orientation, and Linkers

Parental mAbs and DVD-Ig were transiently expressed in HEK293 cells to evaluate expression profiles and obtain enough purified material to determine biophysical and biochemical/drug-like properties and in some cases rodent and cyno PK profiles. The key take-home messages can be summarized as follows: (i) Transient transfection profiles may allow us to select "stable" VD combinations and their correct orientation. (ii) Although linker combinations also impact expression profile, the VD combination and orientation are the key factors determining expression profiles and overall stability of the DVD-Ig molecule.

Inner and Outer VD Function

In all instances, the VD2 (outer domain) functions (affinity and potency) are maintained in all DVD-Ig. This is expected as the VD2 CDRs are exposed. As shown in Fig. 5, the VD1 (inner domain) functions may be also retained – the key aspect is

the nature of the in vitro assay employed to determine VD1 functions. In in vitro functional assays where both the target and the DVD-Ig are in solution, observations show the least loss of VD1 functions, suggesting that overall solution, movement, accessibility, and flexibility of both target molecules are required. In fact, cryo-EM-based studies showed remarkable overall flexibility of DVD-Ig molecules [15, 16], and co-crystal structures of DVD-Ig bound to the VD1 target have shown that this interaction pushes the VD2 (outer domain) to one side [16]. However, in some instances, VD1 (inner domain) functions may be impacted by several factors such as target biology, e.g., size and oligomeric state (monomer, dimer, trimer, etc.), location (soluble or membrane receptor), and epitope. However, VD1 functions can be restored/regained by either placing the right VD at VD1 position or by adjusting the linker length/linker combinations.

Drug-Like Properties

As with any other biotherapeutic, selecting a candidate molecule with all desired properties of a therapeutic candidate is a challenge. As described above, within a DVD-Ig molecule, multiple potential interactions between VD1, VD2, and an additional set of linkers may occur simultaneously. Although understanding all these interactions is difficult, using our panel of DVD-Igs, we have developed methods to identify key features important for selecting DVD-Ig with good drug-like properties. Since in a DVD-Ig format, the outer VD2 is supported by the inner VD1, the relative intrinsic stability of the two VDs and choice of the VD to place close (proximal) to C_H1/CL are the critical factors.

We applied multiple analytical techniques typically used for characterizing mAbs to interrogate the physical and chemical stability characteristics of DVD-Ig. In addition, a novel chromatographic based technique was used to interrogate DVD-Ig's stability in biologically relevant serum solution (Sect. 3). Overall our studies using an in vitro serum stability assay (Fig. 12) show that VDs with low intrinsic stability (weak VH/VL interactions) when placed at the outer VD2 position result in DVD-Ig molecules that have higher aggregation (overall are less stable), faster in vivo clearance, and short half-life. Interestingly, the same VD when placed close (proximal) to C_H1/CL is perfectly capable of supporting VD2 and resulting in stable DVD-Ig with good drug-like properties. These observations suggest that selecting the right VDs and placing them in the right position are important for making DVD-Ig with good drug-like properties. As product development proceeds, lessons learned from developability screening or drug-like property studies inform the selection and chronology of stability studies. Considerations on the product's performance under various storage conditions, expression, and manufacturability provide CMC guidance on how the quality of a DVD-Ig drug varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and to establish a shelf life for the drug product and recommended storage conditions.

3 Analytical Methods to Interrogate Drug-Like Properties

Since the beginning of development of monoclonal antibodies (mAb) in the 1980s, the pharmaceutical industry implemented a customized approach for assessing drug-like properties associated with success rates of each new biologic molecule that proceeded from discovery to development. Although structurally similar to IgG antibodies, DVD-Ig is more complex which influences drug-like properties, including aggregation propensity at higher concentrations and solubility at low temperatures. Molecules with superior drug-like properties such as resistance against unfolding and aggregation as well as high physicochemical stability are more easily developed into manufacturable and deliverable therapeutics [17]; however sometimes this comes at the cost of other properties such as target affinity [12] or requires intravenous delivery at low protein concentrations to get an efficacious dose [18]. Table 2 highlights the scope of each of the typical assays used to assess physicochemical stability attributes.

Below we discuss briefly the methodology used in DVD-Ig candidate selection process that starts with in silico liability screening methods, followed by subsequent

Table 2 Physicochemical characterization methods used for DVD-Ig (list of assays not inclusive)

Analysis	Assay	Information
Primary structure	Intact LC-MS analysis Reduced LC-MS analysis	Confirmation of expected mass Detection of potential sequence heterogeneity
	Peptide mapping by LC-MS	Detection of primary modifications
Purity	SDS-CE under nonreducing conditions SDS-CE under reducing conditions	Detection of aggregates and fragments Detection of mobility differences
Thermodynamic stability	Differential scanning calorimetry	Thermodynamic stability and measured unfolding temperatures within expected ranges
Accelerated stability	Universal buffer platform assays (pH 4, 6, 8)	Comparable degradation (aggregation and fragmentation) rates
Physical stability	Freeze/thaw stability	Minimal loss of monomer upon freeze/thaw
	Sedimentation velocity analytical ultracentrifugation	Solubility and size c(s) distribution in solution
Secondary structure	Far UV CD spectroscopy	Measure differences in beta sheet signature
Tertiary structure	Near UV CD spectroscopy	Measure tertiary folding
Charge/size ratio	Capillary zone electrophoresis	Measure small differences in the surface charge to size
Hydrophobicity	Analytical HIC chromatography	Measure differences in hydrophobicity
Size distribution	SEC-MALLS	Measure molecular weight distribution for aggregates, monomer and fragments

developability risk assessment to rank order candidates according to predefined benchmark criteria based on prior knowledge. Due to the structural similarity between mAb and DVD-Ig formats, we were able to leverage the mAb analytical screening platform [17] and apply it to support DVD-Ig candidate selection and nomination. Lead molecules are those that possess least liabilities and show overall best drug-like properties and, hence, are considered to have the lowest development risk.

3.1 Solubility Assessment

While some DVD-IgG molecules have high solubility (>100 mg/mL), others have shown enthalpic- and entropic-driven behaviors such as gelling, opalescence, phase separation, and precipitation, with many of these phenomena occurring at storage temperature conditions around 5 °C.

Solution conditions such as pH and ionic strength impact the colloidal and conformational stability, similar to what is observed in the parental mAbs, and therefore need to be chosen carefully. Low solubility is manifested at pHs closer to the DVD-Igs isoelectric point (pI). Typically, DVD-Ig's pI is within the parental mAbs range, and the formulation pH is about two pH units lower to avoid deamidation and hydrolysis modifications. Additionally, different from the mAb formulations, in case of a DVD-Ig formulation, a moderate ionic strength (~1–30 mM) has been found to either increase or decrease solubility, depending on the specific DVD-IgG. Thus, selection of buffering components is a critical parameter of formulation development for biological entities.

3.2 Size Homogeneity

The increased mass of a DVD-Ig over conventional IgG proteins translates to higher molecular weight oligomeric and aggregate species. We have demonstrated that size exclusion chromatography (SEC) methods using conventional gel filtration columns are compatible with the larger DVD-Ig proteins although orthogonal testing was also performed to support initial observations from SEC analysis.

DVD-Ig aggregates, monomer, and fragments could be readily discriminated by SEC-MALLS (multi-angle laser light scattering), as depicted in Fig. 6 where a DVD-Ig process intermediate sample containing elevated aggregate and fragment levels was tested. Analysis of UV and static light scattering signals demonstrates that this process intermediate contains low levels of high molecular weight aggregates only detected by light scattering (HMW dotted trace), 6.3% dimer (Peak #2391 kDa), 91.1% DVD-Ig monomer (Peak #1 Mw 192 kDa), and two low molecular weight species (LMW 1.5% and 1.1%). SEC analysis of fully purified DVD-Ig resulted in a symmetric main peak (98%) with low levels of aggregate (1.4%) and fragment (0.6%).

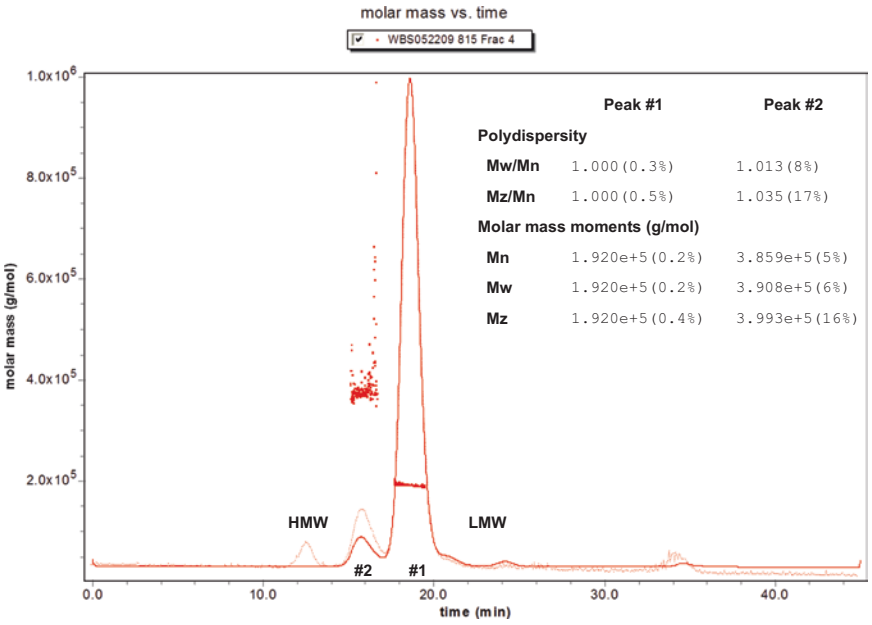

Fig. 6 DVD-Ig process intermediate sample separated by size exclusion chromatography with UV and multi-angle laser light scattering detection

Extended characterization studies using sedimentation velocity analytical ultracentrifugation as an orthogonal technique to size exclusion chromatography have proven to be well suited for DVD-Ig analysis. Data shown in Fig. 7 demonstrate that the DVD-Ig molecule has hydrodynamic properties well characterized by sedimentation velocity analytical ultracentrifugation. A single main species (98.85%) was detected with a sedimentation coefficient (7.505 S) consistent with a DVD-Ig monomer. The sedimentation coefficient for DVD-Ig proteins (~7.5 S) is greater than conventional monoclonal antibodies (~6.4 S) as expected from the increased mass from the additional variable domain. Dimer was detected at 0.9%, and very low levels of fragment and 0.24% high molecular aggregate were also present. Results obtained to date from other DVD-Ig molecules are similar with c(s) distributions consistent with the elevated molecular weight yet retaining the ability to discriminate between monomer and aggregate species.

3.3 Monomer Stability and Aggregate Levels

To explore aggregation levels for DVD-Igs, 59 DVD-Igs produced from a HEK293 transient expression system were evaluated for percent monomer following a protein A affinity capture purification step with no additional aggregate

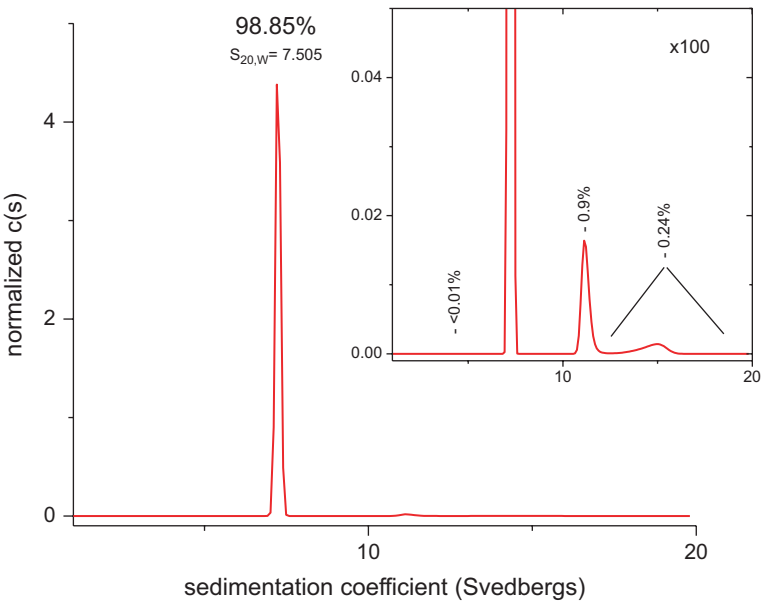

Fig. 7 C(s) Distribution for a DVD-Ig measured by sedimentation velocity analytical ultracentrifugation

removal step. These 59 molecules demonstrated a wide variation in the percentage of monomer under these following conditions: for 25 DVD-Igs, monomers were at >95%; for 13 DVD-Igs, monomers were at 90–95%; for 6 DVD-Igs, monomers were at 85–90%; for 4 DVD-Igs, monomers were at 80–85%; and finally for 11 DVD-Igs, monomers were at <80%. The higher percentage of aggregates observed with certain DVD-Ig appears to be molecule-specific, and many other engineered DVD-Ig molecules have levels of aggregates similar to typical monoclonal antibodies.

3.4 Purity Assessment

Purity determination techniques using either traditional SDS-PAGE or capillary gel electrophoresis (CGE) are broadly compatible with DVD-Ig proteins. As expected from increased molecular weight, there are differences in migration times for DVD-Ig proteins as compared to conventional monoclonal antibodies. However DVD-Ig proteins and monoclonal antibodies are both equally well suited for purity determination using these techniques. An example of CGE analysis of a DVD-Ig is provided in Figs. 8 and 9. Under nonreducing conditions, integration of the electropherogram UV signal detects a single major species (96.7%) with a retention

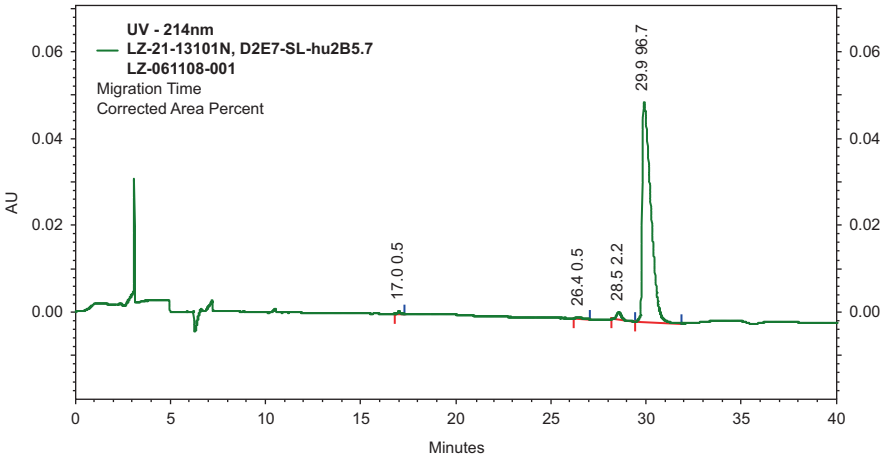

Fig. 8 Analysis of a DVD-Ig by capillary gel electrophoresis (CGE) performed under nonreducing conditions

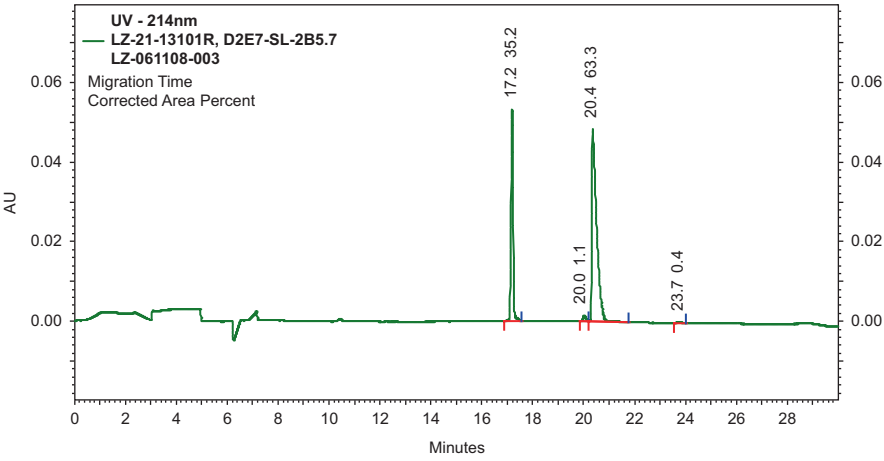

Fig. 9 Analysis of a DVD-Ig by capillary gel electrophoresis (CGE) performed under reducing conditions

time of 29.9 minutes which is consistent with a monomeric DVD-Ig. Minor species including low molecular weight fragments are detected between 15 and 28.5 minutes. Under reducing conditions, single light chain (17.2 minutes) and heavy chain (20.4 minutes) peaks that account for 98.5% of the total UV absorbance signal are detected. A shoulder off of the heavy chain peak (20.0 minutes) likely represents a low percentage (1.1%) non-glycosylated species that is frequently observed when this technique is used to analyze IgG monoclonal antibodies.

3.5 *Hydrophobicity, Charge, and Serum Stability Relationship with Domain Orientation*

Hydrophobic interaction chromatography was used to measure the effects of domain orientation on surface hydrophobicity (Fig. 10). Some domain pairings exhibited measurable changes in surface hydrophobicity depending on domain orientation. However, other pairings appeared to be relatively insensitive to being engineered in the outer vs. inner DVD-Ig domain. This was also the case for charge differences measured by capillary zone electrophoresis (Fig. 11).

A significant disadvantage that many in vitro screening methods have is that these approaches do not precisely mimic the conditions encountered by the biologic in vivo. When biologics are administered by either intravenous [IV], subcutaneous [SC], or intramuscular [IM] routes of administration, the drug encounters a distinctly different local environment. This environment is characterized by a highly volume occupied or molecularly crowded environment where both attractive and repulsive forces drive molecular interactions that can only occur under these conditions. These interactions can influence the physical stability of biologics in vivo resulting in altered pharmacokinetic properties. Developing an understanding of how candidate biologics behave not only in dilute buffer systems but also in crowded environments, such as those encountered in vivo, would better inform the candidate selection process for protein therapeutics. Therefore a novel chromatographic stability method was developed to screen for drug-like properties by analyzing in vitro the time-dependent changes of a fluorescently labeled DVD-Ig candidate when incubated in a biological fluid which the drug is likely to encounter in vivo.

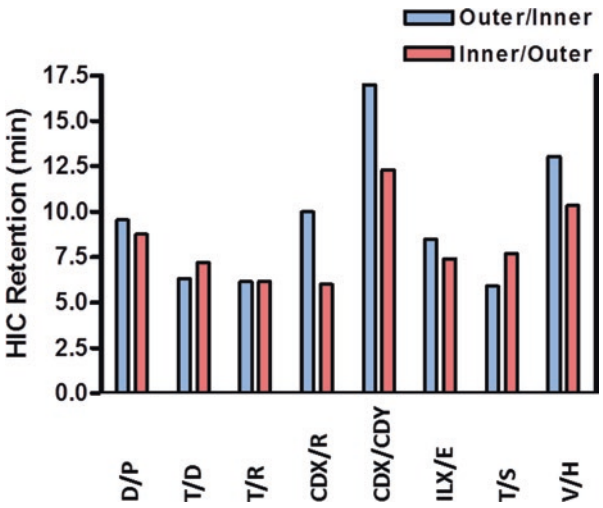

Fig. 10 Analysis of changes in surface hydrophobicity as a function of DVD-Ig domain orientation using hydrophobic interaction chromatography (HIC)

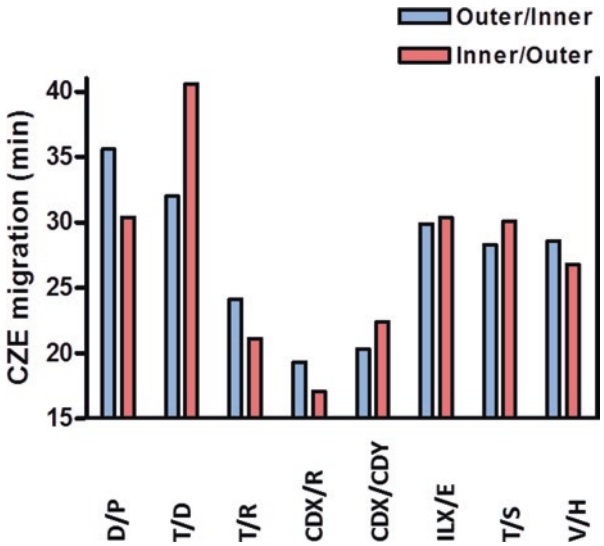

Fig. 11 Analysis of changes in surface charge as a function of DVD-Ig domain orientation using capillary zone electrophoresis (CZE)

This method selectively monitors changes to DVD-Igs that occur over time in complex fluids such as serum. Changes include the formation of high molecular weight (HMW) and low molecular weight (LMW) species from monomeric candidates when incubated in serum, as shown in Fig. 12. Our experience has shown that these changes are frequently unique to the conditions encountered in serum and do not occur in dilute buffer systems. Cumulatively, as depicted from Figs. 10, 11, and 12, these data suggest that although the primary amino acid composition is identical for the pairs of DVD-Ig domains constructed in each of the two possible orientations, the physicochemical properties may differ little between the two constructs and show differentiation in the in vitro biological stability assay only.

3.6 Methods to Interrogate Primary, Secondary, and Tertiary Structure

In silico screening methods are used to identify sequence and structural liabilities that might cause chemical or physical degradation (deamidation, aggregation, colloidal stability) that can be further interrogated via mass spectrometry for delineating structure-based chemical stability attributes. A systematic analysis of DVD-Igs, from primary sequence annotations to three-dimensional structure alignment, is an appropriate approach for investigation of protein structure aided by computational power and prior knowledge. However, the outer variable domain in the DVD-Ig format is not supported by a C_H1/C_L domain and as a result provides additional complexity that is challenging to be comprehensively interrogated by theoretical-based

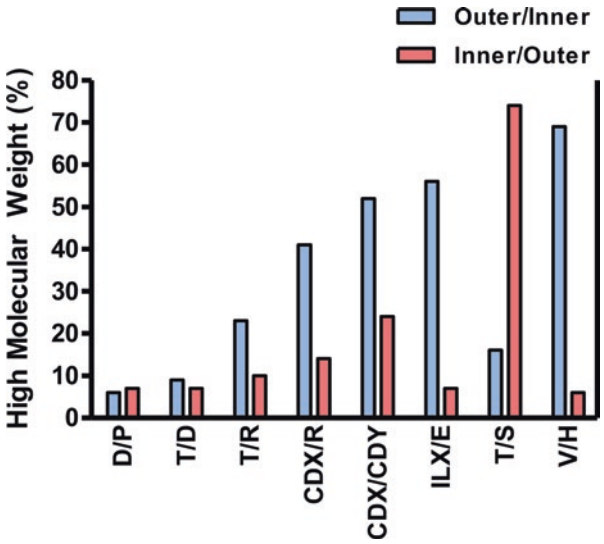

Fig. 12 Analysis of changes in HMW in serum as a function of DVD-Ig domain orientation using a novel SEC technique for assessing biological stability

computational screening. In addition, by adjusting the length and sequence of the linkers connecting inner and outer domains, the steric influence of the outer variable domain on the inner domain may be altered, making it possible to tune the binding affinity of the inner variable domain.

The structural domains of a properly folded DVD-Ig can each have unique thermodynamic properties. Differential scanning calorimetry (DSC) provides experimental insight into the intrinsic thermodynamic stability of the molecule and provides measures of the temperature and the amount of energy necessary to unfold the protein. For conventional monoclonal antibodies, DSC data can usually be fitted to three unique thermal transitions representing the unfolding events for VH/VL, C_H2, and C_H3. For DVD-Ig molecules, the data can often be fitted to four unique transitions where the additional unfolding event is likely a result of the presence of the additional pair of CDRs. As shown in Fig. 13, via differential scanning calorimetry (DSC), the thermal unfolding of a DVD-Ig protein can be fitted to four unique thermal transitions with transition midpoint values of 61 °C, 69 °C, 76 °C, and 84 °C. Furthermore, analyzing in comparison the DSC thermographs between the DVD-Ig and its parental mAb with the same VDs as shown in Fig. 13-insert, when thermally denatured a VD placed as an outer variable domain, it may lose conformational stability than the other domains (inner VD, C_H2, and C_H3) which exhibit similar conformational stability when compared to their antibody counterparts. A decrease in stability of the outer variable domain can result in a higher aggregation propensity of the whole DVD-IgG as compared to the aggregation profile of a typical antibody. In addition, X-ray crystallography and computer modeling have shown that the outer variable domain is conformationally mobile and may reside on the side or in line with the Fab [16].

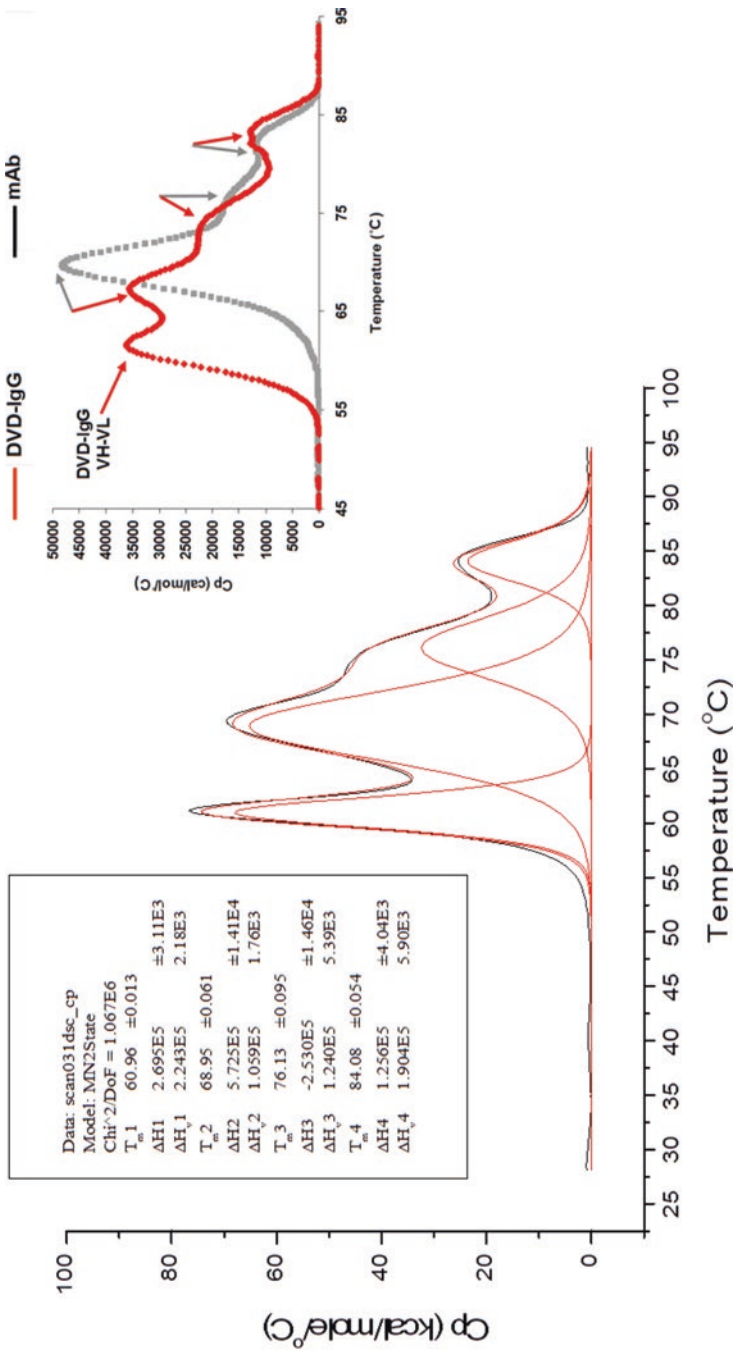

Fig. 13 Analysis of a DVD-Ig conformational stability using DSC

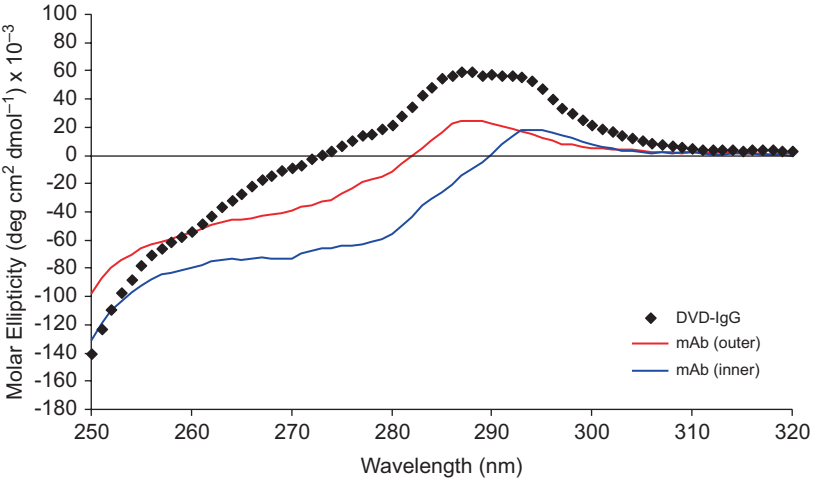

Fig. 14 Near UV-CD spectra of a DVD-Ig and the parental mAbs with same variable domains

The observation that the outer variable domain is conformationally mobile imparts an additional layer of complexity when calculating surface potential and hydrophobicity because these properties will change depending on the orientation of the outer variable domain. For example, dipole moments which arise from surface charge can induce conformational changes in the outer variable domain. Additionally, the outer variable domain flexibility may contribute to the unfolding cooperativity of DVD-IgG individual domains as observed by DSC (Fig. 13) and to the various secondary structure elements that may require a minimal ellipticity in the 260–290 nm region to suggest optimal exposure of aromatic residues to the solvent and indicating absence of a compact tertiary structure (Fig. 14).The near UV-CD profile of the DVD-IgG is shown together with the near-UV CD spectra for the two parental mAbs, where the VD of the red mAb comprises the outer VD and the VD of the blue mAb comprises the inner VD of the DVD-Ig. The DVD-Ig is less "S-shaped" and more linear than that of the mAbs, indicating that it is not as structurally compact. All of this would affect the strength of intermolecular interactions which govern solubility, aggregation, and viscosity.

3.7 Impact of Biophysical and Biochemical Attributes on DVD-Ig Colloidal Stability and Formulation Development

Many treatment indications, where a biologic is the preferred therapeutic and administration is via subcutaneous or intramuscular route and then the volume limitation, typically less than 1.5 mL per injection, require administration of a high-concentration formulation of the drug product (>100 mg/mL). Development of stable, easy-to-use, high-concentration liquid protein formulations poses several

challenges including those associated with the issues of aggregation and solubility. Non-ideality leads to enhanced protein-protein attractive interactions which increases the propensity of the molecules to aggregate.

High-Concentration Stability

One of the ultimate aims of any formulation scientist is to achieve sufficient shelf life stability for a drug product formulation. DVD-Ig stability is a major priority during the development of biopharmaceuticals, and aggregation is a critical stability parameter for safety and efficacy. Like with mAb aggregates, the DVD-Ig aggregates may differ not only in size, ranging from dimers to oligomers to large visible particles, but also in conformation consisting of unfolded, partly unfolded, native monomers, or combinations thereof. Large native-like aggregates, with essentially intact secondary and tertiary structure, may cause immune reactions, especially when also chemically modified [19].

DVD-Ig stability is driven by the long- and short-range electrostatics and described by potential energies surrounding two interacting charged molecules. More and more measurements and predictions for the interactions arising from the charge-charge, permanent, and induced dipole moment of the molecule are needed to be cross-examined for enabling a stable high concentration. Under dilute conditions, long-range electrostatic repulsions drive the colloidal stability role unless the molecules have a tendency to self-associate to form small oligomers for lowering the overall system energy. On the other hand, in concentrated solutions, the dominant potentials are the short-range forces that fall off rapidly with distance due to a higher-order inverse dependence on center-center distance. It is important to consider that only charge-charge and excluded volume hard sphere interactions are repulsive in nature. The contribution of most of the electrostatic attractive forces, independent of the range of the force, is anticipated to decrease with an increase in the ionic strength of the solution. Charge-charge and van der Waals interactions (including dipole interactions) have been shown to vary significantly on the addition of the salt due to effects such as charge shielding and preferential anion binding [20]. The hydrophobic interactions are short-range attractive forces that are unaffected by the ionic strength of the solution especially in the range 0–20 mM; however, high salt concentrations may enhance hydrophobic attractions by preferential exclusion mechanism. Hydrophobic interactions have been observed to be the dominant colloidal instability driver causing protein aggregation in various high and low concentration formulations, when low repulsive charge-charge electrostatic interactions are easy to be overcome by hydrophobic interactions [21].

Freeze/Thaw Stability

Freezing of protein solutions is an operation frequently performed during drug manufacturing in order to minimize protein instability during storage and transportation. The storage of drug substance at subzero temperatures mitigates potential risks

associated with liquid storage, such as degradation and shipping stress, making it a favorable solution for long-term storage. However, slower (generally uncontrolled) rates of freezing and thawing of drug substance in conventional multi-liter storage containers can lead to greater cryoconcentration (exclusion of solute molecules) resulting in zones of higher protein and excipient concentrations and changes to the desired formulation pH and excipient concentration [22]. These conditions can negatively impact product quality, thus changing the target product profile.

Freeze/thaw studies can provide valuable knowledge on the molecule even when performed from an early formulation image. The study helps evaluate the impact of freeze/thaw rate, mode of freezing, drug substance container and concentration, and formulation on the drug substance quality. Samples can be subjected to freeze/thaw cycles in which rates of freezing and thawing are passively controlled. Samples are frozen by placing them at −80 °C and thawed at room/water bath or refrigerated temperatures repeatedly for desired number of cycles. Additionally, these studies can inform freeze-dried or lyophilized formulations suitability assessments. Repeated freeze/thaw cycles during the various manufacturing steps mean exposure of the protein to non-optimal thermal conditions, potentially leading to denaturation and aggregation. During freezing, the physical environment changes dramatically, inducing several possible stresses, like formation of ice water interfaces, low temperature destabilization, and changes in solute concentration and pH. Differences in the protein concentration also impact the freeze/thaw profile, leading to higher incidence of aggregation. Air-liquid interface studies conducted by shaking the protein solution in the presence of headspace has shown that proteins exhibit surface activity causing denaturation when exposed to liquid-air or liquid-liquid interfaces. Exposure to a hydrophobic environment causes it to unfold, resulting in self-association and aggregation. Addition of another variable domain to a mAb makes the DVD more susceptible to aggregation.

Forced Degradation Studies

Forced degradation studies can be used to investigate liabilities hard to be identified at early development or discovery stages. Some liabilities manifest during stability studies in form of modifications as provoked by different stress conditions and subsequently identified by mass spectrometry on peptide level, as shown in Table 3 for recommended degradation studies that apply to DVD-Igs.

4 Concluding Remarks

In this chapter we have discussed lessons learned from developing the DVD-Ig bispecific platform. While the DVD-Ig deviates from the IgG format in order to become more functionally capable of engaging two targets simultaneously, its

Table 3 Recommended degradation studies

Stress factors	Conditions
Elevated temperature	Accelerated stress; improper shipment, storage or handling deviations; specific production processes
Freezing/ thawing	Accidental freezing during storage or shipment; storage of frozen (bulk) material; lyophilization
Mechanical stress	Production (pumping, filtration, stirring); shipment; handling (e.g., shaking)
Light	Exposure to daylight or artificial light during production, shipment, storage, or handling; UV detection during downstream processing
Oxidative stress	Contact with oxygen (air, dissolved O_2) excipients, e.g., peroxide impurities in polysorbate, metal ion traces from production equipment or excipients; light
pH changes	Production (downstream processing); freezing; formulation; dilution in infusion liquids; viral inactivation

modular design closely mimics the architecture of naturally occurring IgG antibodies. As such, the DVD-Ig allows for the translation of existing development platforms built around IgG therapeutics (expression, purification, and manufacturing platforms) with minimal impact on CMC development timelines and cost. We present in this chapter cumulative analytical observations gathered from characterizing a panel of DVD-Igs alongside their parent mAbs. Analytical outcomes from investigating the elements of structural complexity of the DVD-Ig molecule allow us to conclude that domain orientation has an impact on physical stability in biological fluid that correlates with PK outcomes. However, effects of domain stability in dilute buffer solutions are inconsistent. By correlating the mAb development experience with DVD-Ig structural complexity, this chapter provides the basis for evaluating the stability in vitro and in vivo and selecting stable DVD-Ig molecules with good manufacturability attributes and desired PK properties and therapeutic function. Although we have learned much about the DVD-Ig architecture and how to develop therapeutics based on this platform, many attributes of each individual therapeutic candidate need to be understood empirically and on a case-by-case basis.

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Chapter 6
High-Throughput Conformational and Colloidal Stability Screening of Biologic Molecules

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1 Introduction

1.1 Background and Purpose of Investigation

Monoclonal antibodies are the most common therapeutic proteins that are either commercially available or in clinical development [1]. Other categories of common therapeutic proteins in development include bispecific antibodies and enzymes. Although the molecular robustness of therapeutic proteins is embedded in their primary amino acid sequences, clinical efficacy and stability are more dependent upon their complex higher-order structures. These higher-order structures are maintained by an array of intrinsic and mostly noncovalent interactions that are easily perturbed by environmental factors. Proteins may unfold, exposing hydrophobic regions and aggregate. Additionally, the surfaces of proteins are often an anisotropic patchwork of charged and hydrophobic areas that contribute to colloidal associations or repulsions in solution. Furthermore, aggregates resulting from conformational unfolding or colloidal interactions may lead to immunogenicity after parenteral administration. Advanced automated, high-throughput cell culture fermentation and purification technologies may produce hundreds of candidates, in small quantities, against a common therapeutic target. Therefore, high-throughput profiling methods are

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needed for screening large numbers of candidates for robustness and selecting those with the highest likelihood of withstanding various unit operations during development as well as having appropriate long-term stabilities [2, 3].

Size-exclusion liquid chromatography (SEC) is an essential tool for detecting (separating) and measuring high molecular weight (HMW) and low molecular weight (LMW) species in a protein sample. Often, an experiment is conducted to evaluate the stability of a protein under specified environmental and aqueous conditions. Samples are collected at predetermined time points and analyzed for HMW and LMW species by SEC [4, 5]. The current generation of liquid chromatography systems may be configured to evaluate 96 or 384 samples in less than 10 minutes per sample. The chromatographic profiles (often detected at 280 nm) may be analyzed using sophisticated software that deconvolutes complicated (non-baseline separated) peaks representing the relative compositions of HMW, LMW, and native protein species. Experience with molecular profiling by SEC shows that analyzing concentrated protein solutions (≥25 mg/mL) stressed at appropriate conditions (either incubated at elevated temperatures or freeze/thaw cycling) provides useful discriminatory results compared with testing more dilute solutions. Proteins at higher concentrations are more likely to reveal intrinsic liabilities resulting from both short-range and long-range colloidal interactions [2, 6]. Consequently, HMW species resulting from partial unfolding or surface hydrophobic patches are more likely to be detected at higher concentrations. Unfortunately, dialyzing and concentrating small amounts of dilute protein solutions are slow and labor-intensive processes. The main challenge for implementing effective high-throughput molecular profiling is to identify appropriate methods and experimental conditions that can provide similar discriminatory outcomes to SEC while testing more dilute protein solutions. Dilute samples (1–20 mg/mL) may be more readily prepared using commercially available liquid handling systems [2].

Rather than collecting information about each sample, a more effective strategy for screening large numbers of proteins may be to identify and eliminate the least stable candidates first. Ideally, the screening method must be able to provide similar discriminatory results compared with SEC of concentrated samples (≥25 mg/mL) while testing more dilute samples. Currently, the best technique that matches these requirements is in-tandem differential scanning fluorimetry (DSF) and differential static light scattering (SLS) as a function of increasing temperature. Simultaneous detection assesses protein conformational stability via fluorescence emission of exposed tryptophans and aggregation state using static light scattering (SLS) [7–9]. Several recent literature references cite DSF/SLS as useful for high-throughput protein formulation and selection screening [10–12].

Our primary goal was to compare thermally derived DSF/SLS parameters with SEC results for a set of monoclonal and bispecific immunoglobulins. The purpose was to compare the risk assessments for aggregation propensity from both approaches. Our secondary goal was to determine whether DSF/SLS of dilute solutions provides comparable results to %HMW measured at 40 °C for 21 days at 100 mg/mL. All of the proteins in this study were evaluated in simple buffer without stabilizing excipients under pH conditions (two units below the isoelectric points)

that increase charge-mediated repulsions in solution to probe intrinsic properties [6]. For this evaluation, we randomly selected a set of nine mAbs and eight DVD-Igs engineered with the IgG1 framework. Detailed descriptions of the bispecific DVD-Ig architecture may be found in earlier reports [13, 14]. The variable regions of the mAbs and DVD-Igs represented significant sequence diversity against a range of therapeutic targets. Prior to the comparisons of outcomes between SEC and DSF/SLS, we measured the concentration dependencies of the temperature-dependent emission and light scatter profiles. This was important to show that emission and light scattering information obtained from dilute and concentrated protein solutions were similar. Finally, we compared the key thermal parameters from DSF/SLS with %HMW and propose an aggregation risk assessment strategy.

1.2 Introduction to Simultaneous Differential Scanning Fluorimetry and Light Scattering

The first generation of differential scanning fluorimetry techniques used thermal ramping instruments such as PCR temperature cyclers. The protein sample would be combined with a florescent dye such as Sypro Orange. The dye would interact with the protein and fluorescence intensity, and peak wavelength would shift based on the degree of protein unfolding and hydrophobic exposure in solution as a function of temperature. Since the fluorescent dyes were themselves very hydrophobic, the major drawback of this technique was interactions with the native state that may affect the unfolding rate or extent of the sample. Additional issues include quenching of the dye fluorescent intensity in the presence of certain excipients and thereby affecting differentiation between samples based on emission spectra [12, 15, 16]. Static light scattering (SLS) was accomplished separately using 96-well plate readers. The sample plate for SLS was often prone to microbubbles or trace particles that affected the results. More sample and time were needed to obtain DSF/SLS information when both techniques were accomplished separately [5, 11].

Currently, DSF/SLS may be accomplished label-free while using one instrument. The addition of hydrophobic florescent dyes remains optional. The DSF/SLS instrument measures the fluorescence emission of intrinsic tryptophan(s) that becomes exposed during thermal unfolding of the protein. The elevated temperature also induces aggregation which is detected by light scattering of the accumulating fraction of oligomers in solution [10]. One commercial DSF/SLS instrument is equipped with two lasers. One laser (260–280 nm) serves to excite intrinsic tryptophan residues and for sensitive detection of low levels of aggregates. The other laser (about 470 nm) is used for light scattering and detection of larger particles or higher aggregate concentrations [7, 8]. DSF/SLS may be used as a stand-alone protein characterization technique for thermally induced conformational stability assessment that also provides concentration-dependent light scattering information [9]. The typical DSF/SLS analysis requires less than 10 µL of sample at a wide concentration range (0.1–100 mg/mL) that is loaded into a glass capillary and arranged into

a microarray. The microarray is heated at a constant rate, both lasers simultaneously excite the samples, and fluorescent emission and light scattering are detected by a cooled closed-circuit detector (CCD) imaging spectrograph [7, 9].

Under thermal stress, the complex three-dimensional structure of the protein typically unfolds cooperatively to reveal a sigmoidal two- or three-state profile. The melting point (Tm) is determined from the midpoint of each transition, and the Tonset of unfolding (Tonset) is obtained as the fluorescence signal changes by 2–5% compared to the baseline (folded state) [9, 12]. The fluorescence signal may be represented as the ratio of tryptophan intensity between 350 and 330 nm or between fully unfolded and exposed compared with fully folded. Conversely, the fluorescence may be denoted as the barycentric mean which may better represent the shift in tryptophan fluorescence intensity based on changes in the polarity of the aqueous environment [7, 9].

Accumulation of aggregates as a result of thermal stress is detected by scattering at both 266 nm and 473 nm. The fine aggregates and low concentrations are detected at 266 nm, whereas scattering by larger aggregates and at higher fractions is detected at 473 nm. The light scattering profile is often sigmoidal in shape and may precede or follow the fluorescence profile. However, the shape of the light scattering signal is very sensitive to the size, fraction, and behavior of aggregates and may appear as a peak or remain flat depending if oligomers adhere to the capillary or drop out of solution [7]. One commercial DSF/SLS instrument uses back-reflection of static light scattering to detect the presence of aggregates. The back-reflected light passes through the sample twice. The light that passes through the capillary is back-reflected leading to a cancelling in the initial incident light signal and provides a better assessment of total aggregates in solution. Light that interacts with aggregates is scattered and not back-reflected. The difference between the initial light intensity and the back-reflected intensity that reaches the detector is used for quantitation. In Rayleigh static light scattering including back-reflected light, intensity is proportional to the product of aggregate concentration (C_{agg}), aggregate refractive index (m) raised to the fourth power, and aggregate diameter (d) raised to the sixth power:

$$I_{BR} \propto C_{agg} \times m^4 \times d^6$$

The benefit of the back-reflection detection is that it maintains the high data point density of the simultaneous dual-UV fluorescence detection to accurately determine the aggregation onset ($T_{onset\text{-}Agg}$ or Tonset) without compromising data quality [9, 17].

In summary, current DSF/SLS instruments are able to simultaneously measure intrinsic label-free fluorescence and light scattering, at two wavelengths (266 and 473 nm), respectively, in 48 samples over 2 hours using no more than 10 μg of protein. The measurement is accomplished by loading approximately 10 uL of protein solution into specialized glass capillaries that are further arranged into a microarray for analysis. Therefore, commercial DSF/SLS instruments can simultaneously determine parameters such as T_m, $T_{onset\text{-}SLS}$ (or Tagg), and $T_{onset\text{-}DSF}$ (or Tonset) during one experiment using small amount of materials ($\leq$0.1 mg) that are more readily available from high-throughput cell culture and purification.

2 Methods and Experimental Design

We selected a group of nine IgG1 mAbs and eight bispecific DVD-Igs that demonstrate a range of stabilities in solution. The concentration dependencies of the thermal parameters Tonset and Tagg were evaluated between 1 mg/mL and 100 mg/mL for the model proteins. In addition to the values, we compared the results from two DSF/SLS instruments as well as changes in the fluorescence emission and light scattering profiles for selected candidates. Finally, we compare the thermal parameters Tonset and Tagg for the model proteins with aggregation after incubation at 40 °C for 21 days and measured by SEC. .

2.1 Materials

All model monoclonal antibodies (mAb, molecular weights approximately 150 kD) and model bispecific antibodies (DVD-Ig, molecular weight approximately 200 kD) were prepared at the AbbVie Bioresearch Center (Worcester, MA). The bispecific DVD-Ig was designed by combining the complementarity determining regions (CDRs) of two precursor IgG1s into one dual-targeting protein using naturally occurring peptide linkers to attach the domains to the constant framework. Specific DNA templates were created and cloned into an appropriate expression vector. The model proteins were expressed in Chinese hamster ovary (CHO) cells and grown in bioreactors. After an appropriate incubation period, the CHO cells were harvested and lysed, and the supernatants were purified by a multistep chromatography process and diafiltered into 15 mM histidine buffer pH 5.2–6.0 (two units below pI of protein). The final quality of the nine model IgG1s and eight DVD-Igs met purity standards adherent with regulatory requirements. The monomer purity of all proteins was >95% by SEC, and the MW was confirmed by nonreduced intact mass spectrophotometer analysis (Agilent 1290 UPLC and Agilent 6545XT AdvanceBio LC/Q-TOF) as the front end. Following confirmation of identity, all proteins were manually concentrated to ≥50 mg/mL (Allegra X22R/Beckmann Coulter centrifuge) in appropriate spin columns (Amicon Ultra 15 30K MWCO) [13, 14].

2.2 Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) was accomplished using an Agilent 1100 instrument, and separation of protein species of differing molecular weights was accomplished with a TSKgel Super 3000SWXL, 5 μm, 4.6 × 300 mm column (at 25 °C) (Tosoh Bioscience). The mobile phase consisted of 100 mM Na_2HPO_4/100 mM Na_2SO_4, PH 6.8, adjusted to a flow rate of 0.8 mL/min. The

samples were held at 4 °C in type 1 glass low-volume inserts within standard vials, and 20 μL was injected onto the column for each analysis as programed in the sample schedule using the associated ChemStation software. The run time for each analysis was 20 minutes. Detection of species was accomplished using a photodiode array UV detector that measured absorbance at both 214 nm and 280 nm, and the relative percent of each species was calculated relative to total peak area.

2.3 Simultaneous Differential Scanning Fluorimetry and Static Light Scattering

The UNIT (Unchained Labs, Pleasanton, CA) and Prometheus (NanoTemper Technologies GmbH, Munich, Germany) were used for simultaneous DSF/SLS analysis of proteins derived from CHO cells described above. Approximately 10 μL of sample was loaded into special capillaries at concentrations ranging from 1 mg/mL up to 100 mg/mL as specified. The thermally derived fluorescence and light scattering transitions were detected over a range of 25–95 °C at a heating rate 1 °C/minute. Fluorescence emission is reported as the barycentric mean (BCM) or ratio 350 nm/330 nm of for the Prometheus. Light scattering intensity from the UNIT is reported from 266 nm, whereas backscatter light intensity is reported from the Prometheus. The melting point (Tm) is identified from the midpoint of the fluorescence emission profile slope. The onset of melting (Ton) or onset of aggregation by SLS (Tagg at 266 nm or 473 nm) is identified using the analytical suites of the instrument software that detect the first significant deviation from the profile baseline.

2.4 Statistical Analysis

Without any assumptions about the nature of the correlation between two variables, the Spearman rank-order correlation (SROC) was used to determine whether there is a positive or negative monotonic relationship between aggregation (%HMW) and either Tonset or Tagg:

$$r_S = 1 - \frac{6\Sigma d_j^2}{n\left(n^2 - 1\right)} \tag{1}$$

The SROC is represented by r_S, where n is the number of %HMW and Tonset-DSF or Tonset-SLS observations and d is the difference between the ranked values for both variables [11].

3 Results and Discussion

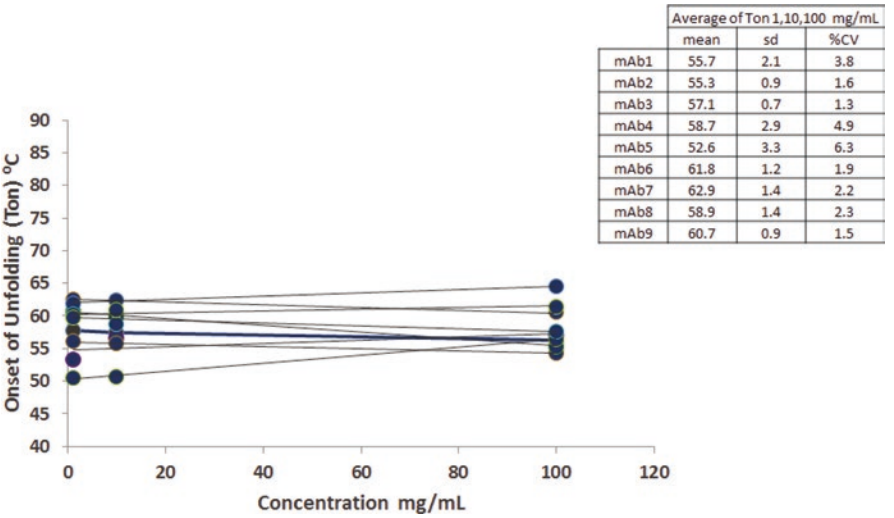
3.1 Differential Fluorescent Emission and Static Light Scattering Profiles as a Function of Concentration

Monoclonal Antibodies

The fluorescent emission (DSF) profiles of nine IgG1 mAbs showed a linear and relatively independent relationship between the onset of unfolding (Tonset) and concentration between 1 mg/mL and 100 mg/mL. Instead of reporting correlation coefficients, we calculated the average and coefficient of variation (%CV) between the Tonset values obtained at the three concentrations. Except for one example, where CV ≈ 6%, the CV < 5% for eight of the nine mAbs indicates generally low variability between values obtained within the concentration range. In addition to the low variability in Ton with concentration, approximately four of the mAbs trended slightly lower in Tonset as concentration increased. At higher concentrations, increased interactions leading to conformation changes may be possible at increasing temperature [6]. Interestingly, mAb5 with CV > 5% trended higher with increased concentration. Rather than improved stability, this may reflect a change in the fluorescence emission profile with increased concentration or changes in the polarity of the exposed tryptophan environment [12]. For the eight mAbs with CV < 5%, the Tonset ranged between 55 °C and 63 °C. The mAb with CV > 5% had Tonset of 53 °C (Fig. 1a).

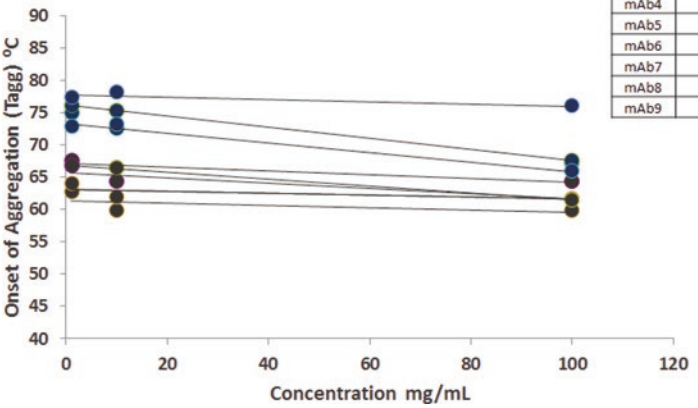
The aggregation temperature (Tagg) obtained from the light scattering (SLS) profiles for the nine mAbs were linear but generally showed more variability with concentration. Four of the nine mAbs had a CV > 5%, and mAb5 was not included in this group. Although most Tagg values trended lower at higher concentrations, there were more examples of trending towards higher values with concentration compared to unfolding. For the five mAbs with CV < 5%, the Tagg ranged between 61 °C and 77 °C. The four mAbs with CV > 5% had Tagg between 64 °C and 73 °C (Fig. 1b). Consequently, within all cases Tonset of unfolding preceded the start of aggregation Tagg.

At 100 mg/mL, the fluorescence emission profile may not reach a plateau. This is likely the result of fluorescence detector saturation at the higher concentrations. Therefore, the Tm values that are determined from the linear portion of the profiles (within a transition) may be over or under estimated because the midpoint cannot be accurately detected in some cases. Also the number of transitions in the emission profile, which define whether complete unfolding follows a two- or three-state mechanism, may be less pronounced or shifted at higher compared with lower concentrations. This may reflect the more complex array of interactions that occur at higher protein concentrations and temperatures and the effect on tryptophan environment. Nevertheless, the emission profiles of mAbs at 1 mg/mL and moderately increased concentrations such as 10–15 mg/mL are approximately similar. As a



	Average of Ton 1,10,100 mg/mL		
	mean	sd	%CV
mAb1	55.7	2.1	3.8
mAb2	55.3	0.9	1.6
mAb3	57.1	0.7	1.3
mAb4	58.7	2.9	4.9
mAb5	52.6	3.3	6.3
mAb6	61.8	1.2	1.9
mAb7	62.9	1.4	2.2
mAb8	58.9	1.4	2.3
mAb9	60.7	0.9	1.5

Fig. 1a Tonset of unfolding (Ton) of mAbs as a function of concentration (mg/mL). The Tonset of unfolding is generally unchanged as a function of mAb concentration. Significant change in Tonset is demonstrated as CV > 5% of the average between 1, 10, and 100 mg/mL. Most mAbs have a CV < 5% and trend slightly lower with increasing concentration. One mAb with CV > 5% trends higher with increasing concentration (mAb5). Trending of Tonset with concentration reflects changes in the emission spectrum attributed to protein conformation state and tryptophan environment. The Tonset values were acquired using the UNIT DSF/SLS instrument (Unchained Labs)



	Average of Tagg 1,10,100 mg/mL		
	mean	sd	%CV
mAb1	66.1	1.6	2.4
mAb2	71.5	4.0	5.6
mAb3	60.7	1.7	2.9
mAb4	64.2	2.5	3.8
mAb5	62.5	1.2	1.9
mAb6	70.6	4.1	5.9
mAb7	72.9	4.7	6.5
mAb8	63.9	3.5	5.4
mAb9	77.1	1.1	1.4

Fig. 1b Tonset of aggregation (Tagg) of mAbs as a function of concentration (mg/mL). The Tagg changes more significantly with concentration compared with Ton for this set of mAbs. Similarly, significant change in Tagg is demonstrated as CV > 5% of the average between 1, 10, and 100 mg/mL. Of the 9 mAbs evaluated, 4 have a CV > 5% indicating a more significant change in Tagg with concentration. The Tagg will be sensitive to concentration as well as the size of aggregates in solution. The Tagg values were acquired using the UNIT DSF/SLS instrument (Unchained Labs). The Tagg at 1 mg/mL was acquired at 266 nm and at 473 nm for the higher concentrations

result, the onset of unfolding (Tonset) is a more reliable parameter to use for assessment of conformational changes across a wide range of concentrations compared with Tm (Fig. 2).

Similar to the DSF profile, the SLS profile may not reach a plateau. The SLS profile is strongly sample specific and dependent on concentration. Therefore, using a Tm-like determination from the profile may not provide reliable information for relative comparisons. Changes in the SLS profile are anticipated at higher concentrations because light scattering is affected by the concentration and shape of colloids in the solution. Once unfolded, the mAbs may form a large number of small oligomers, coalesce into larger precipitates, or adsorb to the capillaries. And these oligomer phases are dynamic which change as temperature continues to increase (Fig. 2) [9, 10, 17].

The Tm and Tonset values were mostly equivalent between both instruments at the three concentrations. For this subset of three mAbs, the Tagg determined at 10 mg/mL showed an approximate 5°–10° difference between instruments. This difference in Tagg was less pronounced at 100 mg/mL. Additionally, Tagg could not be determined at 1 mg/mL for the three mAbs using the second instrument. Scattered and back-reflected light may be less sensitive to low levels of oligomers (in dilute samples). Also the software algorithms that calculate Tagg in both instruments may use different criteria resulting in minor variability. Although differences in profiles and calculated values exist between both instruments, the same conclusions regarding sample stability are obtained (Table 1).

Dual-Variable Domain Immunoglobulins

The fluorescent emission (DSF) spectra of eight DVD-Igs (based on IgG1 architecture) showed linear but relatively more dependent relationships between the onset of unfolding (Tonset) and concentration in the range of 1–100 mg/mL compared with mAbs. Similarly, instead of reporting correlation coefficients, we calculated the average and coefficient of variation (%CV) between the Ton values obtained at the three concentrations. Five of the eight DVD-Igs showed a strong dependence of Tonset with concentration, CV 6–8%. Six of the eight DVD-Ig Ton values trended lower with increasing concentration, whereas two out of eight trended higher. Not surprisingly since DVD-Igs have approximately 30% higher molecular weights and are more complex proteins compared with mAbs, higher concentrations appeared to exacerbate interactions leading to conformation changes especially at increased temperature. For the DVD-Igs with CV < 5%, the Tonset ranged between 50 °C and 65 °C, and for the ones with CV > 5%, the Tonset ranged from 46 °C to 64 °C (Fig. 3a).

The aggregation temperature (Tagg) obtained from the light scattering (SLS) profiles for the eight DVD-Igs was also linear, and all trended lower at higher concentrations. Four of the eight DVD-Igs had a Tagg CV > 5%, but only three of these also had a CV > 5% for Tonset. For the four DVD-Igs with CV < 5%, the Tagg ranged between 52 °C and 61 °C. The four DVD-Igs with CV > 5% had Tagg

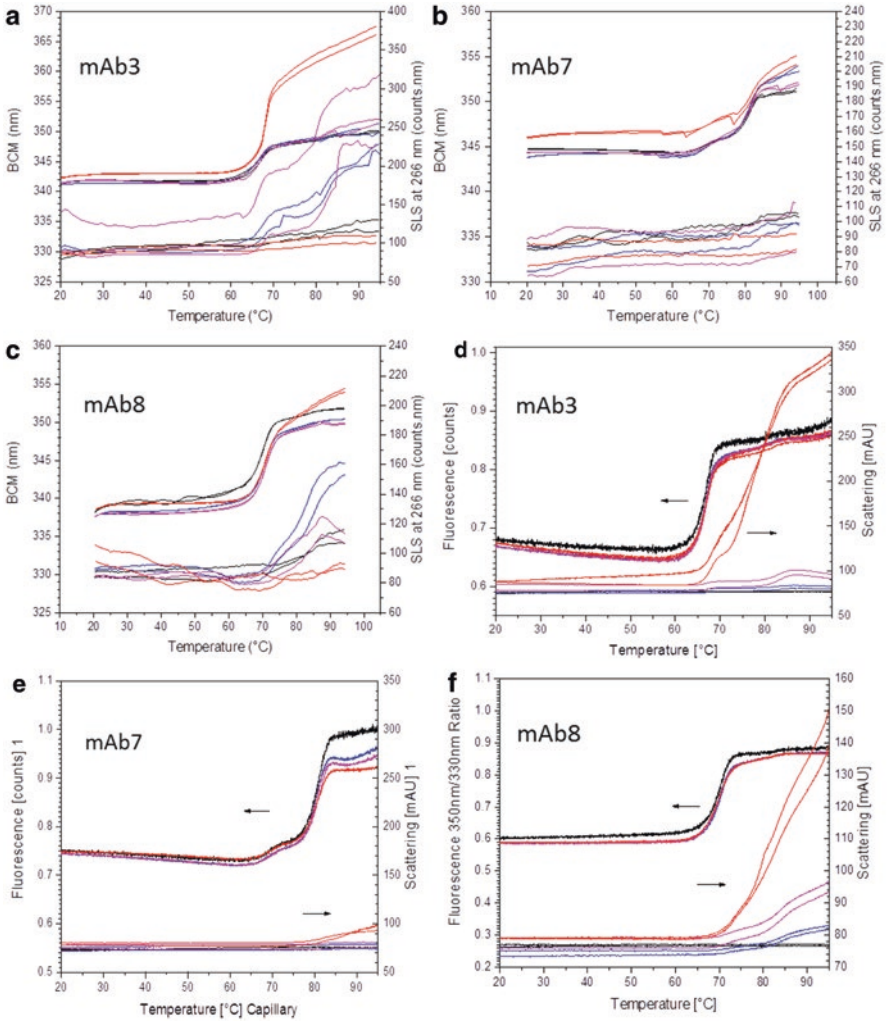
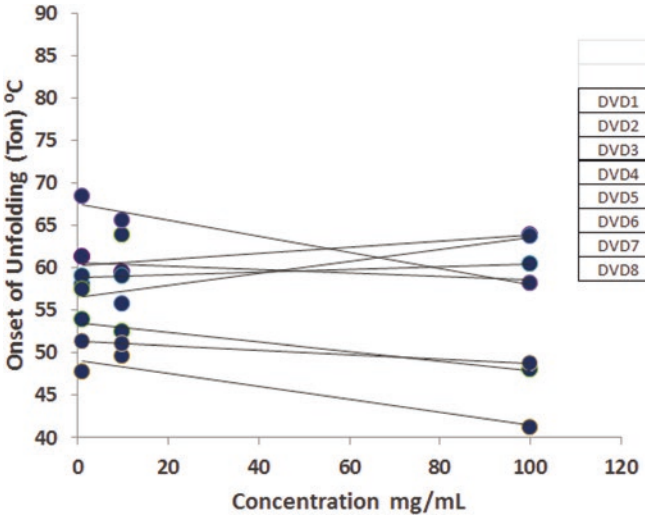


Fig. 2 Concentration dependence of emission spectra and light scattering profiles of mAbs and comparison between two instruments. The mAbs in these profiles had the highest thermal parameter dependence on concentration within the group. Profiles (**a**–**c**) were acquired using the UNIT (Unchained Labs), and profiles D–F were acquired using the Prometheus (NanoTemper). Black lines represent 1 mg/mL, red lines represent 100 mg/mL, and blue and purple lines represent 10 and 20 mg/mL, respectively. Fluorescence emission spectra appear nearly identical between both instruments. Light scattering (bottom) profiles in (**a**–**c**) were acquired at 266 nm which was appropriate for fine aggregates and low concentrations. Conversely, acquisition of scattering profiles and Tagg at 473 nm is appropriate for larger aggregates and higher concentrations (not shown). The scattering profiles (**a**–**c**) (266 nm) show more variability between replicates, higher signal-to-noise ratio, and higher error in the calculated Tagg. The 100 mg/mL scatter profiles at 266 nm are flat, whereas at 473 nm, they are flat at 1 mg/mL. The Prometheus instrument provides the profile resulting from back-reflected light (bottom **d**–**f**). The profiles from ed light were low or flat at 1 mg/mL for these mAbs suggesting that aggregates were not detected

Table 1 Comparison of thermal parameters for mAbs

mAb	DSF / SLS	1 mg/mL			10 mg/mL			100 mg/mL		
		Tagg °C	Ton °C	Tm °C	Tagg °C	Ton °C	Tm °C	Tagg °C	Ton °C	Tm °C
mAb3	1	62.7 ± 0.3	57.7 ± 1.1	64.2 ± 0.6	59.7 ± 0.3	57.4 ± 0.8	63.8 ± 1.1	59.7 ± 0.7	56.3 ± 0.3	68.6 ± 1.1
	2	nd	59.6 ± 0.2	66.7 ± 0.0	65.4 ± 0.3	59.6 ± 0.1	66.6 ± 0.1	61.5 ± 0.0	59.4 ± 0.4	66.8 ± 0.0
mAb7	1	76.1 ± 0.3	62.1 ± 0.2	68.8 ± 0.3	75.2 ± 1.1	62.3 ± 0.6	67.5 ± 0.2	72.5 ± 0.4	64.5 ± 0.5	67.5 ± 0.7
	2	nd	65.4 ± 0.9	69.4 ± 0.2	80.7 ± 0.3	64.4 ± 0.1	69.3 ± 0.2	75.2 ± 0.3	65.3 ± 0.1	69.5 ± 0.1
mAb8	1	66.5 ± 1.7	60.6 ± 0.5	67.1 ± 0.2	66.3 ± 0.7	58.9 ± 0.5	65.3 ± 0.4	61.4 ± 0.2	57.6 ± 0.8	67.5 ± 0.7
	2	nd	58.8 ± 1.1	69.4 ± 0.0	75.9 ± 0.6	59.3 ± 0.3	69.3 ± 0.2	65.2 ± 0.3	58.9 ± 0.1	68.5 ± 0.1

1. UNIT, Unchained Labs (Tagg 1 mg/mL at 266 nm; Tagg 10 and 100 mg/mL at 473 nm)
2. Prometheus, NanoTemper (Tagg from back-reflection profile)
3. nd = not detected



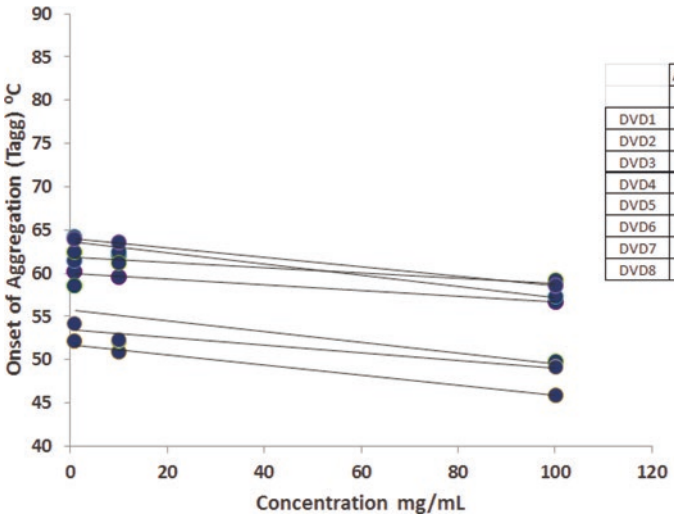
	Average of Ton 1,10,100 mg/mL		
	mean	sd	%CV
DVD1	61.5	2.3	3.7
DVD2	46.2	4.4	9.4
DVD3	59.2	4.1	6.9
DVD4	59.5	0.9	1.5
DVD5	51.4	3.0	5.9
DVD6	59.8	3.6	6.0
DVD7	64.0	5.3	8.2
DVD8	50.4	1.4	2.7

Fig. 3a Tonset of unfolding (Tonset) of DVD-Igs as a function of concentration (mg/mL). The Tonset of unfolding for the DVD-Igs was more dependent on concentration compared to mAbs (63% had CV > 5%). The Tonset values trended lower or higher with concentration, reflecting conformational changes that affect tryptophan fluorescence intensity. Also, the values were somewhat lower compared with the mAbs with three proteins having Ton <50 °C. The Tonset values were acquired using the UNIT DSF/SLS instrument (Unchained Labs)

between 50 °C and 62 °C (Fig. 6). In six out of eight examples, Tonset preceded the start of aggregation Tagg; however, in most cases the fluorescence emission and light scattering parameters were only separated about 2 °C, and in two examples, Tagg preceded Tonset by about 2 °C (Fig. 3b).

The SLS profiles for the DVD-Igs significantly vary based on sample concentration. At moderate concentrations, the SLS profiles of the DVD-Igs increase abruptly suggesting a rapid accumulation of oligomers during the early phases of thermal protein unfolding but differ from 100 mg/mL in plateau rather than Tagg. The SLS profiles from more dilute concentrations such as 1 mg/mL may not show an increase in aggregate formation with temperature and remain unchanged. The shape of the SLS profile, relationship to the DSF profile, and Tagg values changed more significantly between more dilute and moderate concentrations compared with moderate to higher concentrations. Consequently, the aggregation propensities of DVD-Igs at 100 mg/mL by SLS may be estimated by testing solutions at 10 mg/mL. Nevertheless, once unfolded, the DVD-Ig may also form a large number of small oligomers, coalesce into larger precipitates, or adsorb to the capillaries, and this would affect the SLS profile (Fig. 4) [9, 17].

The Tm and Tonset values showed more variability between instruments for this subset of three DVD-Igs compared with the mAbs. Differences in Tonset and Tm values, across the three concentrations, obtained by both instruments ranged between 5° and 8°, whereas Tagg values remained within 5 °C. Similar to the mAbs,



	Average of Tagg 1,10,100 mg/mL		
	mean	sd	%CV
DVD1	58.8	1.8	3.1
DVD2	49.6	3.3	6.6
DVD3	60.8	1.7	2.8
DVD4	61.2	3.6	5.9
DVD5	53.5	4.5	8.4
DVD6	60.9	1.7	2.8
DVD7	62.0	3.0	5.9
DVD8	51.8	2.5	4.9

Fig. 3b Tonset of aggregation (Tagg) of DVD-Ig as a function of concentration (mg/mL). The Tagg for all the DVD-Igs trended lower as concentration increased, and the dependence was significant in 50% of the proteins. The Tagg values for two of the DVD-Igs (DVD1 and DVD7) preceded the Tonset, and an additional four proteins had Ton and Tagg values within a two-degree difference. This suggests that aggregation is more dependent on conformational changes for the larger and more complex DVD-Ig formats. The Tagg values were acquired using the UNIT DSF/SLS instrument (Unchained Labs). The Tagg at 1 mg/mL was acquired at 266 nm and at 473 nm for the higher concentrations

the Tagg could not be detected at 1 mg/mL for the three DVD-Igs using the second instrument. Consequently, instrument-dependent differences result from the software algorithm that calculates Tagg. Except for one example (DVD8 at 10 mg/mL), both instruments provide conclusions that are equivalent regarding sample stability (Table 2).

3.2 Relation of mAb and DVD-Ig Aggregation to Thermal Fluorescent and Light Scattering Parameters

For the nine IgG1 mAbs, the Tonset values ranged 53–65 °C, and the Tm values ranged 65–79 °C. Although the mAbs were randomly selected from an available pool, they appear to generally have good conformational stabilities. We found no direct relationships between aggregate levels (%HMW) and Tonset, Tm, or aggregation rates (k/month, 40 °C, and 100 mg/mL) for the mAbs in this study, $r_S \approx 0.1–0.2$. Similarly, we found no direct relationship between aggregate levels (%HMW) and Tagg, $r_S < 0.1$. As a result, parameters obtained from thermal denaturation are not directly correlated with accelerated storage stability for this set of mAbs.

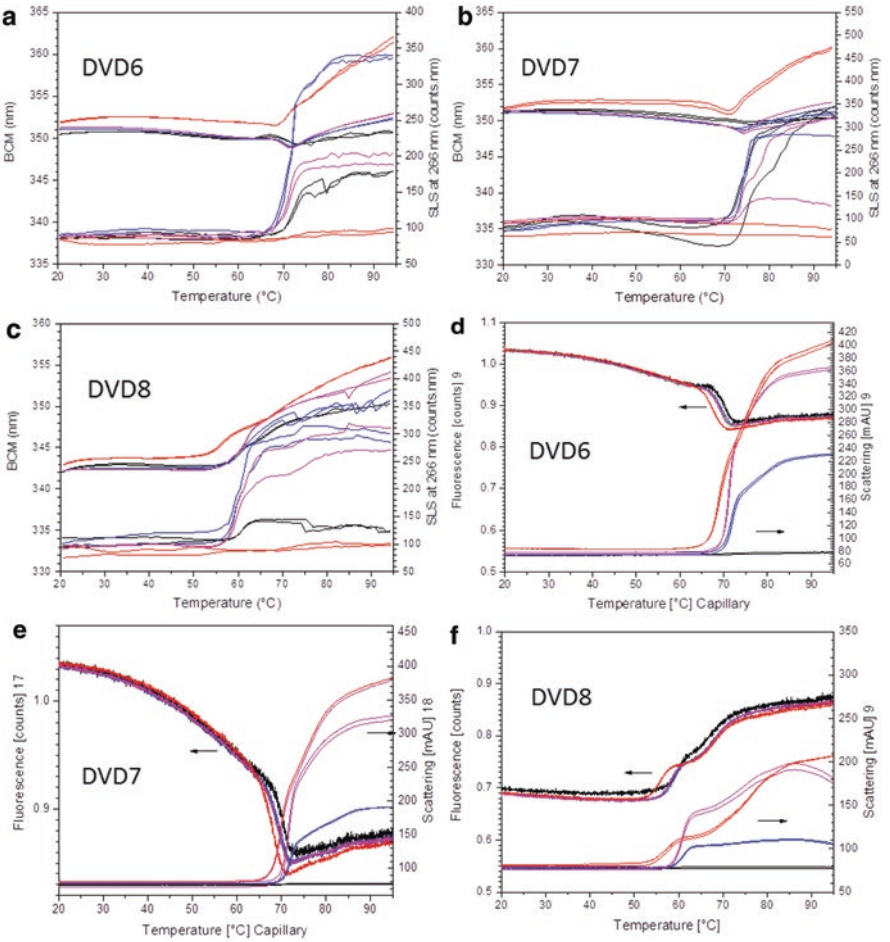


Fig. 4 Concentration dependence of emission spectra and light scattering profiles of DVD-Igs and comparison between two instruments. The DVDs representing these profiles had among the highest thermal parameter dependence on concentration within the group. Profiles (**a**–**c**) were acquired using the UNIT (Unchained Labs), and profiles (**d**–**f**) were acquired using the Prometheus (NanoTemper). Black lines represent 1 mg/mL, red lines represent 100 mg/mL, and blue and purple lines represent 10 and 20 mg/mL, respectively. Unlike the mAbs (Fig. 2), the fluorescence emission spectra appear slightly varied between both instruments; however, the calculated parameters were not significantly different. Light scattering (bottom) profiles (**a**–**c**) were acquired at 266 nm which was appropriate for fine aggregates and low concentrations. Conversely, acquisition of scattering profiles and Tagg at 473 nm is appropriate for larger aggregates and higher concentrations (not shown). The scattering profiles (**a**–**c**) (266 nm) show more variability between replicates, higher signal-to-noise ratio, and higher error in the calculated Tagg. The 100 mg/mL scatter profiles at 266 nm are flat, whereas at 473 nm, they are flat at 1 mg/mL. The Prometheus instrument provides the profile resulting from back-reflected light (bottom **d**–**f**). The profiles from back-reflected light are low or flat at 1 mg/mL suggesting that aggregates were not detected

Table 2 Comparison of thermal parameters for DVD-Igs

DVD	DSF / SLS	1 mg/mL			10 mg/mL			100 mg/mL		
		Tagg °C	Ton °C	Tm °C	Tagg °C	Ton °C	Tm °C	Tagg °C	Ton °C	Tm °C
DVD6	1	62.5 ± 0.2	65.3 ± 0.7	76.1 ± 1.1	61.2 ± 0.1	63.9 ± 1.2	76.9 ± 0.7	59.1 ± 0.1	58.2 ± 0.4	69.2 ± 0.5
	2	nd	66.4 ± 0.1	69.9 ± 0.2	64.2 ± 0.2	66.0 ± 0.1	69.0 ± 0.2	57.5 ± 0.3	64.3 ± 0.0	67.4 ± 0.0
DVD7	1	64.2 ± 0.3	66.3 ± 0.6	75.1 ± 0.2	63.4 ± 0.1	65.5 ± 0.3	75.9 ± 0.6	58.6 ± 0.3	63.3 ± 0.7	71.9 ± 1.1
	2	nd	68.0 ± 0.1	69.9 ± 0.1	65.4 ± 0.1	65.9 ± 0.1	69.1 ± 0.3	57.2 ± 0.4	62.6 ± 0.3	67.8 ± 0.1
DVD8	1	54.1 ± 0.9	51.2 ± 0.3	61.1 ± 0.3	52.2 ± 0.1	51.1 ± 0.5	57.3 ± 0.7	49.1 ± 0.3	48.8 ± 0.6	59.1 ± 0.3
	2	nd	54.2 ± 0.0	60.0 ± 0.1	56.4 ± 0.3	54.3 ± 0.0	58.9 ± 0.1	50.8 ± 0.6	52.0 ± 0.0	55.8 ± 0.0

1. UNIT, Unchained Labs (Tagg 1 mg/mL at 266 nm; Tagg 10 and 100 mg/mL at 473 nm)
2. Prometheus, NanoTemper (Tagg from back-reflection profile)
3. nd = not detected

Since proteins aggregate through colloidal and conformational pathways, one or the other mechanism will dominate under accelerated storage conditions [18]. However, during DSF/SLS, proteins denature and aggregate as a result of increased temperature. As a result neither Tagg nor Tonset represent events that are directly related to aggregation under accelerated storage conditions. Nevertheless, some proteins that have poor conformational stability may denature and aggregate with increasing temperature as well as aggregate under accelerated storage.

The Tagg values were compared against %HMW to determine if the lack of correlation was the result of several outliers. We noticed that all of the Tagg values were approximately ≥60 °C. Furthermore, one third of the mAbs exceeded 5% HMW. Consequently, we created a risk assessment space by dividing the graph into quadrants using 5% HMW and Tagg 60 °C as acceptance criteria limits. Additionally, we label each quadrant according to risk assessment where the majority of mAbs with Tagg ≥ 60 °C and % HMW ≤ 5% are in the low-risk group. The quadrant representing Tagg ≤ 60 °C and HMW ≥ 5% is labeled high risk, and the two remaining quadrants represented moderate risk for aggregation propensity (Fig. 5a). We treated the Tonset vs %HMW values in a similar manner and noticed that low-risk boundaries are appropriately established at Tonset ≥ 55 °C and % HMW ≤ 5%. In this example although most of the mAbs fell into the low-risk area, a significant fraction was distributed between both moderate risk quadrants (Fig. 5b).

In this study, the risk assessment space for aggregation propensity is based on complimentary %HMW and thermal parameters from only nine IgG1 mAbs. The usefulness of the risk assessment space increases as additional complimentary data are included and the results become distributed across all quadrants. This approach to estimating risk assessment is more helpful when the candidates share structural similarities such as all being engineered using an IgG1 framework. As a result, based on the risk assessment space established with these nine mAbs, an IgG1 candidate with a Tonset = 60 °C and Tagg = 65 °C may be estimated to have a low to moderate risk for aggregation during accelerated storage. Furthermore, the acceptance criteria that define the boundaries of the risk assessment space may be adjusted based on additional data [2].

Fig. 5 (continued) are supported by experience and prior knowledge. 1, 10, and 100 mg/mL for each mAb are shown in the displays. The relationships visually confirm that there is no dependence between %HMW and either Tagg or Ton. The Tagg and Tonset values are primarily divided between low and moderate risk for aggregation. These mAbs have equivalent IgG1 frameworks but differ in complementarity domain sequences. They have been engineered to remove large hydrophobic surface areas and pronounced chemical liabilities while optimizing biological activity. Nevertheless, differences in solution properties and stability are still evident. Consequently, a candidate mAb with a similar IgG1 framework, produced in a high-throughput manner and determined to have a Tagg = 65 °C and Tonset = 60 °C, may be estimated to have a primarily low to moderate risk for aggregation propensity during long-term storage. Risk assessment diagrams with correlations for many hundreds of mAbs along with more representative distribution would provide the estimate of aggregation propensity

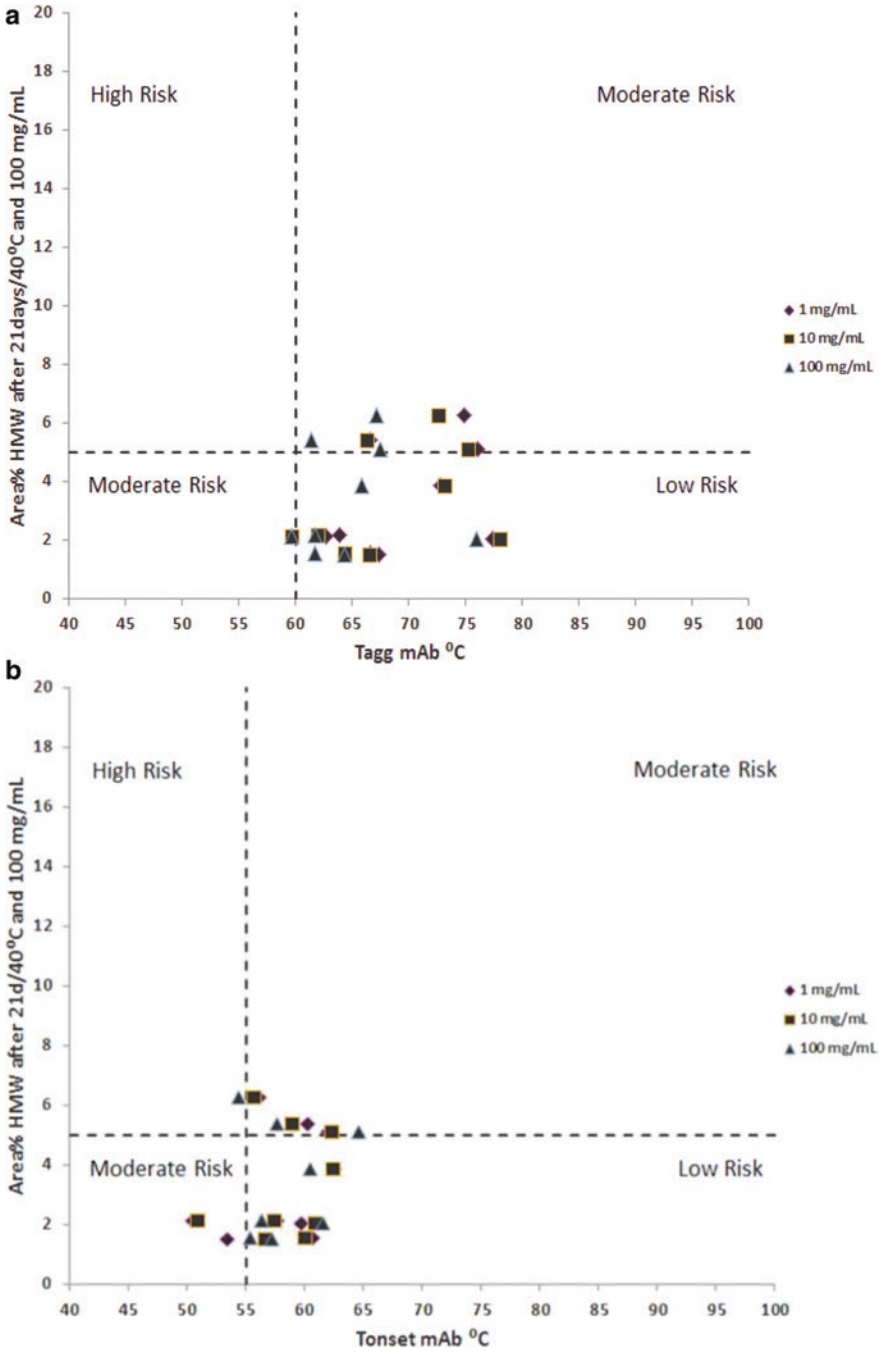


Fig. 5 Relationship between %HMW and Tagg and Ton for mAbs. Risk assessment distributions for the IgG1 mAbs in the set evaluated for this study. The risk assessment is based on acceptance criteria of 5% HMW and 60 °C for Tagg (**a**) and 55 °C for Ton (**b**). The acceptance criteria

The bispecific DVD-Igs are novel formats which, as a group, demonstrated overall lower solution stabilities than mAbs. For eight DVD-Igs, the Tonset ranged 41–68 °C, and the Tm ranged 58–79 °C. Compared with mAbs, the thermal parameters occurred over wider ranges and had more pronounced changes with concentration. The Tagg ranged 46–64 °C and in some cases preceded Tonset. Although this was a relatively small pool of candidates, we found a more significant inverse relationship between aggregate levels (%HMW) and Tonset and Tagg for the DVD-Ig in this study, $r_S \approx 0.5–0.7$. The DVD-Igs represent larger and more structurally complex proteins compared to mAbs. As a result Tagg and Tonset are likely to represent events that occur under accelerated storage conditions that lead to aggregation. Consequently, aggregation of the DVD-Igs under accelerated storage conditions may be more dependent upon partially unfolded states rather than colloidal interactions in the native state.

While charting %HMW against Tagg, we noticed that most values were approximately ≥55 °C. Furthermore, 50% of the DVD-Igs exceeded 10% HMW. As a result, we used 10% HMW and Tagg 55 °C as acceptance criterion limits or boundaries of the risk assessment space. Similarly, we label each quadrant according to risk assessment where the DVD-Igs with Tagg ≥ 55 °C and % HMW ≤ 10% are in the lower-risk group for aggregation propensity. The quadrant representing Tagg ≤55 °C and HMW ≥ 10% is labeled high risk, and the two remaining quadrants representing intermediate profiles are labeled as moderate risk for aggregation propensity (Fig. 6a). Of the DVD-Ig in this study, a significant number (3/8) were shown to be high risk for aggregation propensity with very high oligomer levels and low conformational stabilities. One candidate fell into the intermediate category, and the remainder of these bispecifics were classified as lower risk for aggregation. The results for Tonset paralleled Tagg for the bispecifics in this study. Following DSF/SLS screening of a set of DVD-Igs (engineered with the IgG1 framework) in the absence of SEC data, we would select those with Ton and Tagg ≥ 55 °C with a reasonable assumption of low to moderate risk of aggregation during accelerated storage conditions (Fig. 6b).

4 Summary and Conclusion

The goal of this study was to determine if DSF/SLS may be used instead of SEC for molecular profiling and developability assessment of aggregation propensity. Although SEC remains the standard analytical technique for identifying HMW

Fig. 6 (continued) pronounced chemical liabilities while optimizing biological activity. Solution stability for these more complex proteins may be more dependent on conformation compared with colloidal properties. As a result, thermally induced unfolding and aggregation of DVD-Ig may be better at predicting outcomes during accelerated storage conditions compared with mAbs. Consequently, a candidate DVD-Ig determined to have a Tagg = 50 °C and Tonset = 45 °C appears to be very likely to have a high risk for aggregation during long-term storage. Risk assessment distributions with correlations for many hundreds of DVD-Igs along with results distributed across all quadrants would provide a more confident aggregation propensity evaluation

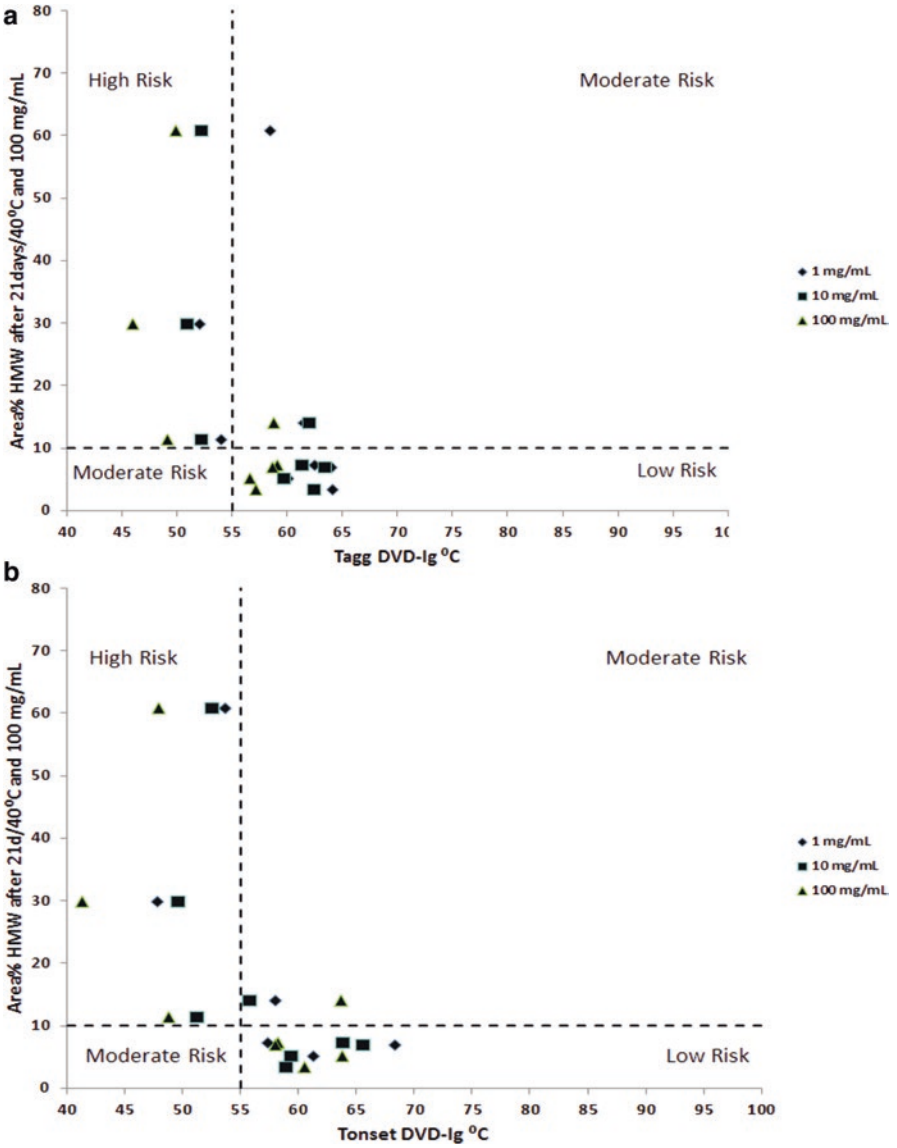


Fig. 6 Relationship between %HMW and Tagg and Tonset for DVD-Igs. Risk assessment distributions for the DVD-Igs in the set evaluated for this study. The risk assessment is based on acceptance criteria of 10% HMW and 55 °C for Tagg (**a**) and Tonset (**b**). The acceptance criteria are supported by experience and prior knowledge. 1, 10, and 100 mg/mL for each DVD-Ig are shown in the displays. The relationships visually confirm that there is an inverse dependence between %HMW and both Tagg and Ton. The Tagg and Tonset values are primarily divided between low, moderate, and high risk for aggregation. These DVD-Igs have equivalent IgG1 constant regions but differ in complementarity domain sequences as well as linker lengths between outer and inner domains. Consequently, these bispecific antibodies are more conformationally complex compared to mAbs although they have been engineered to remove prominent hydrophobic surface areas and

species in solution, sample preparation, incubation time, and data analysis continue to motivate the search for alternative methods of high-throughput candidate selection or formulation screening [2]. We set out to investigate the potential of replacing %HMW by SEC with thermal parameters derived from DSF/SLS. We hoped to determine if comparisons with %HMW from SEC were affected by sample concentration during DSF/SLS analysis and endeavored to understand the relationship with Tonset and Tagg parameters.

DSF/SLS is an accessible high-throughput technique that uses only 10 μL of sample over a wide range of protein concentrations [4, 8–10]. This is especially useful when only trace amounts are available during molecular profiling to select developable candidates. Evaluating a wide range of concentrations is in contrast to traditional DSC which has a limited sample concentration range (0.5–2 mg/mL) [12]. Tonset of unfolding and Tagg have been reported to be reliable parameters for assessing thermal and colloidal stability by DSF/SLS [10, 12]. Although Tonset and Tagg are relatively invariant with concentration, significant changes may imply concentration-dependent stability of the analytes.

All proteins aggregate in solution through a combination of conformational or colloidal pathways. The dominant pathway is dictated by the intrinsic properties of the protein [18]. DSF/SLS induces structural changes in the protein during a constant heating rate. As a result aggregation that is measured by SEC that occurs under accelerated (25 °C and 40 °C) conditions may compare poorly with parameters obtained from a thermal ramping experiment [5]. The set of IgG1 mAbs in this study showed poor correlations between Tagg and Tonset with %HMW by SEC. Nevertheless, a risk assessment space may be constructed using appropriate boundaries for transition temperatures and %HMW based on experience and prior knowledge. This diagram may be used as a reference when estimating the aggregation propensity based on thermal parameters. More data refines the empirical boundries and provides more confidence for estimating protein aggregation propensity [2].

We have seen that this approach may be applied to mAbs as well as novel format bispecific DVD-Igs. The DVD-Igs show a stronger inverse dependency between aggregation and thermal denaturation compared to mAbs. This may be the result of a stronger dependency on conformational stability (partial unfolding) in solution compared with the IgG1 mAbs in this study. A previous report showed a stronger dependency of Tonset with aggregation rate for a set of eight mAbs that demonstrated a range from 48 °C to 64 °C and aggregation rates between 1% and 3.5%/month [10]. By comparison, the nine mAbs in this study ranged in Ton between 53 °C and 63 °C and aggregation rates between 0.6% and 2.6%/month without a correlation between the variables. This clearly underscores the need for evaluating a significantly larger and structurally diverse sample population to better understand the relationship between aggregation and parameters from DSF/SLS.

We have demonstrated the utility of DSF/SLS for comparing individual mAbs and DVD-Igs for aggregation propensity. However, DSF/SLS may also be used and often applied to screen formulation conditions for any protein. Solution conditions such as pH or excipient concentrations are varied and the relative changes in Tagg and Tonset or Tm evaluated. The relationship between Tagg and Tonset is

also important as different conditions may accelerate or delay denaturation and aggregation separately [5, 7–9, 11, 19].

NOTE: All of the analysis and preparation for this manuscript were conducted at the AbbVie Bioresearch Center, Worcester, Massachusetts.

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Chapter 7
An Empirical Phase Diagram: High-Throughput Screening Approach to the Characterization and Formulation of Biopharmaceuticals

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1 Introduction

The stability of macromolecule-based drug products and vaccines, in contrast to traditional small-molecule drugs, is more often a significant problem for the pharmaceutical and biotechnology industry and often leads to major issues in development, manufacturability, storage, and shipment. The integrity of these biopharmaceuticals is often compromised due to effects of temperature, pH, contaminants, and excipient incompatibility as well as the intrinsic instability of the molecules themselves. This can jeopardize not only therapeutic efficacy but also the ability to store and ship such agents in a pharmaceutically acceptable manner. Thus, regulatory agencies require that companies have a thorough understanding of their

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product's manufacturing control points prior to launch as well as the ability to demonstrate long-term storage stability. It is therefore important to develop a product in which a comprehensive understanding of the ramifications of nonoptimal conditions during both manufacturing and storage is available.

While an empirical approach is often used to understand the stability and develop appropriate formulations of macromolecular systems, results of such studies often lead to less than optimal formulations. This can create a variety of difficult problems in the drug development process. Furthermore, many smaller companies attempting to develop biopharmaceuticals have neither the resources nor the time to invest in prolonged investigations. Therefore, it is desirable to have in place a set of processes or systems that can produce a detailed understanding of stability while at the same time introducing both efficiency and speed to the preformulation and formulation processes. This review focuses on one such systematic approach developed in our lab for the rapid characterization and formulation of biopharmaceuticals. Additionally, several new approaches are also described here for comparison purposes.

A schematic representation of the "empirical phase diagram/high-throughput screening" approach described here to optimize macromolecule formulation is shown in Fig. 1 and can be briefly described as follows. Extensive studies using a variety of techniques sensitive to different properties of the target macromolecules are performed to characterize the physical, chemical, and biological properties of the drug candidate under a range of solution conditions. The data generated in the process is combined to construct a more intuitive visual picture of the results, which is known as an empirical phase diagram (EPD). This not only permits a relatively straightforward interpretation of the complex data sets usually generated but also

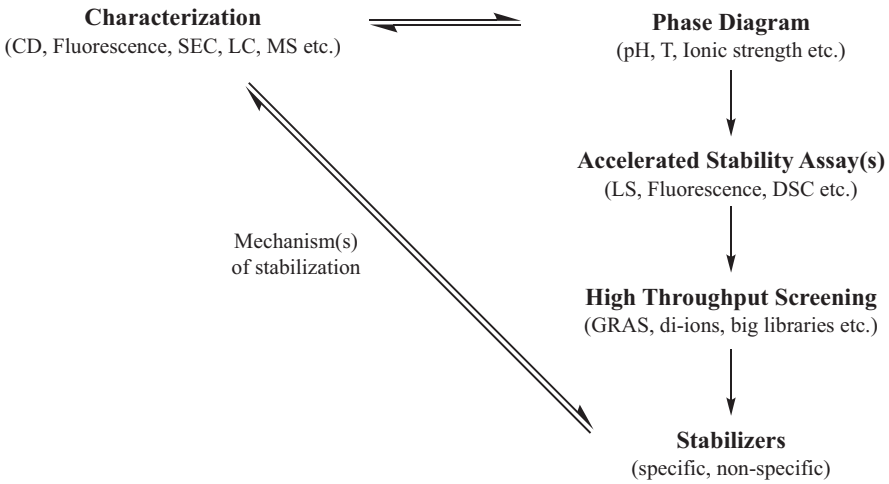

Fig. 1 A general approach to the preformulation studies of macromolecules employing EPDs in conjunction with high-throughput screening to identify stabilizers

graphically depicts zones of defined structural behavior and regions of change of the molecule or complex of interest. This information is then used to design high-throughput screening assays to select potential stabilizers for the biopharmaceutical. The potential excipients thus obtained are then tested for their ability to stabilize the macromolecule against physical and chemical degradation and are further optimized to obtain a desirable formulation. In this review, this approach to optimize macromolecule formulation is described in more detail, and a few specific examples of studies using this method undertaken within the authors' laboratory are provided. This review has been updated from the original version in the first edition of this book with recent work using the EPD approach [1].

2 Biophysical Characterization of Biopharmaceuticals

Optimization of a formulation requires a detailed understanding of the macromolecule's properties under a variety of pharmaceutically relevant stresses. Despite the individual structural and functional differences, biopharmaceuticals ranging from simple peptides, proteins, and nucleic acids to more complex systems such as viruses, viral-like particles (VLPs), and bacteria are primarily comprised of proteins, nucleic acids, lipids, and polysaccharides as bioactive components. The functions of all such macromolecules are usually dictated by their structures. Thus, in most cases, finding the optimal conditions that maintain and/or enhance the structure/stability of macromolecules to be used as drugs or vaccines is the main objective of preformulation studies. In this regard, factors such as pH, temperature, ionic strength, buffer system, drug concentration, solute binding, shear forces, presence of contaminants, and adjuvants in the case of vaccines can all alter the intra- and intermolecular interactions that maintain the structures of macromolecules [2], resulting in a loss of their biological function. A wide variety of experimental techniques can be used to characterize these bioactive components, although in this review we will confine ourselves to the characterization of proteins and nucleic acids, since these are the two classes of macromolecules most commonly used in biopharmaceuticals. Further information about these methods is provided in another chapter of this book.

Far-UV circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy are the two techniques most commonly employed to monitor the secondary structures of proteins and DNA, although Raman spectroscopy is also used occasionally. Similarly, UV-visible absorption, fluorescence, and near UV-CD are powerful spectroscopic techniques for detecting alterations in tertiary structure. Note that despite the superior resolving power of nuclear magnetic resonance (NMR) and X-ray crystallography, these techniques will not be addressed in this article since they are still primarily used indirectly in formulation development. Techniques such as dynamic light scattering (DLS), optical density measurements, and size exclusion chromatography (SEC) are commonly employed to monitor the aggregation

behavior of proteins and nucleic acids. A brief description of these representative techniques, pertinent to the examples presented in this review, is provided below.

The circular dichroism of a macromolecule arises from the differences in absorption of left- and right-handed circularly polarized light due to the presence of optically active chromophores. Both proteins and nucleic acids contain a chromophore (the peptide bond and the purine and pyrimidine bases, respectively), that is often arranged in a regular array to produce dramatic optical activity. The far-UV CD spectra (below 260 nm) of proteins are very sensitive to changes in protein secondary structure with alterations of 2–3% in helix or sheet content easily detected. Secondary structural elements of each protein exhibit characteristic far-UV CD signals, with the α-helix exhibiting a strong double minimum at 222 and 208–210 nm and a stronger maximum at 191–193 nm. In contrast, β-sheet structures typically manifest a weaker single minimum between 215 and 217 nm and a stronger positive maximum between 195 and 200 nm [3]. Certain rarer forms of β-structure as well as turns and irregular structure also manifest characteristic spectra. Changes in protein structure often cause loss of signal intensities or distortion of the peaks inducing shifts in wavelength maxima of the CD spectra. For example, alteration from a typical spectrum of an α-helix to that characteristic of β-sheet when the temperature is elevated may suggest formation of intermolecular β-sheets during protein association at high temperatures [4]. CD spectra are also very sensitive to secondary structure changes in nucleic acids. Due to the differences in base-stacking interactions, the major secondary structures found in DNA, such as the A, B, C, D, T, and Z forms, can be identified by their unique CD spectra between 190 and 300 nm. Typically, the protein and plasmid DNA concentrations used for CD studies are in the range of 100–200 μg/mL and 50 μg/mL, respectively, using a 0.1 cm pathlength cell [5]. This technique has also been shown to be useful in the investigation of nonviral gene delivery complexes in which changes in DNA structure can be easily seen as various cationic delivery agents complex to nucleic acids (both DNA and RNA) [5, 6].

Intrinsic and extrinsic fluorescence spectroscopy are often the methods of choice to study alterations in the tertiary structures of proteins. The dominant natural fluorophore in proteins is usually the indole ring of tryptophan, which absorbs near 290 nm. In addition, the emission spectrum of tryptophan is highly sensitive to the polarity of its immediate environment. Thus, upon unfolding of a protein and subsequent exposure of the tryptophan residues from the usual apolar environment of a protein's interior to the polar aqueous solvent, a red shift in the emission peak is typically observed. Also, specific environmental changes can unpredictably perturb the intensity and quantum yield of tryptophan fluorescence due to its unique interactions with water, oxygen, solutes, peptide bonds, and other amino acid side chains [7]. One phenomenon commonly observed is a blue shift in emission maxima due to protein association arising from an increased burial of indole sidechains. It is also possible to obtain light scattering data during such experiments by monitoring the scattered light seen at the excitation wavelength. The preferred method to do this is to use a second photomultiplier located at 180° to the fluorescence detector, but one

can also simply scan through the entire emission spectrum to obtain such data. Thus, information can be obtained about association/dissociation phenomena simultaneous with fluorescence emission data. Because of their sensitivities to the polarity of microenvironments, extrinsic probes have also become important tools with which to monitor protein structural changes. The fluorescent probes most commonly used to detect tertiary structural changes of proteins are 8-anilino-1-naphthalenesulfonate (ANS) and its dimeric analogue (bis-ANS). These hydrophobic probes are essentially nonfluorescent in aqueous solution but become strongly fluorescent in a less polar environment. ANS is, thus, commonly used to identify partially folded intermediate forms of proteins such as molten globule states [8, 9]. When ANS binds to apolar sites in a protein which become accessible due to structural perturbations, a blue shift of the fluorescence emission maxima and an increase in the fluorescence intensity are usually observed. One needs to be careful in interpreting ANS fluorescence data since ANS may be able to induce structural changes in the protein [10, 11]. Furthermore, potential interactions between the negatively charged ANS and oppositely charged residues on the protein can further complicate interpretation [12]. Fluorescent dyes can also be used to detect the presence of specific types of interactions between proteins. Some of the more common dyes used for this purpose are Congo Red [13], Thioflavine S [14], and Thioflavine T [15, 16]. These dyes are selective for intermolecular beta-sheet interactions that are present among proteins that form beta amyloid structures [17]. As such, these dyes are used as indicators of aggregation, as well as probes of intermolecular beta-sheet contacts that occur between subunit-subunit interfaces of viral capsid proteins [18] and other multimeric protein complexes [19]. Usually, the protein concentrations used for fluorescence studies are about 100–200 μg/mL. In general, one wishes to keep the absorbance at the excitation wavelength below 0.1 to prevent inner filter (self-absorption) effects.

The intrinsic fluorescence of the DNA and RNA bases is too weak for practical applications. The existence of numerous nucleic acid probes, however, enables extrinsic fluorescence to be widely used in this context. Commonly used DNA fluorescent probes include ethidium bromide and other high-affinity dyes such as YOYO-1, TOTO-1, acridine orange, propidium iodide, Hoechst, etc. [5]. Such dyes can be used through their direct interaction with nucleic acids (e.g., in the helix grooves or between the bases) or in displacement assays in which dyes are competitively removed by ligands of interest [9]. Because of the high sensitivity, the DNA concentration used in extrinsic fluorescence study is often in the nanomolar range [5].

UV absorption spectroscopy can also be used to probe changes in tertiary structure of a protein. For this purpose, high-resolution derivative UV absorption spectra are resolved into five to seven peaks originating from Trp, Tyr, and Phe residues. This method can simultaneously monitor changes in the microenvironment of all three aromatic residues, thus providing a more global picture of the behavior of protein tertiary structure than the fluorescence-based techniques. When the native state of a protein changes, the microenvironment surrounding each individual

aromatic residue is subject to alteration. This can produce shifts in the positions of the absorption peaks. In general, these absorbance peaks shift to lower wavelengths (blue shift) when the aromatic residues become more solvent exposed and vice versa. For these studies, the protein concentration should be selected to keep the absorbance at 280 nm below 1.0. To obtain the high resolution needed, use of a diode array spectrometer is highly recommended with an interpolative technique such as splining to obtain the necessary effective resolution [20]. In addition, this technique can also be used to detect significant protein aggregation induced by various stresses, such as pH, temperature, and high concentration, by measuring changes in optical density (turbidity) in the near UV region (e.g., 320–400 nm). Temperature- and/or pH-dependent changes in OD (optical density) are often used to provide profiles of protein stability that can easily be used in microtiter-plate-based high-throughput screening assays and subsequent formulation development, as will be discussed below. The UV absorbance spectra of nucleic acids can also be deconvoluted into contributions from individual bases, but this is not generally as useful as in proteins. Derivative spectra can, however, still be used to detect structural changes since alterations in the interaction between the bases produce large changes in absorbance [10, 17]. Thus, absorbance-detected thermally induced melting of the double helix and the effects of other environmental perturbations are commonly used to monitor nucleic acid structural stability, in both DNA- and RNA-based complexes as well as viruses.

Other techniques such as infrared spectroscopy (FTIR), dynamic light scattering (DLS), and differential scanning calorimetry (DSC) are also often employed in the construction of phase diagrams. A detailed description of these techniques can be found elsewhere [20, 21] and in chapter 8 in this volume.

3 Empirical Phase Diagrams

3.1 Construction of Empirical Phase Diagrams (EPDs)

A diverse collection of data is generated from the various biophysical, calorimetric, and hydrodynamic techniques employed to characterize different structural features of macromolecules and their complexes, as discussed above. An internally consistent interpretation of such complex data sets obtained is often quite difficult. To address this problem, all the data sets can be mathematically incorporated into a color map, known as the empirical phase diagram (EPD). The EPDs permit an objective analysis of such data sets, and one can predict the state of a macromolecule under various conditions of interest. This initially involves a somewhat different way of thinking about macromolecules or macromolecular complexes such as viruses. For example, the most common way to think of a protein is as a collection of atoms present in different locations in a three-dimensional space. This is the picture that typically arises from crystallography or NMR data. Here, we replace this conventional view with a

more abstract (but quite useful) picture in which a macromolecule is represented by a vector in a high-dimensional experimental space. This vector changes as environmental conditions such as pH, temperature, and ionic strength are varied. Thus, this approach provides a highly information-rich picture of a macromolecular system whose behavior can be used to analyze changes in stability/structure with corresponding changes in environmental conditions.

To reiterate, in this method large data sets obtained from a variety of experimental techniques are used to establish a color map ("EPD") of the physical stability of a target molecule or complex over a wide range of conditions such as pH, temperature, ionic strength, concentration, freeze thaw cycles, agitation, and redox potential. Formally, this is accomplished by constructing a vector of n dimensions at each combination of stress conditions examined (e.g., pH, temperature, ionic strength, concentration, etc.). The dimensions of this vector are defined by the accumulated data (i.e., fluorescence emission maxima and intensities, CD intensities, second-derivative UV absorbance peak positions, etc.). The experimental data sets are represented as n-dimensional vectors in a temperature/pH phase space (or other state conditions), where n refers to the number of variables (i.e., number of different types of data) included in the calculation (e.g., $n = 9$ for a data set that includes six UV absorption peaks, a CD signal at 222 nm, and intrinsic and ANS fluorescence emission peak positions). The data from each technique at individual values of pH and temperature serve as the basis for the individual vector's components. After normalization of the data to values between −1 and 1, a principle component analysis (PCA) is performed. In PCA, an $n \times n$ density matrix combining all the individual vectors is then constructed, and n sets of eigenvalues and eigenvectors of the density matrix are calculated. The complete data set is subsequently truncated and re-expanded into three dimensions consisting of eigenvectors corresponding to the three largest eigenvalues (i.e., the experimental measurements that contribute the most to the final vector). The resultant three-dimensional vectors are then converted into a colored plot with each vector component corresponding to a color using an arbitrary red/green/blue (RGB) color scheme. All of the necessary mathematical calculations are easily performed with commercially available software packages such as MATLAB (The Mathworks, Inc., Natick, MA) or Mathematica (Wolfram Research, Champaign, IL). A more detailed discussion of the mathematical theory and calculation process for the construction of macromolecular phase diagrams can be found elsewhere [22, 23].

EPDs can be generated using either a single technique, such as high-resolution derivative UV spectroscopy [22, 24] which generates multiple sets of at least semi-independent data, or employing multiple independent techniques and resultant heterogeneous data sets such as CD, fluorescence (intrinsic and extrinsic), differential scanning calorimetry, and/or dynamic light scattering (DLS) [24, 25]. In fact, the phase diagram approach was originally used to display data sets obtained from a protein's second-derivative UV spectroscopy studies [22]. This was based upon the assumption that the (usual) six peaks seen behave somewhat differently in response to environmental perturbation due to widely dispersed locations of the three differ-

ent types of aromatic sidechains found in proteins. For example, Phe residues are typically buried and Tyr interfacial and Trp indole sidechains dispersed throughout the structure. The multi-technique-based approach, however, has the potential to provide more definitive apparent phase boundaries than the UV absorption-based diagrams since the individual techniques provide more independent information about the different levels of protein structure. In addition, it also provides information about the aggregation behavior of the macromolecules, although turbidity studies can be used to provide related information in the single method UV absorbance approach. Moreover, a multiple-technique-based phase diagram may detect subtle conformational transitions that may be undetected by the data used to construct the UV absorbance-based phase diagrams [24]. No matter which approach is used, the resulting colored maps define regions of color that correspond to different physical (not necessarily thermodynamic) states of the macromolecules under the stress conditions investigated. Changes in physical state are thus demarcated by changes in color, producing pseudo-phase boundaries. That is to say, changes in color correspond to transitions between various physical states of the macromolecule. It needs to be emphasized that these are not necessarily equilibrium transitions (no reversibility is implied) and thus these are not equilibrium (i.e., thermodynamic) phase diagrams, hence the use of the word empirical to describe them. Furthermore, their use is primarily empirical, and employing them in a more fundamental analysis of macromolecular systems needs to be very carefully considered. The pseudo-phase boundaries, however, are the crucial product of the phase diagram since they can be used to give us a fairly precise idea of the conditions under which various physical (and indirectly chemical) degradation events occur. Thus, this technique in no way describes absolute physical states of molecules or their complexes. The empirical phase diagram, rather, describes changes in molecular states that are simply calculated from the coherence/incoherence of the data accumulated. Such empirical maps can provide an initial view of the stability of a macromolecule that can in turn be used to design high-throughput screening assays for potential stabilizers [25, 26] (see below). By employing high-throughput instrumentation (e.g., CD, fluorometer, DLS, DSC etc.), EPDs can be constructed in a few days to a few weeks for a wide variety of environmental variables.

The EPD approach has so far been successfully employed to examine a wide variety of vaccine candidates and recombinant proteins as well as other macromolecule pharmaceuticals (Table 1) [4, 11, 22, 24–38]. These include peptide and protein pharmaceuticals (human FGF-1, interferon-β-1a, monoclonal antibodies, botulinum A neurotoxin and its complex, etc.), recombinant protein-based vaccines (anthrax rPA, ricin toxin A, malaria EBA-175, *Clostridium difficile* toxins, *Mycobacterium tuberculosis* ID93, etc.), viruses and virus-like particle (VLP)-based vaccines (Norwalk VLPs, adenovirus types 2 and 5, respiratory syncytial virus, rotavirus, measles virus, Ebola VLP, Marburg VLP, influenza VLP, etc.), a protein adjuvant (*E. coli* heat-labile toxin), DNA and bacterial vaccines, as well as gene delivery vectors. These studies are summarized in Table I where references to the individual studies can be found. Phase diagrams have also been constructed employing a wide variety of environmental variables such as pH and temperature,

Table 1 Examples of EPD-based characterization of biopharmaceuticals

Variable	Biopharmaceutical	Techniques employed	No. of phases observed	Conditions maintaining native-like structure	Reference
pH vs temperature	Recombinant bovine granulocyte colony stimulating factor (bGCSF)	2nd-derivative UV	6	pH 5–7, 10–50 °C	[22]
	Recombinant human interferon-β-1a ($I = 0.1$)	4th-derivative UV, CD, intrinsic and ANS fluorescence	≥3	pH 4, 10–55 °C; pH 5–6, 10–57 °C pH 7, 10–55 °C; pH 8, 10–50 °C	[24]
	Recombinant human interferon-β-1a ($I = 1.0$)		≥3	pH 4, 10–50 °C; pH 5–6, 10–60 °C pH 7, 10–55 °C; pH 8, 10–52 °C	
	rPA of *B. anthracis*	CD, intrinsic and ANS fluorescence	5	pH 4, 10–30 °C; pH 5, 10–35 °C pH 6–8, 10–40 °C	[25]
	Hsc70	2nd-derivative UV, CD, and intrinsic fluorescence	6	pH 5–8, 10–40 °C	[11]
	Gp96	2nd-derivative UV, CD, and intrinsic fluorescence	5	pH 6, 10–40 °C pH 7–8, 10–55 °C	[11]
	EBA-175 RII	CD, intrinsic and ANS fluorescence	4	pH 5–8, 10–50 °C	[25]
	Fibroblast growth factor 20 (FGF 20)	4th-derivative UV	≥3	pH 5 and 5.5, 10–40 °C pH 6–8, 10–50 °C	[27]
	Recombinant human gelatin (8.5 kDa)	CD, light scattering, and intrinsic fluorescence	≥3	Undefined	[35]

(continued)

Table 1 (continued)

Variable	Biopharmaceutical	Techniques employed	No. of phases observed	Conditions maintaining native-like structure	Reference
	Recombinant human gelatin (25 kDa)	CD, light scattering, and intrinsic fluorescence	≥3	Undefined	
	Recombinant human gelatin (50 kDa)	CD, light scattering, and intrinsic fluorescence	≥3	Undefined	
	Recombinant human gelatin (100 kDa)	CD, light scattering, and intrinsic fluorescence	≥3	Undefined	
	Ricin toxin A-chain	CD, intrinsic and ANS fluorescence	3	pH 4, 10–30 °C; pH 5–7, 10–45 °C pH 8, 10–42 °C	[26]
pH vs temperature	Pramlintide (synthetic analogue of human amylin)	2nd-derivative UV, intrinsic fluorescence, and $OD_{350\ nm}$	≥4	pH 5–8, 10–50 °C	[36]
	C. botulinum Type A neurotoxin	CD and intrinsic and ANS fluorescence	5	pH 5–8, 10–40 °C	[28]
	C. botulinum Type A holotoxin complex	CD and intrinsic and ANS fluorescence	5	pH 3–5, 10–40 °C	
	Human fibroblast growth factor 1	2nd-derivative UV	≥4	pH 5–8, 10–40 °C	[27]
	C. difficile toxoid A	CD, intrinsic fluorescence, ANS fluorescence, and $OD_{350\ nm}$	4	pH 6–8, 10–50 °C	[37]
	C. difficile toxoid B		5	pH 5.5–7.5, 10–50 °C	
	IgG mAb1 (100 mg/mL)	CD, intrinsic fluorescence, ANS fluorescence, and light scattering	≥3	pH 6–8, 10–70 °C	Unpublished data
	IpaD from *Shigella flexneri*	2nd-derivative UV, CD, intrinsic and ANS fluorescence	≥8	pH 7–8, 10–30 °C	Unpublished data
	SipD from *Salmonella* spp.	2nd-derivative UV, CD, intrinsic and ANS fluorescence	≥8	pH 7–8, 10–35 °C	Unpublished data
	BipD from *Burkholderia pseudomallei*	2nd-derivative UV, CD, intrinsic and ANS fluorescence	≥4	pH 4–8, 10–30 °C	Unpublished data

pH vs temperature	PcrV from *Pseudomonas aeruginosa*	2nd-derivative UV, CD, intrinsic and ANS fluorescence	≥4	pH 5–8, 10–45 °C	Unpublished data
	LcrV from *Yersinia* spp.	2nd-derivative UV, CD, intrinsic and ANS fluorescence	≥5	pH 5–8, 10–50 °C	Unpublished data
	MxiH from *Shigella flexneri*	2nd-derivative UV, CD, and ANS fluorescence	≥4	pH 5–7, 10–40 °C	Unpublished data
	PrgI from *Salmonella typhimurium*	2nd-derivative UV, CD, and ANS fluorescence	≥4	pH 5–7, 10–45 °C	Unpublished data
	BsaL from *Burkholderia pseudomallei*	2nd-derivative UV, CD, and ANS fluorescence	≥4	pH 5–7, 10–45 °C	Unpublished data
	Adenovirus type 5 (low ionic strength)	2nd-derivative UV, intrinsic fluorescence, PI fluorescence, $OD_{350\,nm}$, and DLS	≥4	pH 6–8, 10–40 °C	[38]
	Adenovirus type 5 (high ionic strength)		≥3	pH 4–8, 10–40 °C	
	Adenovirus type 2	2nd-derivative UV, CD, intrinsic fluorescence, PI fluorescence, $OD_{350\,nm}$, and DLS	4	pH 5–8, 10–37 °C	[38]
	Adenovirus type 4	CD, intrinsic fluorescence, PI fluorescence, light scattering, $OD_{350\,nm}$, and DLS	5	pH 3, 10–30 °C; pH 4, 10–40 °C pH 5, 10–45 °C pH 6–8, 10–40 °C	[65]
	Measles virus	CD, intrinsic fluorescence, ANS fluorescence, laurdan fluorescence, DLS, and light scattering	6	pH 7, 10–20 °C	[66]

(continued)

Table 1 (continued)

Variable	Biopharmaceutical	Techniques employed	No. of phases observed	Conditions maintaining native-like structure	Reference
pH vs temperature	Respiratory syncytial virus	2nd-derivative UV, CD, intrinsic fluorescence, ANS fluorescence, and $OD_{350\ nm}$	≥3	pH 6 and 8, 10–40 °C	[29]
	Rotavirus	CD, intrinsic fluorescence, light scattering, and DLS	4	pH 6–7, 10–70 °C pH 8, 10–80 °C	[67]
	Recombinant vaults expressed in Sf9 insect cell line	CD, intrinsic fluorescence, ANS fluorescence, and light scattering	6	pH 4–8, 10–45 °C	[68]
	Norwalk virus-like particles	2nd-derivative UV, CD, intrinsic and ANS fluorescence	4	pH 3–7, 10–55 °C	[30]
	Attenuated *V. cholerae*	CD, extrinsic (*Bac*Light kit) fluorescence, and DSC	4	pH 7 and 8, 10–40 °C	[69]
Ionic strength vs pH	Plasmid DNA pMB 290	CD, YOYO-1 fluorescence, and DLS	≥3	pH 5–8, 0–150 mM	[34]
	DNA/DOTAP lipoplexes (low charge ratio)	CD, YOYO-1 fluorescence, and DLS	≥3	pH 5–8, 50–150 mM	
	DNA/DOTAP lipoplexes (high charge ratios: 4)	CD, YOYO-1 fluorescence, and DLS	≥3	pH 5–8, 0–150 mM	
	DNA/DOPE-DPTAP liposomes(low charge ratio)	CD, YOYO-1 fluorescence, and DLS	≥3	pH 4–6, 50–150 mM pH 7–8, 10–75 mM	
	DNA/DOPE-DPTAP liposomes(high charge ratio)	CD, YOYO-1 fluorescence, and DLS	≥3	pH 5–8, 0–150 mM	
	DNA/PEI polyplexes (low charge ratio)	CD, YOYO-1 fluorescence, and DLS	≥3	pH 5–8, 50–150 mM	
	DNA/PEI polyplexes (high charge ratio)	CD, YOYO-1 fluorescence, and DLS	≥3	pH 5–7, 25–125 mM	

Concentration vs temperature	IgG1κ mAb1	2nd-derivative UV, CD, intrinsic fluorescence, and $OD_{350\ nm}$	≥2	From 0.1–140 mg/mL, Temperature 10–50 °C	[70]
	IgG1κ mAb2	2nd-derivative UV, CD, intrinsic fluorescence, and $OD_{350\ nm}$	≥2	0.1 mg/mL, 10–75 °C 0.1–10 mg/mL, 10–57 °C ≥100 mg/mL, 10–50 °C	
	Pramlintide (synthetic analogue of human amylin) at pH 4	2nd-derivative UV, intrinsic fluorescence, and $OD_{350\ nm}$	≥6	1.8 mg/mL, 10–30 °C 3.5 mg/mL, 10–35 °C; 5.3 mg/mL, 10–45 °C 7.0 and 8.8 mg/mL, 10–40 °C	[36]
pH vs temperature	$MxiH^{\Delta 5}$	2nd-derivative UV, CD, and ANS fluorescence	≥2	pH 4–8, 10–45 °C	[71]
	$BsaL^{\Delta 5}$	2nd-derivative UV, CD, and ANS fluorescence	≥2	pH 4–8, 10–45 °C	
	$Prgl^{\Delta 5}$	2nd-derivative UV, CD, and ANS fluorescence	≥2	pH 4–8, 10–45 °C	
	Chlamydia trachomatis CT584	2nd-derivative UV, CD, and ANS fluorescence	≥4	pH 4, 10–30 °C; pH 5, 10–35 °C pH 6, 10–40 °C pH 7, 10–55 °C?	[72]
	IgG1κ mAb	CD, Trp fluorescence, SLS, ANS fluorescence	≥4	pH 3, 10–40 °C; pH 4, 10–55 °C pH 5–8, 10–65 °C	[50]
		Adiabatic compressibility, Trp red-edge fluorescence max, coefficient of thermal expansion, rotational correlation time	≥4	pH 4, 10–55 °C pH 5–8, 10–65 °C	

(continued)

Table 1 (continued)

Variable	Biopharmaceutical	Techniques employed	No. of phases observed	Conditions maintaining native-like structure	Reference
	Mouse pneumonitis outer membrane protein	CD, static light scattering, intrinsic fluorescence, $OD_{350\ nm}$	≥3	pH 6–8, 10–50 °C	[73]
	EC5 domain of E-cadherin (I = 0.1 M)	2nd-derivative UV, CD, and intrinsic fluorescence	3	pH 3–8, 10–70 °C	[74]
	EC5 domain of E-cadherin (I = 1 M)		3	pH 3–8, 10–55 °C	
	EC5 domain of E-cadherin (reduced, I = 0.1 M)		≥4	Only partially stable	
	BSA	CD, $OD_{350\ nm}$, intrinsic fluorescence	≥3	pH 4, 10–45 °C; pH 5–8, 10–60 °C	[75]
pH vs temperature	Ebola virus-like particles	CD, intrinsic fluorescence, laurdan fluorescence, DLS	4	pH 3–5, 10–50 °C	[76]
	Marburg virus-like particles		3	pH 3–5, 10–55 °C	
	H1N1 influenza virus-like particles	CD, intrinsic fluorescence, ANS fluorescence, DLS, SLS, laurdan fluorescence	~10	pH 6–8, 10–50 °C	[77]
	sEphB4 (tyrosine kinase receptor)	CD, SLS, DSC, intrinsic fluorescence, ANS fluorescence	4	pH 5–8, 10–50 °C	[78]
	sEphB4-HSA fusion protein		3	pH 3–4, 10–50 °C pH 5–8, 10–55 °C	
	Aldolase	2nd-derivative UV, CD, intrinsic fluorescence, $OD_{320–340\ nm}$	4	pH 5–8, 10–50 °C	[79]
	BSA		6	pH 5–8, 10–55 °C	
	Chymotrypsin		6	pH 4–6, 10–70 °C	
	Lysozyme		3	pH 3, 10–65 °C pH 4–8, 10–70 °C	
	IgG2	CD, SLS, intrinsic fluorescence, ANS fluorescence	4	pH 4, 10–55 °C pH 5–8, 10–65 °C	[80]

	IgG1	CD, adiabatic compressibility, intrinsic fluorescence, ANS fluorescence, SLS, $OD_{350\ nm}$	3	pH 4, 10–45 °C pH 5–6, 10–60 °C pH 7–8, 10–75 °C	[49]
	Glutathione S-transferase from *Necator americanus*	CD, intrinsic fluorescence, ANS fluorescence, SLS	4	pH 5, 10–35 °C pH 6, 10–45 °C pH 7–8, 10–50 °C	[81]
	Hemagglutinin antigen of H1N1 influenza	CD, intrinsic fluorescence, SLS	5	pH 7–8, 10–50 °C	[82]
	Anthrax protective antigen	CD, $OD_{350\ nm}$, intrinsic fluorescence, ANS fluorescence	3	pH 5, 10–30 °C pH 6, 10–40 °C pH 7–8, 10–45 °C	[83]
	2-FHis anthrax protective antigen		2	pH 5, 10–40 °C pH 6, 10–45 °C pH 7–8, 10–50 °C	
	Surface adhesion A protein from *Streptococcus pneumoniae*	CD, intrinsic fluorescence, ANS fluorescence, SLS	3	pH 4, 10–35 °C pH 5–8, 10–55 °C	[84]
	Serine-threonine protein kinase from *Streptococcus pneumoniae*		3	pH 3, 10–45 °C pH 4, 10–55 °C pH 5–8, 10–70 °C	
	Cell wall separation protein from *Streptococcus pneumoniae*	CD, 2nd-derivative UV, ANS fluorescence, SLS	3	pH 5, 10–35 °C pH 6, 10–30 °C	
	FGF-1	CD, intrinsic fluorescence, ANS fluorescence, SLS	2	pH 5–8, 10–40 °C	
	FGF-1 + heparin		3	pH 5–8, 10–55 °C	
	FGF-1 L26D/H93G		2	pH 5–8, 10–40 °C	
	FGF-1 C83T/C117V/K12V		2	pH 5–8, 10–40 °C	

(continued)

Table 1 (continued)

Variable	Biopharmaceutical	Techniques employed	No. of phases observed	Conditions maintaining native-like structure	Reference
pH vs temperature	FGF-1 P134V/C117V		2	pH 5–8, 10–40 °C	[85]
	FGF-1 K12V/C117V		2	pH 4, 10–30 °C pH 5–8, 10–40 °C	
	FGF-1 A66C (oxi)		2	pH 5–8, 10–40 °C	
	FGF-1 K12V/C117V/P134V		2	pH 4, 10–30 °C pH 5–8, 10–40 °C	
	FGF-1 C83T/C117V/L44F/F132W		2	pH 5–8, 10–40 °C	
	FGF-1 SYM6DD/K12V/P134V		2	pH 4, 10–30 °C pH 5–8, 10–40 °C	
	FGF-1 Symfoil-4P		3	pH 3–8, 10–80 °C	
	FGF-1 SYM10 ΔΔ		2	pH 3, 10–50 °C pH 4–5, 10–65 °C pH 6–8, 10–75 °C	
	IgG1 (1 mg/mL)	CD, intrinsic fluorescence, ANS fluorescence, SLS	3	pH 5, 10–65 °C pH 6–8, 10–70 °C	[86]
	IgG1 (100 mg/mL)		3	pH 5, 10–55 °C pH 6–8, 10–65 °C	
	IgG1	CD, intrinsic fluorescence, ANS fluorescence SLS	3	pH 4, 10–45 °C pH 5–8, 10–65 °C	[87]
	IgG1 partially deglycosylated		3	pH 4, 10–40 °C pH 5, 10–55 °C pH 6–8, 10–60 °C	
	IgG1 completely deglycosylated		3	pH 5, 10–45 °C pH 6–8, 10–60 °C	
	Human Pentraxin Protein (PTX-2)	CD, intrinsic fluorescence, ANS fluorescence	3	pH 6, 10–65 °C pH 6.5–8.5, 10–75 °C	[88]

Temperature vs ionic strength	mAb FAb fragment	CD, intrinsic fluorescence, ANS fluorescence, SLS	5	pH 5–8, 10–65 °C	[89]
			3	I 0–1 M, 10–70 °C I 1–2 M, 10–75 °C	
pH vs temperature	H56 from *Mycobacterium tuberculosis*	CD, intrinsic fluorescence, SLS	4	pH 7–8, 10–90 °C	[90]
	H1 from *Mycobacterium tuberculosis*		3	pH 7.5–9, 10–30 °C	
	IpaB/IpgC complex from *Shigella*	CD, intrinsic fluorescence, SLS	3	pH 3, 10–45 °C pH 4, 10–60 °C pH 5–8, 10–70 °C	
pH vs temperature	IpaB from *Shigella*		6	pH 6–8, 10–50 °C	[91]
	IpgC from *Shigella*		5	pH 6–8, 10–40 °C	
	Pneumolysoid L460D from *Streptococcus pneumoniae*	CD, intrinsic fluorescence, SLS	5	pH 5–8, 10–45 °C	[92]
	TSP-2 from *Schistosoma mansoni*	CD, intrinsic fluorescence, ANS fluorescence	3	pH 5–8, 10–50 °C	[93]
	IgG1-Fc diglycosylated	Intrinsic fluorescence, ANS fluorescence, $OD_{350\ nm}$	5	pH 5.5–6, 25–50 °C	[94]
	IgG1-Fc nonglycosylated (DD)		4	pH 5.5–6, 25–50 °C	
	IgG1-Fc monoglycosylated		5	pH 5.5–6, 25–50 °C	
	IgG1-Fc nonglycosylated (DN)		5	pH 5–5.5, 25–50 °C	
	IgG1-Fc nonglycosylated (QQ)		5	pH 5.5–6, 25–35 °C	
	Inactivated polio vaccine, serotype 3 (IPV3)	CD, intrinsic fluorescence, SLS	6	pH 6–7, 10–45 °C	[95]

(continued)

Table 1 (continued)

Variable	Biopharmaceutical	Techniques employed	No. of phases observed	Conditions maintaining native-like structure	Reference
	DB fusion + OPOE	CD, intrinsic fluorescence, SLS	4	pH 3–8, 10–55 °C	[96]
	DB fusion + LDAO		3	pH 3–8, 10–90 °C reversible	
	Oncolytic herpes simplex virus	CD, intrinsic fluorescence, SLS	3	pH 5.5–8, 10–50 °C	[97]
	IgG1Fc high mannose in 0.15 M NaCl	Intrinsic fluorescence, ANS fluorescence, $OD_{350\ nm}$	3	pH 3–8, 10–90 °C, % native area 51%	[57]
	IgG1Fc Man5 in 0.15 M NaCl		3	51%	
	IgG1Fc GlcNac in 0.15 M NaCl		4	40%	
	IgG1Fc N297Q in 0.15 M NaCl		4	26%	
	IgG1Fc high mannose in 10% (w/v) sucrose		3	56%	
	IgG1Fc Man5 in 10% (w/v) sucrose		3	55%	
pH vs temperature	IgG1Fc GlcNac in 10% (w/v) sucrose		4	51%	
	IgG1Fc N297Q in 10% (w/v) sucrose		4	48%	
	FGF-1 WT	Intrinsic fluorescence, ANS fluorescence, SLS	4	pH 5–8, 10–45 °C	[98]
	FGF-1 (C16S/A66C/C117A)		4	pH 6–8, 10–45 °C	
	FGF-1 (C16S/A66C/C117A/P134A)		4	pH 5–8, 10–45 °C	
	FGF-1 (C16S/A66C/C117A/P134V)		4	pH 6–8, 10–45 °C	

	Acrylodan-Labeled Glucose-Binding Protein Sm4-AC	CD, intrinsic fluorescence, ANS fluorescence, SLS	3	pH 5–8, 10–45 °C	[99]
	Escherichia coli heat-labile toxin, LT (R192G/L211A) protein adjuvant	Intrinsic fluorescence, DSC, SLS	6	pH 5.5–8, 10–50 °C	[100]
	Lumazine synthase derived from *Bacillus anthracis*	Intrinsic fluorescence, extrinsic fluorescence, DSC, CD	5	pH 6, 25–40 °C pH 7, 25–50 °C pH 8, 25–50 °C	[101]
	ID93, a vaccine candidate against *Mycobacterium tuberculosis*	CD, intrinsic fluorescence, SLS, $OD_{350\,nm}$	5	pH 7–8.5, 10–40 °C	[102]
Protein concentration vs ionic strength	Lysozyme	UV-Vis spectrophotometer	5	Cluster 1: undersaturated zone Cluster 2–5: supersaturation zone	[103]

temperature and API (active pharmaceutical ingredient) concentration, temperature and excipient concentration, pH and ionic strength, etc. A few representative examples of such phase diagrams and their use as formulation tools are discussed below.

3.2 EPDs Using a pH/Temperature Phase Space

Employing a Single Technique

The power of the phase diagram approach employing a single technique which produces multiple experimental parameters is well illustrated with the relatively unstable recombinant protective antigen (rPA) of anthrax [25] which is used in vaccines against this dangerous organism. Employing a UV-visible diode array spectrophotometer, UV absorption spectra up to 0.01 nm resolution can be obtained using interpolation techniques that permit very precise resolution of the spectra into distinct peaks by derivative analysis [22]. A protein's second-derivative UV absorbance spectrum typically displays six distinctive negative peaks from its three different types of aromatic amino acids (Fig. 2), all of which can be monitored as a function of temperature at various pH values (e.g., pH 3–8) to provide a comprehensive overview of the tertiary structure of a protein. Structural changes in the protein induced by changes in temperature and pH can be characterized by alterations in the positions of these peaks. Because the three different aromatic residues are usually extensively dispersed throughout a protein, they potentially provide a very sensitive

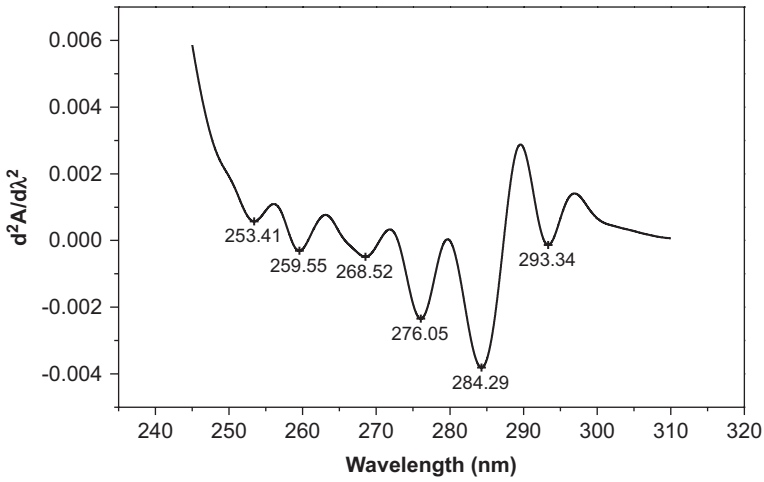

Fig. 2 A second-derivative UV absorbance spectrum of a protein displaying six distinctive negative peaks from the three different types of aromatic amino acids

monitor of the three-dimensional structure of a protein and possible conformational changes as it is stressed. Representative plots of changes in peak positions of the Phe, Tyr, and Trp residues of rPA as a function of temperature at six different pH values are shown in Fig. 3. One difficulty with such an approach involves the large amount and complex behavior of the data generated. To better visualize such data and provide a more intuitive picture of the protein's behavior, the EPD approach becomes extremely helpful. A T/pH phase diagram of rPA developed from high-resolution second-derivative UV absorption spectroscopy studies is shown in Fig. 4.

To generate this phase diagram, second-derivative peak position data was obtained over the pH range of 3–8 and a temperature range of 10 to 82.5 °C using a 20 mM citrate-phosphate buffer (I = 0.1 adjusted with NaCl) (Fig. 3). This buffer was chosen to provide as flat a pH response as possible over this wide temperature range. At 10 °C, the negative spectral peaks occurred at approximately 253 nm (peak 1, Phe), 259 nm (peak 2, Phe), 268.5 nm (peak 3, Phe), 276 nm (peak 4, Tyr), 284 nm (peak 5, Tyr/Trp), and 291.6 nm (peak 6, Trp). The six-peak position data obtained as a function of temperature and pH from second-derivative UV absorption spectroscopy was used to construct an EPD of rPA using the multidimensional vector approach described above (Fig. 4). Thus, data at each unique value of pH and temperature was mapped to a vector consisting of the six derivative peak positions corresponding to that point. This particular phase diagram was constructed employing MATLAB software. The phase diagram of rPA constructed in this manner suggests that the rPA exists in a similar physical state at lower temperatures (10–40 °C) over the entire pH range (3–8) studied. At temperatures greater than 45 °C, a major structurally disruptive transition occurs at pH 6–8. The structural changes occur at much lower temperatures at the lower pH values (pH 4–5) indicating that the protein is more thermally stable in the pH range of 6–8. From this data, the region of maximum stability appears to be at pH 6–7 (up to 50 °C). The transitions at various pH values in this phase diagram appeared to occur at slightly higher temperatures than those observed by other biophysical methods (Fig. 5). Thus, to further extend this approach to obtain a more global insight into the structural as well as the colloidal stability of the macromolecule, multiple techniques were also employed for construction of EPD of anthrax rPA, as discussed below. It should be mentioned that this approach can also be applied to other techniques such as one-dimensional NMR, DSC, or Raman spectroscopy in which multiple peaks (e.g., resonance, endotherms, spectral peaks) can be monitored as a function of environmental perturbations.

Employing Multiple Techniques

The EPD approach is a general method that can use vectors of any finite dimension for the construction of diagrams [24]. Theoretically, there is no limit to the number of variables (data sets) that may be included in EPDs because of the truncation procedure (which could, of course, be more than three terms currently employed for

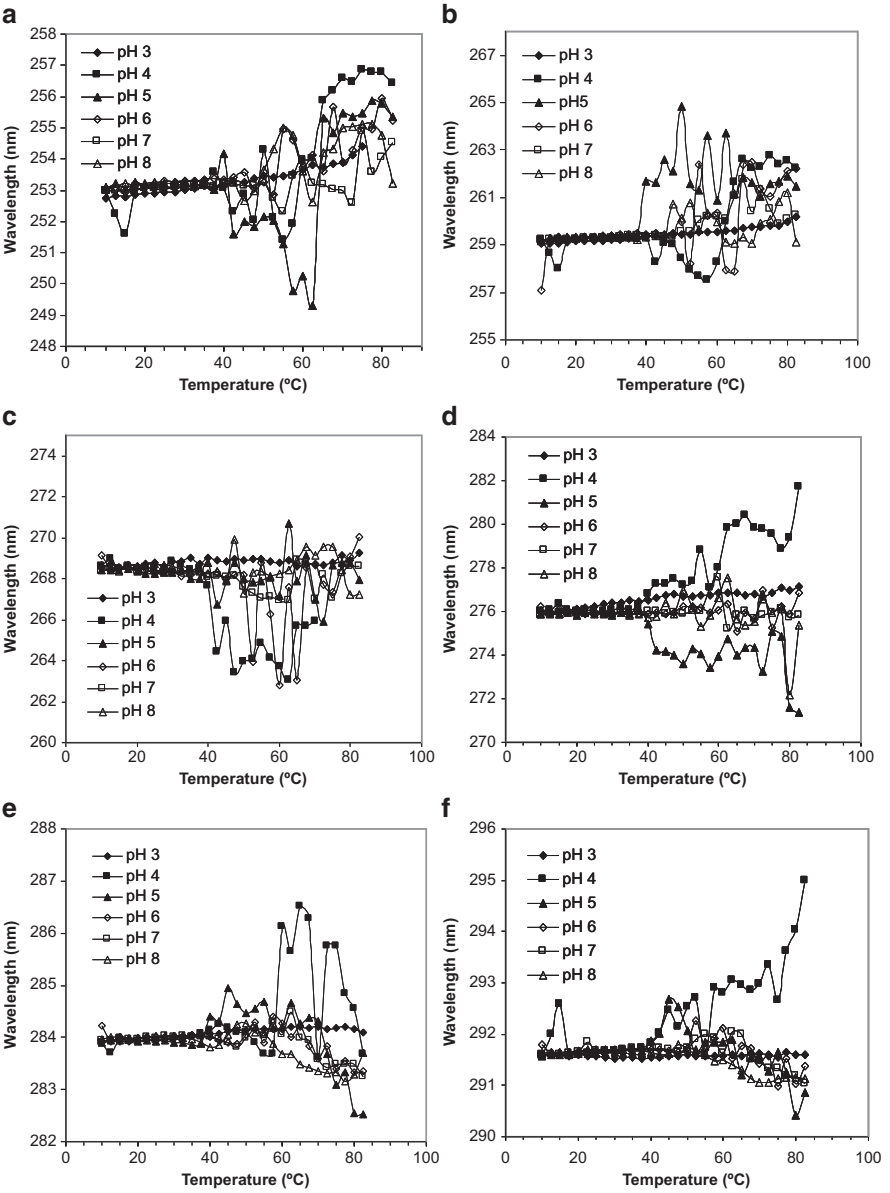

Fig. 3 Second-derivative UV absorbance studies of rPA as a function of temperature and pH. The wavelength positions of six peaks are recorded: (**a**) Peak 1, Phe; (**b**) peak 2, Phe; (**c**) peak 3, Phe; (**d**) peak 4, Tyr; (**e**) peak 5, Tyr/Trp; and (**f**) peak 6, Trp. Error bars are not included to allow better visualization but are generally of the order ±0.04 nm

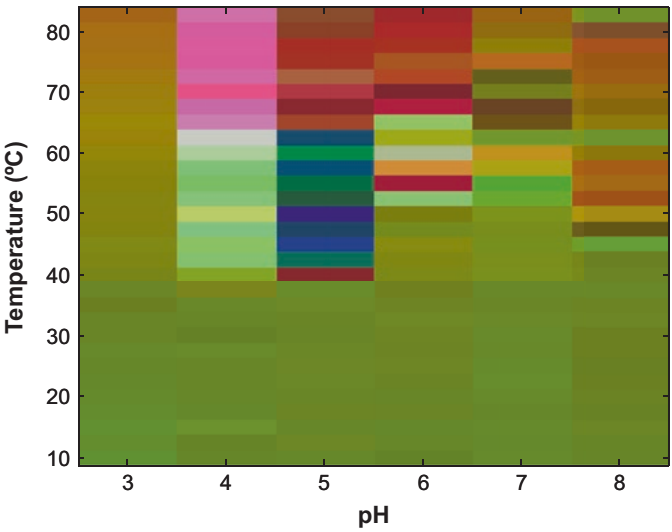

Fig. 4 EPD of rPA on a temperature versus pH axes constructed using high-resolution second-derivative UV spectroscopy data. The data employed to generate these images was obtained over the pH range of 3–8 (20 mM citrate-phosphate buffer, $I = 0.15$ M adjusted with NaCl) and temperatures from 12 to 82.5 °C in 2.5 °C increments. Blocks of continuous color represent homogenous phases, conditions under which the raw data-derived vectors behave similarly

color display purposes). To detect more subtle changes and to incorporate additional structural information that may not be detected by the UV absorption-based technique, data from multiple techniques have more commonly been used to construct EPDs [26, 27]. When heterogeneous data sets from multiple techniques are used, great caution should be taken to keep experimental variables like protein concentration, heating rates, equilibration time at each temperature point, etc. as similar as possible. An example of a T/pH multiple-technique-based phase diagram for anthrax rPA with the corresponding phases is shown in Fig. 6 [25]. This phase diagram was generated employing Mathematica software and utilizing far-UV CD and intrinsic and ANS fluorescence data (Fig. 5a–c). In this case, each (T, pH) condition is mapped to a vector consisting of molar ellipticity at 222 nm obtained from CD, intrinsic tryptophan fluorescence spectral center of mass, and ANS fluorescence intensity data. The advantage of this approach is that the data employed reflects more global aspects of secondary and tertiary structure change as well as the exposure of the apolar binding sites of protein as a function of pH and temperature. This information can then be employed to assign the origin of the behavior of the protein with each apparent phase observed. The diagram for rPA obtained in this manner suggests that rPA adopts at least five distinct behaviors (Fig. 6). Again note that these do not correspond to true "thermodynamic phases" in the formal use of the concept but are simply empirical (non-thermodynamic) states defined by the experi-

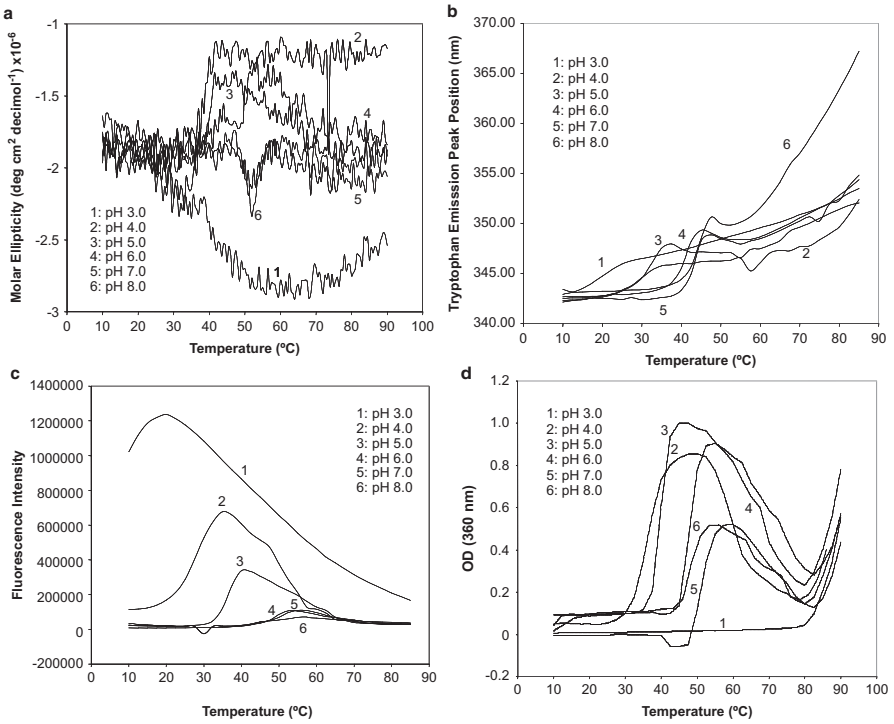

Fig. 5 Physical stability of rPA as a function of pH and temperature in 20 mM citrate-phosphate buffer (I = 0.15 M adjusted with NaCl) as determined by CD thermal unfolding monitored at 222 nm (**a**); intrinsic Trp fluorescence peak positions employing a spectral center of mass procedure (**b**); ANS fluorescence intensity (**c**); turbidity measurements, OD at 360 nm (**d**). The thermal traces represent an average of two measurements in which each data point has a standard error of less than 0.5

mental methods employed. The properties of the phases can be established through interpretation of the biophysical measurements themselves. Combining the multi-technique phase diagram with knowledge of the behavior of the protein based on the individual measurements, it appears that the pinkish region in the lower, right-hand corner of the diagram is the state of most continuous stability (Fig. 6). This correlates well with CD, intrinsic and ANS fluorescence, and the second-derivative UV absorbance data. The midpoints of the transitions observed by the biophysical measurements generally correspond to the color changes between the phases. A second phase (blue/purple) is apparent at pH 3 at temperatures below 45 °C and also seems to encompass pH 4 at temperatures greater than 30 °C and pH 5 at temperatures greater than 35 °C. This state appears to have significant molten globule character since the tertiary structure changes appear to occur before the secondary structure alterations. As the temperature increases at pH 3, the protein rapidly enters another phase near 50 °C. A third phase appears to be present at pH 5–8 in the higher temperature region. This depicts a phase in which the protein is severely structurally

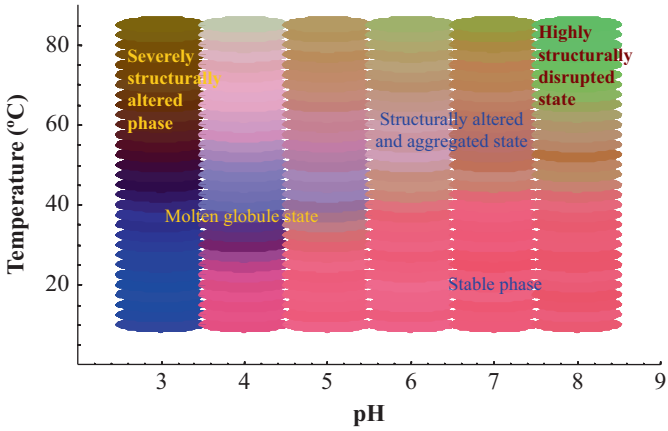

Fig. 6 EPD of rPA on a temperature versus pH plane based on intrinsic and ANS fluorescence, light scattering ($OD_{360\ nm}$), as well as CD data. The data employed to generate these images was obtained over the pH range of 3–8 and at temperatures from 12 to 85 °C in 2.5 °C increments. Blocks of continuous color represent uniform phases, conditions under which the raw data-derived vectors behave similarly

altered and tends to aggregate and may also involve molten globule behavior. The aggregation behavior of the protein was further confirmed by light scattering measurements (optical density at 360 nm), as shown in Fig. 5d. At pH 8, a completely different phase appears at very high temperatures which we assume corresponds to an even more structurally disrupted form. The information obtained from the phase diagram can be further used to select conditions for the development of a high-throughput assay to screen for potential stabilizers, as illustrated with rPA as an example (see Sect. 5).

3.3 EPDs Using Other Environmental Variables

Temperature Versus Concentration

EPDs can be constructed using any environmental variable that can be quantified. An example of a EPD of a monoclonal antibody (mAb) using temperature and protein concentration as independent variables is shown in Fig. 7 [31]. The behavior of the immunoglobulins at ultrahigh concentrations has become of great interest due to a need to deliver low volume/high content doses of this class of proteins. This diagram is constructed using multidimensional vectors at each environmental condition (temperature and mAb concentration) from various normalized data comprising six second-derivative UV absorbance peaks, tryptophan emission peaks, turbidity (OD) at 350 nm, and CD molar ellipticity at 218 nm. Note that in this case the con-

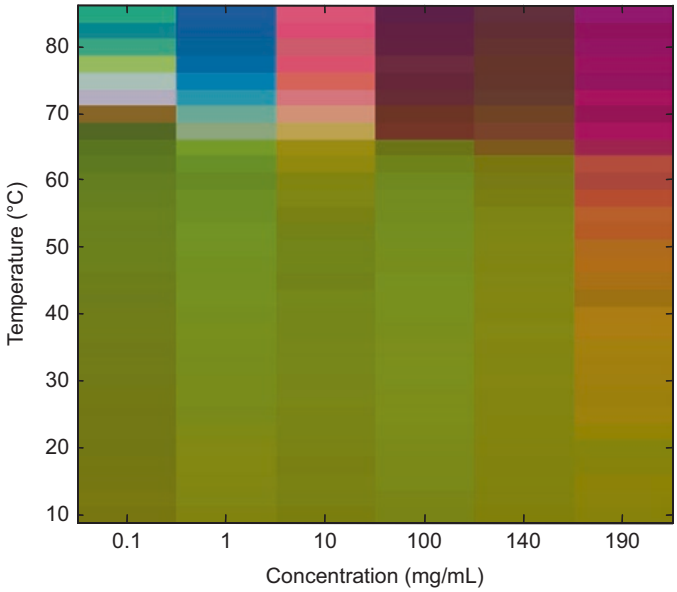

Fig. 7 EPD of a monoclonal antibody on a temperature versus protein concentration plane. Each temperature/concentration point on the diagram is constructed from data obtained from fluorescence emission peak position; $OD_{350\ nm}$; second-derivative UV absorbance peak positions of Phe, Tyr, and Trp; and CD molar ellipticity. Note that the concentration axis is not linear

centration data are represented on a nonlinear scale, making interpolation to other conditions somewhat more difficult.

One of the purposes of this study was to develop methods that can be employed to directly study the physical structure and thermal stability of proteins at high concentrations and then use a combination of these methods to construct a phase diagram to examine the effect of increasing protein concentration (0.1 to ~200 mg/mL) on thermal stability. Although highly concentrated protein formulations offer increased promise for more convenient treatment of a broad range of diseases, there remains a big obstacle to their development stemming from a lack of available methods to determine macromolecular structure, stability, and aggregation propensity within highly concentrated solutions [31, 39]. Currently, most of the analytical methods employed in the development of highly concentrated protein formulations require dilution of the samples before acquiring data, and subsequent extrapolation of stability indicating parameters obtained from these studies may not be representative of the more highly concentrated solutions. Thus, biophysical characterization of a highly concentrated protein formulation requires the use of novel approaches. A detailed description of these methods (e.g., front face fluorescence, short pathlength CD, FTIR, DSC, etc.) is beyond the scope of this chapter and can be found elsewhere [31]. Data obtained from the abovementioned techniques was used to generate the temperature vs concentration phase diagram of the mAb (Fig. 7).

The information obtained from the individual experimental methods permits assignment of physical meaning to the colored phases observed in the diagram over the temperature and concentration range of the study (data not shown [31]). Briefly, the phase diagram of this monoclonal antibody (Fig. 7) suggests that, at temperatures below 50 °C, the antibody exists in a very similar state over the entire concentration range examined. Further inspection of the biophysical data collected in this study indicates that the protein is in a native or near native state under these conditions. At temperatures above 65 °C and at low concentrations, the immunoglobulin appears to be in a slightly unfolded and aggregated state. At higher concentrations, the phases at elevated temperatures correspond to different unfolded, gelled, and precipitated states. In addition, the apparent phase boundaries occur at somewhat decreased temperatures as the protein concentration is increased. Thus, for the first time, a method was developed to directly detect the structural changes of a protein at high concentrations. Additionally, using this approach, the thermal stability of the protein could be correlated to corresponding changes in its concentration. This study suggests that the decrease in structural stability observed at higher concentrations of mAb is probably the result of aggregation or more limited self-association upon heating in crowded solutions and not due to a decrease in the intrinsic structural stability of the mAb [31].

Ionic Strength Versus pH

The phase diagram approach has also been applied to determine changes in the physical stability of nonviral gene delivery complexes under different solution conditions [34]. In the past, the characterization of nonviral gene delivery systems has been complicated due to their size, complexity, and heterogeneity. This issue has been addressed with the creation of EPDs using several different physical techniques in combination. A representative ionic strength–pH EPD of a DNA/carrier complex is illustrated in Fig. 8. This DNA/carrier complex was prepared by using a mixture of a cationic lipid (1,2-dioleyl-3-trimethyammonium-propane, DOTAP) and a helper lipid (1,2-dioleyl-sn-glycero-3-phosphoethanolamine, DOPE). Three experimental approaches (DLS, CD, and YOYO-1 dye fluorescence) were selected for this study to monitor different structural aspects of the particles related to size, DNA conformation, and the extent of DNA condensation, respectively [34]. The electrostatic nature of the interaction between the plasmid DNA and the cationic carriers prompted the selection of environmental variables pH (4–8, at 1 unit interval) and ionic strength (10–150 mM, at 25 mM intervals). An EPD for (DOTAP/DOPE)/DNA complexes formulated at a high charge ratio indicates the presence of distinct structural forms of the complexes (Fig. 8). The regions of similar color represent at least somewhat homogenous phases, while structural changes of gene delivery complexes are marked by corresponding changes in colors. In general, the EPD suggests the presence of at least three different physical states. At very low salt concentration (10 mM), a small and distinct phase is seen across pH 4–6 (pink), while a second phase is observed at salt concentrations greater than 100 mM and

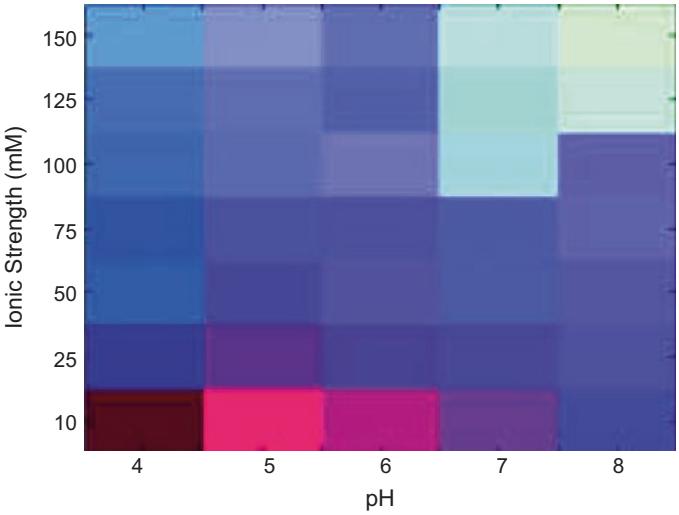

Fig. 8 Ionic strength–pH EPD of a nonviral gene delivery complex. The EPD is generated from dynamic light scattering, CD, and fluorescence studies. Regions of similar color represent similar structural behavior, while the change of color defines the conditions under which the structure of the gene delivery complex alters

pH 7–8 (light blue/green). Presence of a third continuous phase is apparent in moving from pH 4–6 at 25–150 mM salt to pH 7–8 at salt concentrations below 100 mM (blue phase). The pH-dependent stability of these complexes may be attributed to varying ionization states of the carrier. The helper lipid DOPE, for example, is less ionized at high pH (7 and 8) and thus forms less stable complexes with DOTAP than at lower pH values. The light blue/green phase observed in the EPD corresponds to the presence of larger species as determined by DLS studies. Furthermore, protonation of the nucleic acid bases and phosphate groups at low pH values and condensation of the DNA plasmid mediated by salt could account for the observed structural alterations [40]. Thus, a comprehensive picture of the behavior of complexes with a corresponding identification of apparent phase boundaries in terms of relevant variables is obtained. Such an approach permits development of screening assays to identify potential stabilizers. Additionally, the correlation between the physical aspects of complexes with their ability to transfect cells can be explored. The EPD approach therefore provides a convenient and powerful tool to characterize the structure of the complexes over a wide range of experimental variables especially for cases in which the use of higher-resolution techniques such as NMR or X-ray crystallography are difficult or impossible to apply. It should be noted that EPD analysis of gene delivery complexes reveals the presence of more gradual, subtle structural alterations than in the EPDs described above for other biopharmaceuticals (e.g., proteins, viral particles etc.), in which well-defined changes in structure were reflected by abrupt color changes.

4 New Approaches to EPDs

The EPD approach for the rapid preformulation of vaccines and macromolecule-based pharmaceuticals described above is primarily based on the static properties of the macromolecules involved. One defining characteristics of large molecules, however, is their dynamic nature. These internal motions range from small alterations of the side chains and the peptide backbone to large-scale movement of structural domains relative to each other. The relationship between this property and their stability and formulation is somewhat controversial but is expected to be profound [41]. The phase diagram approach can be expanded to include dynamic data such as that obtained by isotope (hydrogen/deuterium) exchange rates (k), red-edge excitation spectra (REES) ($\Delta\lambda$), coefficients of thermal expansion (α), and compressibility (c) as well as fluorescence lifetime anisotropy measurements. Such data can be used to construct EPDs using the vector approach described above and should better reflect dynamic aspects of macromolecular structure. To elaborate, proteins in the presence of deuterium will undergo an exchange of amide hydrogen atoms for deuterium atoms at a rate dependent on the dynamic exposure of the peptide backbone. This rate of exchange is a function of both solvent penetration and local unfolding [42, 43]. For this reason, extended measurements of the rate of hydrogen/deuterium exchange using NMR, mass spectrometry [44, 45], or FTIR among other techniques can be related to the internal amplitude motions within the protein that render interior regions accessible to the solvent. Red-edge fluorescence is performed by exciting the target molecule at various excitation wavelengths and monitoring shifts in the wavelength emission maximum [46]. A red shift of the emission maximum reflects changes in the solvent relaxation time that occur with restricted motion of the fluorophore. These studies can thus be employed to probe the mobility of the fluorophore (intrinsic or extrinsic) as a function of stress variables such as pH, temperature, and ionic strength. A related technique can employ an extrinsic solute quencher such as acrylamide, iodide, or cesium ion to probe the accessibility of a fluorophore [47]. Another fluorescence based-approach sensitive to the dynamic nature of macromolecular behavior combines lifetime measurements and polarized light to obtain anisotropic information about the fluorophore that can be related to its mobility in terms of its rotational correlation times. A third technique, pressure perturbation calorimetry, can also be used to determine the coefficient thermal of expansion of a macromolecule [48]. When pressure is increased or decreased above a solution, heat is either absorbed or released, and this heat is proportional to the pressure change and the corresponding response of macromolecules present to these changes. This permits the calculation of volumetric properties of the protein. Since these measurements can be made over a wide range of temperatures, it is possible to calculate the coefficient of thermal expansion and volume change as a function of temperature of a macromolecule or particle. Another approach, ultrasonic spectroscopy, employs attenuation of sound measurements to determine the complementary parameter of compressibility [48]. In preliminary studies, we have found that "dynamic" EPDs provide information above and beyond that obtained by the static

approach [49, 50]. The utility of this approach in formulation activities has, however, yet to be established. In contrast, the use of H/D exchange using mass spectrometry is rapidly becoming an important technique for therapeutic protein development [44]. It can establish the effect of excipients on the flexibility of mAbs as well as the sites which they contact other proteins [45].

EPDs can also be constructed using chemical stability data of proteins and vaccine antigens to further expand the utility of this approach. Typically, one- or two-dimensional reversed-phase HPLC coupled to mass spectrometry is used for this purpose. The conditions such as high pH and temperature (to induce deamidation) and the presence of oxidizing agents are used to accelerate chemical degradation processes, and the resultant rate constants are used as input parameters. Lower resolution methods such as isoelectric focusing or RP-HPLC alone can be used as well. Data from such analyses can either be combined into physically based phase diagrams or used separately as an independent EPD to provide a much more comprehensive view of the degradation of systems of interest. Comparisons of physically based EPDs with chemically based ones should be of special interest in establishing any relationship between the two phenomena.

In addition to color-based EPDs, a number of other data visualization tools can be used to visualize complex data sets. The most common of these is the radar chart. Although the original color-based EPD method has been widely employed for formulation screenings of numerous macromolecules (mAbs, vaccine candidates, nucleic acids, viruses, virus-like particles, etc.), it still suffers from several drawbacks including (1) lack of a direct correlation between colors and structural states of macromolecules, (2) no indication of contribution of individual techniques and the corresponding data set, and (3) difficulty in data interpretation by color-blind individuals (Fig. 9a). To address these issues, we describe below three approaches with improved data visualization features [51]. The first is called a three-index EPD in which the color itself relates to a defined structural state of macromolecules [51]. “Three-index” indicates that this EPD is constructed by summation of data collected at three structural levels: secondary structure changes, tertiary structure changes, and alterations in quaternary (aggregation) structure under stressed conditions. The degree of structural change within the range of stress conditions is reflected by an empirically derived parameter designated as the structural index (secondary index (SI), tertiary index (TI), and aggregation index (AI)). In most cases, a structural index is calculated simply by normalization of the structural data to a number within the range of 0–1. For SI and TI, the number 1 represents the native state, and the number 0 the maximally conformationally altered state. This is flipped for AI with the number 0 indicating no aggregation. To construct a three-index EPD, each index is first mapped to a corresponding RGB color component. This RGB scheme represents an ordered arrangement of red, green, and blue color components that are assigned to SI, TI, and AI, respectively. The value of a structural index determines its color gradation. A range of a structural index from 0 to 1 represents black to a full color of the index. The three index colors are finally summed together to generate a color with meaningful structural information (Fig. 9b). For a macromolecule in its native state, the SI, TI, and AI would correspond to a value of 1

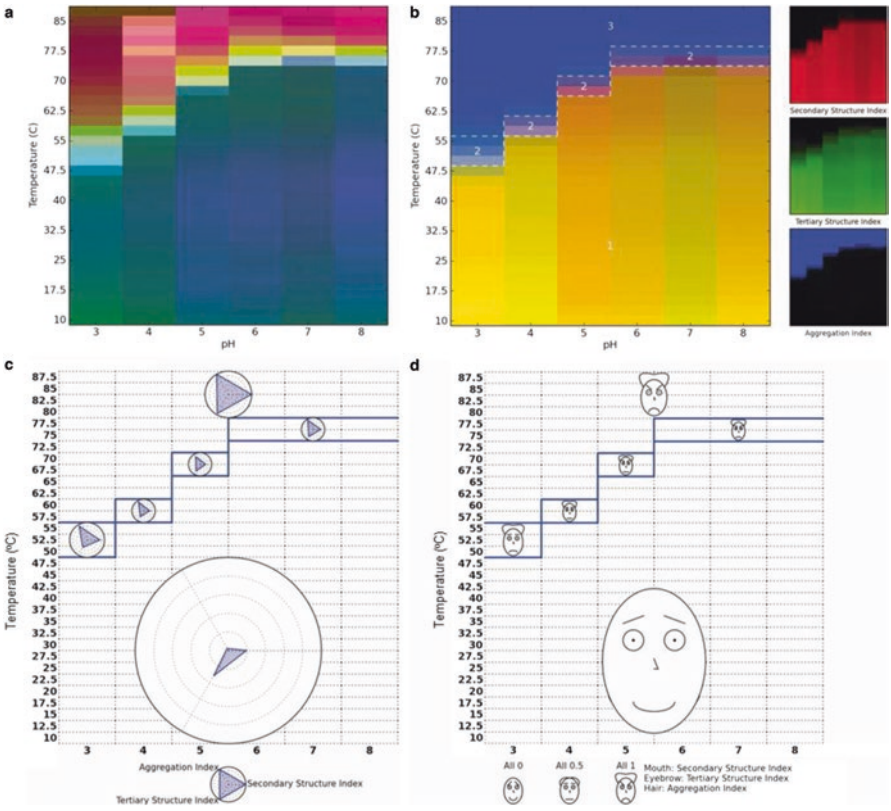

Fig. 9 (**a**) EPD, (**b**) three-index EPD, (**c**) radar chart, and (**d**) Chernoff face diagram for the protein antigen SP1732 as a function of temperature and pH. Three structural regions are observed: (1) native state, (2) molten globular state, and (3) aggregated state

(full red), 1 (full green), and 0 (black), respectively. The summation will generate a yellow color (red + green) indicative of a native state (Fig. 9b, region 1). Likewise, a black color on the EPD means an extensively unfolded state without aggregation. A blue region represents an aggregated state (Fig. 9b, region 3).

The second method employs radar charts instead of colors to manifest phase differences in a phase diagram [51]. The radar chart is a widely used presentation tool for multivariate data. In a radar chart, each variate data is plotted on an individual axis originating from the center of a circular chart. Each axis is usually spaced at even angles. By analogy to the above, each structural index (SI, TI, AI, etc.) is plotted on an individual axis. The center of the circular chart corresponds to a structural index of 0 while the rim a structural index of 1. Unlike the three-index EPD, all three structural indices use the number 0 to represent the native state of macromolecules. Sequential connection of all data points in a radar chart generates a polygon. The native state has a polygon with the minimal area, and an increase in the polygon area indicates a structural change. The signal change on each axis reflects the

contribution of the corresponding structural level to overall structural alterations. While a radar chart can be plotted for each stress condition (pH, temperature, etc.), a clustered radar chart can be generated for a defined phase region by averaging all data points within that region for the purpose of concise data presentation (Fig. 9c). Another advantage of the radar chart over the three-index EPD is that it can display more than three variables.

A third data visualization tool is designated a Chernoff face diagram [51]. Although it is not as widely used as the radar chart diagram, it has some unique features that may occasionally make it a better choice. These two methods are highly similar except one major difference – Chernoff face diagram uses a Chernoff face instead of a radar chart to manifest structural states of macromolecules (Fig. 9d). The idea behind this approach is based on a human's sensitive ability of face recognition. The multivariate data are translated into human faces containing approximately seven key facial features including mustache, nose, mouth, ear, hair, eyes, and the location of eyebrows. Each structure index is assigned to a facial feature and finally mapped together to generate a face integrating key structural properties of macromolecules. The native state is often assigned to a "happy and bald" face for better recognition.

5 Extending the EPD Paradigm Using Data Science and Machine Learning

As described above, the first step in creating an EPD is to represent data obtained from analytical measurements as an n-dimensional vector. Historically, these vectors have contained preselected features from the data (e.g., the position of the six peaks in the second-derivative UV spectrum, Fig. 2) based on our understanding or intuition for which experimental parameters should best correlate with macromolecular state or stability [51]. While this produces useful EPDs, much of the underlying data is discarded in this procedure, and it is not a priori clear if the preselected features actually contain the most useful information to characterize molecular state.

Modern techniques from data science and machine learning may assist in finding additional features of the data that would be useful for characterizing molecular properties that are relevant to pharmaceutical formulation and stability problems. These techniques completely abstract the state of the molecule as a point in a (usually much larger) vector space; for instance, rather than just taking the six peak positions from a second-derivative UV spectrum (i.e., a vector space with $n = 6$), we would take the entire UV spectrum (i.e., a vector space with $n = 1000$). These data theoretically contain everything that the technique itself can determine about molecular state as a function of the independent variables, including information that could be missed by focusing on a small subset of features. That being said, such data sets can be very large; if multiple analytical techniques are combined, there can be thousands to even hundreds of thousands of observations associated with each

sample [52–54]. This necessitates the application of novel computational techniques to determine which of these data are most informative. To explain how this can be done, we will initially focus on an application of this approach to the study of how glycosylation state and formulation variables influence the stability of the Fc of IgG1 [52], as well as more recent applications of these techniques to mixtures of IgG1 Fc glycoforms. We will also discuss the use of this approach to analyze a stability study of the complex polymeric drug substance crofelemer, which, while not a protein, presents many of the same analytical challenges [53–55].

Antibodies are produced in a variety of glycosylation sites within the body. For IgG1 antibodies, a major site of glycosylation is the N297 residue on the Fc fragment. It has been shown that changes in glycosylation state can have important effects on biologically and pharmaceutically relevant properties of antibodies (including modulating Fc receptor specificity and affinity) in addition to being a major determinant of antibody stability. To systematically study these effects, we expressed and purified the IgG1 Fc in a well-defined "high mannose" glycosylation state (HM-FC) [56]. Treatment of HM-Fc with the bacterial enzyme α-1,2 mannosidase produced a variant with five mannose moieties at the end of the glycosylation change, which we called Man5-Fc. Treatment of HM-Fc with endoglycosidase H resulted in a single GlcNAc moiety on the N297 residue (GlcNAc-Fc). We also generated an aglycosylated N297Q mutant (N297Q-Fc). Each of these Fc glycosylation variants was formulated in either a 10% sucrose solution or an NaCl solution with an ionic strength of 0.15 (in addition to a 20 mM citrate-phosphate buffer common to both). These eight sample types (four glycosylation variants each in two different formulations) were subjected to pH values ranging from 4 to 7.5 and temperatures from 25 to 90 °C. Molecular state was monitored using a variety of techniques, including intrinsic Trp fluorescence and extrinsic fluorescence based on the SYPRO orange dye [52, 56, 57].

The resulting data set included over 2000 separate measurements (or "features") for each of the eight samples. To test whether these measurements could be reliably used to distinguish the samples from one another, we performed a classification analysis; a "classifier" is an abstract machine learning function that takes input data (e.g., the peak positions of the intrinsic Trp fluorescence spectrum) and attempts to optimally predict the type of sample it is (e.g., Man5-Fc in 10% sucrose). We found many different classifiers that could distinguish these samples with near 100% accuracy, even when only provided with a small portion of the data (i.e., just the data from pH 6.0 and 7.5). This indicates that techniques like intrinsic Trp fluorescence contain sufficient data on molecular state that they can distinguish even very subtle differences in formulation and glycosylation state [52].

More recently, we applied a similar approach to a more complex problem: determining differences between mixtures of IgG1 Fc glycoforms. This work was based on the four pure glycoforms discussed above; in addition to 100% HM-Fc, Man5-Fc, GlcNAc-Fc, and N297Q-Fc solutions, we created 90/10% mixtures of HM-Fc/Man5-Fc, HM-Fc/GlcNAc-Fc, and HM-Fc/N297Q-Fc, as well as 50/50 mixtures of HM-Fc/Man5-Fc, HM-Fc/GlcNAc-Fc, and HM-Fc/N297Q-Fc, and one mixture

with 25% of each variant. This generated a total of 11 samples, some with subtle differences (say, 100% HM-Fc vs 90/10 HM-Fc/Man5-Fc) and some with more obvious differences (say 100% HM-Fc vs 100% GlcNAc-Fc). These samples were subject to a wide array of analytical measurements, including intrinsic Trp fluorescence, differential scanning calorimetry, UV-visible spectroscopy, and a variety of other methods (unpublished data).

We found that simply combining all of this data together (with over 10,000 features per sample) did not allow us to build classifiers with high accuracy. Thus, while these data contain useful information, simply aggregating all of the data without any filtering or analysis may not allow machine learning functions to determine important differences in molecular state. To overcome this problem, we used a straightforward "Mutual Information" (MI) analysis. The MI calculates how much knowledge of one variable (e.g., the amount of UV absorption for the sample at 280 nm) can tell you about another variable (e.g., whether the sample in question is 100% HM-Fc or 100% Man5-Fc) [52, 53, 58]. We found that choosing only the top 100 most informative measurements (in other words, creating a vector space with $n = 100$ where each element of the space is one of the most informative measurements we made) allowed us to build many different classifiers with near 100% accuracy (data not shown). This suggests that a program where a large number of analytical measurements are made at first, and the most informative measurements determined later, may allow for the construction of mathematical functions that can reliably differentiate even very subtle differences in molecular state.

We have also applied this general approach to the study of crofelemer. Crofelemer is a plant-based drug that is prepared by processing and purifying an oligomeric proanthocyanidin from the sap of the *Croton lechleri* tree. It is an FDA-approved therapeutic for the treatment of noninfectious diarrhea for patients being treated with antiretroviral drugs for HIV [59]. It is a complex macromolecular drug, consisting of polymers of random, alternating sequences of (+)-catechin, (+)-gallocatechin, (−)-epicatechin, and (−)-epigallocatechin. While it is not a protein, the polymers in question have complex chemical and structural properties that are sensitive to formulation parameters like temperature and solution pH. In this study, a variety of crofelemer preparations were exposed to elevated temperatures (25 and 40 °C) for varying lengths of time, and the samples were subjected to extensive analytical characterization (UV absorption spectroscopy, circular dichroism, FTIR, mass spectrometry, among others) [54, 55].

In many cases, the traditional features captured by these analytical techniques did not show significant differences between samples, and the question then became how to find those differences in a massive sea of data. The authors approached this problem by applying an MI analysis to this data and discovered many interesting features of the data that would otherwise have been missed [53, 54]. For instance, while the major peaks in the mass spectrometry experiments were essentially identical for all samples considered, the MI analysis revealed several smaller peaks that had statistically significant differences between the sample types in the stability study. Assignment of these peaks to putative molecular species revealed interesting differences in the production of oxidized chemical species between different

crofelemer preparations and storage conditions [54]. This highlights how novel insights can be gained by searching for the most informative differences between samples, even if those differences are in regions of the data sets that have not yet been assigned a physical or chemical meaning.

While the results discussed above are encouraging, the application of data science techniques to problems in pharmaceutical formulation is still in its infancy. These approaches have the promise to further the conceptual ideas that underlie EPDs by finding the most informative aspects of analytical data sets. Such techniques could be used to improve the visualization of data in EPDs or to identify or predict optimal formulation conditions for pharmaceutical stability and efficacy.

6 Development and Optimization of High-Throughput Stabilizer Screening Assays

The EPD and other alternate approaches described above can be used to comprehensively characterize the solution behavior of biopharmaceuticals under various stress conditions. Although not discussed here, it can also be used to characterize the solid-state behavior of dried formulations using alternate technologies. The identification of "apparent" phase boundaries is one of the most important applications of the approach. This locates conditions under which the macromolecules are marginally stable or undergo critical structural changes, as defined by the regions of abrupt or gradual color change. These conditions can be employed to accelerate degradation pathways to develop high-throughput screening assays for the identification of potential stabilizers for the rational formulation of biopharmaceuticals. The type of assay employed (static and dynamic light scattering for aggregation, intrinsic or extrinsic fluorescence for tertiary structural changes, CD for secondary structural alterations, etc.) is determined by the nature of the relevant physical changes and their potential adaptation to a high-throughput format to decrease analysis time. Techniques such as CD and DSC have also been automated although they have not yet been adapted to microtiter plate-based formats. Recent work by a variety of companies suggests that techniques such as FTIR and fluorescence lifetime anisotropy may soon be available in a high-throughput format.

Once such assays are established, various libraries of compounds can be screened and potential stabilizers identified. Examples of compound libraries that can be screened include comprehensive selections of GRAS agents (generally regarded as safe, i.e., carbohydrates, amino acids, polymers, detergents, etc.) as well as collections of osmolytes, polyanions, and low molecular weight di- and multi-ions [60]. Each compound should be examined over as wide a range of concentration as possible and tested in at least triplicate over a range of positions on a microtiter plate. Data is typically recorded as a function of time based upon the initial optimization of the screening assay. Compounds which effectively stabilize the target system in terms of slowing or inhibiting the magnitude of the parameter change are

selected for further study. If stabilizers are not identified in these small libraries, larger collections of hundreds of thousands of selected compounds used in drug screening protocols can be employed, although issues of safety become significant. Compounds identified in these libraries could also serve as starting point for combinatorial chemical attempts to generate unique stabilizers. Once potential excipients are identified, the concentration and their use in a combination that provide maximum stability can be initially determined by the screening assays. The optimal excipient(s) concentration is then used to verify, at admittedly low resolution, the mechanism of stabilization of the compound. For example, it is possible that a stabilizer may prevent aggregation but not provide conformational stabilization or vice versa; to obtain this information, individual techniques such as fluorescence and CD are used in the presence and absence of potential excipients to determine apparent thermal transition temperatures (Tms). An increase in Tm is taken as direct evidence of conformational stabilization. A typical goal is to obtain at least a 5–15 °C increase in the thermal melting temperature in the presence of compounds tested. Similar studies of aggregation/association behavior using light scattering or some other size sensitive technique are also performed. The information from the conformational and size sensitive methods is then used to select optimal stabilizers. Accelerated stability studies of this type are frequently but by no means always predictive of macromolecule stability behavior under more moderate (e.g., storage) conditions [61, 62]. The major unknown here is whether the mechanism of physical degradation probed by the accelerated stability studies is similar to that seen under more moderate environmental conditions. Hence, long-term stability studies at lower temperatures (e.g., 2–8 °C) need to be performed after the optimization of final formulations with independent considerations of chemical stability (see above) and confirmation of stabilization by relevant biological assays.

Versions of the high-throughput screening approach for the identification of stabilizers described above has been successfully used in a wide variety of applications including formulation studies of peptides, proteins, viruses, virus-like particles, as well as other vaccine antigens (Tables 2 and 3) [4, 25, 26, 32, 46, 60, 63, 64]. For example, the phase diagram generated for anthrax rPA (Fig. 6) suggested that a high-throughput screening assay could be developed at pH 5 at 37 °C using one or more techniques to generate signals reflecting physical degradation. Aggregation was one of the more apparent pathways of physical degradation of this protein and was therefore selected for stabilization analysis [25]. Therefore, a turbidity assay was developed for the rPA to produce conditions that could achieve significant aggregation by monitoring the turbidity of the solution at 360 nm in 96-well microtiter plates. Experiments were performed both with and without potential excipients, and the aggregation seen with protein alone was used as the standard. The maximum OD observed in control samples at 70 minutes was used as a measure of the maximum extent of aggregation since the signal change was complete by this time. Inhibition by various agents was then characterized by their ability to lower the maximum OD obtained. A number of potent inhibitors were identified by this method, with trehalose, sorbitol, mannitol, dextrose, and sodium citrate found to be

Table 2 Stabilizers identified for the selected biopharmaceuticals employing EPD approach from T_m differences

Biophar-maceutical	Stabilizer(s)	Transition temperature				Reference
		Without stabilizer(s) (°C)	With stabilizer(s) (°C)	ΔT_m (°C)	Technique(s) used to measure T_m	
rPA of *B. anthracis*	20% trehalose	49	55	6	CD	[25]
EBA-175 RII	20% sorbitol	52	56	4	Intrinsic fluorescence	[25]
	20% sucrose	52	55	3		
Fibroblast growth factor 20 (FGF 20)	Heparin	55	67	12	DSC	[27]
Ricin toxin A-chain	50% glycerol	50	66	16	Intrinsic fluorescence	[26]
C. difficile toxoid A	20% sorbitol + 20% dextrose	60	67	7	Far-UV CD	[37]
C. difficile toxoid B	10% dextrose + 0.05% Tween 80	56	71	15	Far-UV CD	
IpaD from *Shigella flexneri*	10% trehalose + 5% dextrose	59	63	4	Intrinsic fluorescence	Unpublished data
SipD from *Salmonella* spp.	10% trehalose + 5% dextrose	57	63	6	Intrinsic fluorescence	
BipD from *Burkholderia pseudomallei*	10% trehalose + 5% dextrose	70	75	5	Intrinsic fluorescence	
PcrV from *Pseudomonas aeruginosa*	10% trehalose + 5% dextrose	60	65	5	Intrinsic fluorescence	
LcrV from *Yersinia* spp.	10% trehalose + 5% dextrose	75	78	3	Intrinsic fluorescence	
MxiH from *Shigella flexneri*	20% dextrose	41	47	6	Far-UV CD	Unpublished data
PrgI from *Salmonella typhimurium*	20% trehalose	41	45	4	Far-UV CD	
Respiratory syncytial virus	40% sucrose or trehalose	53	68	15	Far-UV CD	[104]
	40% sucrose or trehalose	55	65	10	2nd-derivative UV	
	40% trehalose	49	52	3	Laurdan fluorescence	

(continued)

Table 2 (continued)

Biophar-maceutical	Stabilizer(s)	Transition temperature				Reference
		Without stabilizer(s) (°C)	With stabilizer(s) (°C)	ΔT_m (°C)	Technique(s) used to measure T_m	
Norwalk virus-like particles	20% sucrose	62 69	64 71	2 2	DSC	[105]
Measles virus	10% mannitol	44	51	7	Laurdan fluorescence	[66]
Attenuated *V. cholerae*	0.15 M arginine or 0.075 M glutamic acid	32.5	55	2.5	Extrinsic (*Bac*Light kit) fluorescence	[69]
Surface adhesion A protein from *Streptococcus pneumoniae*	50 μM manganese chloride	59	70	11	Far-UV CD	[84]
Serine-threonine protein kinase from *Streptococcus pneumoniae*	20% lactose	70	59	3		
Cell wall separation protein from *Streptococcus pneumoniae*	20% lactose	34	44	10		
sEphB4-HSA fusion protein	20% dextrose	T_{m1}: 58 T_{m2}: 66	T_{m1}: 60 T_{m2}: 69	T_{m1}: 2 T_{m2}: 3	DSC	[78]
mAb-B	10% dextrose	60	63	3	Intrinsic fluorescence	[49]
IgG2	20% sorbitol	50	56	6	Intrinsic fluorescence	[80]
Hemagglutinin antigen of H1N1 influenza	20% dextrose	55	59	4	Intrinsic fluorescence	[82]
IgG1	5% dextrose + 10% sorbitol	56	61	5	Intrinsic fluorescence	[86]
Pneumolysoid L460D from *Streptococcus pneumoniae*	20% sorbitol	49	54	5	Intrinsic fluorescence	[92]
mAb FAb fragment	0.067 M spermidine trihydrochloride	T_{m1}: 70 T_{m2}: 75	T_{m1}: 74 T_{m2}: 76	T_{m1}: 4 T_{m2}: 1	DSC	[89]
TSP-2 from *Schistosoma mansoni*	20% dextrose	68	60	8	Intrinsic fluorescence	[93]
Acrylodan-Labeled Glucose-Binding Protein Sm4-AC	20% sucrose + 2.5 mM $CaCl_2$	50	60	10	Intrinsic fluorescence	[99]

Each measurement was conducted in triplicate and had ~1.0 °C of uncertainty

Table 3 Stabilizers identified for the selected biopharmaceuticals employing EPD approach using a high-temperature hold and monitoring aggregation

Biopharmaceutical	Stabilizer(s)	Aggregation			Reference
		Without stabilizer(s)	With stabilizer(s)	Technique(s) used to measure T_m	
H1N1 virus-like particles	10% 2-OH propyl B-CD	–	250% inhibition	OD350	[77]
sEphB4-HSA fusion protein	20% sorbitol	23% aggregate	1% aggregate	HPLC SEC	[78]
Glutathione S-transferase from *Necator americanus*	20% sorbitol	OD 350:0.2	OD 350:0.0	OD350	[81]
mAb-B	10% sucrose	–	82% inhibition	OD350	[49]
IgG1	0.75 M arginine	–	96% inhibition	OD350	[86]
Human pentraxin protein (PTX-2)	0.2 M sodium citrate	10% HWMS	0% HWMS	HPLC SEC	[88]

most effective. A representative example of the aggregation kinetics of the anthrax rPA at 37 °C, both alone and in the presence of selected compounds demonstrating a range of effectiveness, is shown in Fig. 10. This approach permitted efficient screening of a GRAS library and the rapid identification of potent stabilizers against aggregation. After optimization of the concentrations of selected stabilizers, studies were performed to ensure that the compounds also protected against conformational instability. To this end, thermal unfolding experiments in the presence of the potential stabilizers were performed using both intrinsic fluorescence and CD measurements. Representative data are shown in Fig. 11, and the dramatic stabilizing effect of trehalose, one of the most effective inhibitors of aggregation, is clearly seen in terms of an increase in the thermal stability of rPA by almost 10 °C over the unprotected protein. From the first measurement for construction of the EPDs to identification and optimization of stabilizers, the total time required for the general approach described here is typically less than 2 months and can often be accomplished in only a few weeks.

7 Conclusion

A critical step in the successful development of biopharmaceutical products is to find acceptable conditions for maintenance of stability. The less systematically based approaches that have typically been employed in the past for macromolecular stabilization are often both slow and produce less than optimal formulations. The "design of experiment" type of approach that is also sometimes employed suffers

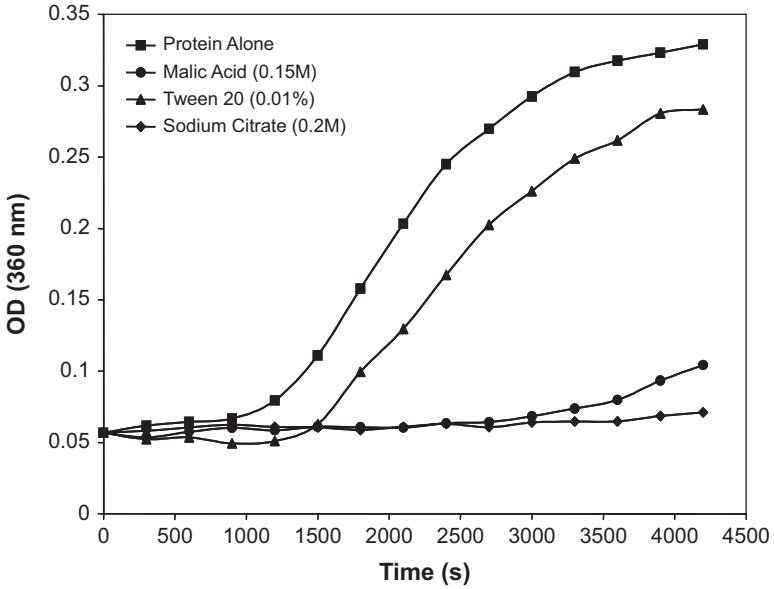

Fig. 10 Aggregation of rPA in the presence and absence of excipients (0.15 M malic acid, 0.01% Tween 20, and 0.2% sodium citrate)

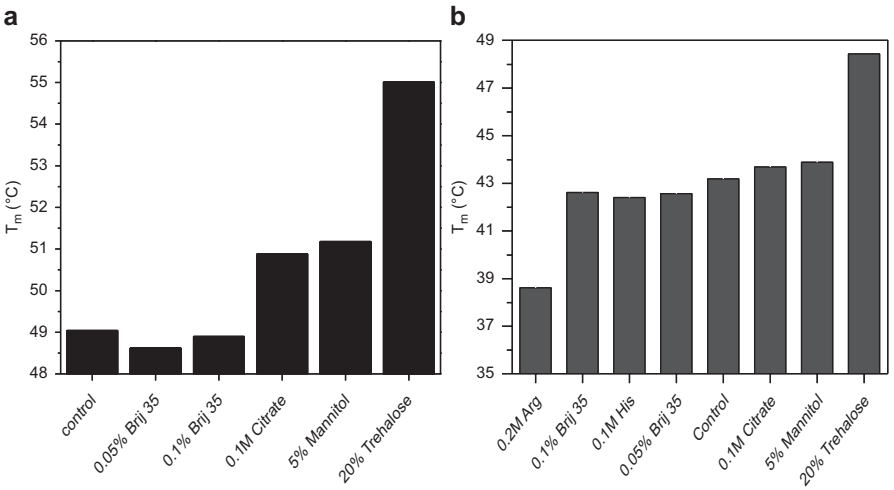

Fig. 11 Increase in rPA thermal transition temperature in the presence of trehalose compared to several other excipients as determined by CD (**a**) and intrinsic Trp fluorescence (**b**). The error bars represent an average of two measurements in which each data point has a standard error of less than 0.5. Note that the appearance of the fluorescence changes at much lower temperatures than those detected by CD implies the existence of a molten globule-like state(s) between these two temperatures [25]

from significant holes in the experimental space examined but does offer the advantage that it can be very sparing in the use of sometimes scarce drug substance. The systematic approach to preformulation and formulation of biopharmaceuticals described here is both rapid and comprehensive and also provides a direct route into the screening of large libraries of potential stabilizers. It typically requires 3–20 mg of target macromolecules. Furthermore, this approach begins to move the formulation process from strategic experimentation to rational design. Although there is no limitation to the number of variables that may be included in the phase diagram because of the truncation procedure used, if more than three different types of measurements are used to describe a molecule's behavior, some information may be lost. Alternatively, other higher-dimensional display methods must be employed to portray the data. Another potential limitation to the EPD approach is the resolution between the colored phases. In some cases, the color differences between phases are not as distinctive as might be desired, making the assignment of different phases difficult. Despite these limitations, the EPD approach appears to provide an intuitively attractive and powerful method to comprehensively characterize and display the solution behavior of biopharmaceuticals under various environmental conditions.

The EPD/high-throughput screening approach has been successfully used to characterize and formulate various biopharmaceuticals. To date this approach has been applied to a wide range of potential vaccine antigens and therapeutic agents including peptides, proteins, and nucleic acids as well as macromolecular assemblies including viruses, viral-like particles, bacteria, and toxins (Tables 1 and 2). With the availability of high-throughput instrumentation, the entire process of data acquisition, analysis, and interpretation described here can be accomplished in a relatively short period of time (weeks to few months), potentially dramatically enhancing both the effectiveness and efficiency of the formulation development process.

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Chapter 8
Biophysical Characterization and the Development of Therapeutic Proteins

Yangjie Wei, Nicholas R. Larson, Gang Hu, Prashant Kumar, and C. Russell Middaugh

A critical element in the development of drugs involves their structural analysis at both the chemical and physical level. With small molecules, this can be accomplished with high precision and resolution with techniques such as crystallography, NMR, and mass spectrometry among other methods. The large size and complexity of macromolecular drugs (e.g., proteins, nucleic acids, vaccines, etc.) usually require a somewhat different approach involving multiple lower resolution methods that can be combined into a multiple faceted picture of the macromolecule. While crystallography, cryo-electron microscopy, and NMR can provide high-resolution structures of proteins, nucleic acids, and some macromolecule complexes, they are usually not applicable to pharmaceutical development due to size, physical state, and other limitations. We will here limit our discussion to methods that are usually available to the non-expert due to their simplicity of operation and availability. Furthermore, we will only consider proteins with an emphasis on monoclonal immunoglobulins with a few examples to better illustrate certain techniques, although much of what we will discuss is more widely applicable. More detailed information can be obtained in the references and in some cases in the figure legends.

When discussing the structure of proteins, it is usual to classify levels of organization into four categories. These are the primary (the amino acid sequence), secondary (reflecting local interactions such as helices, sheets, and turns), tertiary (aspects of the overall three-dimensional structure), and quaternary (subunit and aggregative nature) structures. Different methods can be used to examine and sometimes quantitate various aspects of this classification system. We will employ this convention in our discussion here.

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1 Primary Structure

In the past, the sequencing of proteins was performed using the Edman degradation reaction in an automated instrument. Currently, we almost always use some form of peptide mapping. In this method, a protein is digested with one or more proteases. The digest is separated into individual peptides by chromatography (usually reversed-phase HPLC) and their molecular weights determined by high-resolution mass spectrometry. Subtle changes like those produced by deamidation and oxidation can be analyzed by an initial chromatography step to separate modified forms followed by peptide mapping. The high resolution of modern mass spectrometers (less than 10 ppm) allows single dalton changes as seen in deamination events to be detected. Such methods have become sufficiently routine that they can be used in stability studies (Fig. 1) [1].

It is common that lesser resolution methods are often used across all stages of development. The best known of these is SDS polyacrylamide electrophoresis

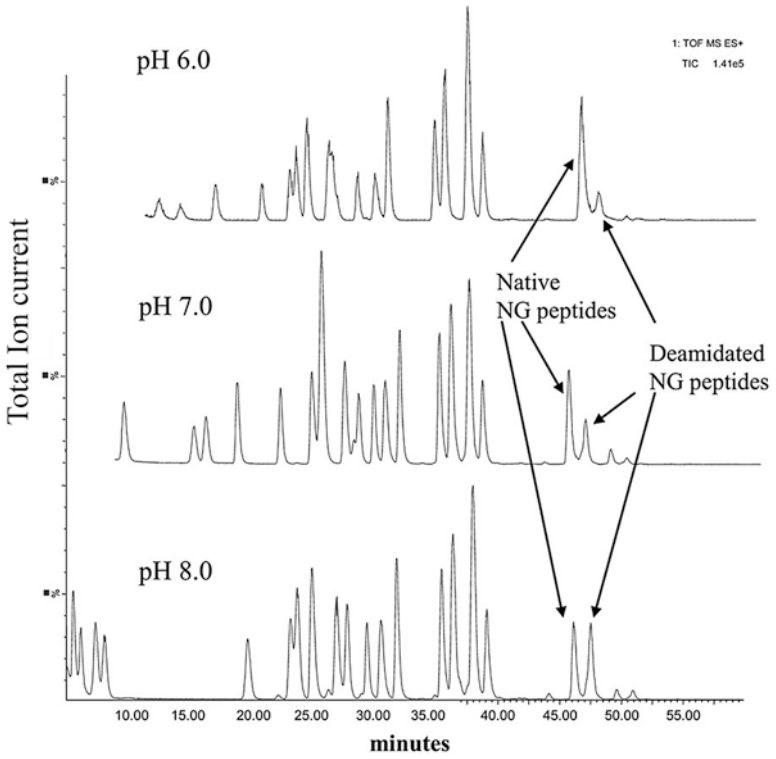

Fig. 1 Recombinant antigen A is a 42 kDa multi-epitope antigen produced during the development of a broadly effective vaccine for group A *Streptococcus*. Shown are peptide maps of antigen A produced by Lys C digestion at pH 6.0, 7.0, and 8.0. Proteolysis was performed for 7 days at 4 °C and then resolved and analyzed by LC/MS as described. (See reference [1])

(SDS-PAGE). In this method, proteins are unfolded by sodium dodecyl sulfate (SDS) into rodlike extended structures whose length is proportional to their molecular weight. The proteins are then separated by electric field-induced passage through a polyacrylamide matrix where they migrate approximately proportional to the log of their molecular weight. The proteins are stained by a dye, and their relative mobilities are obtained. This method is not usually sufficient to detect single amino acid changes but can often resolve small changes in molecular weight and larger changes such as oligomerization if these associations are not disrupted by the SDS. A reducing agent is usually included to aid in the unfolding of the proteins and to separate disulfide-linked subunits. Despite its lack of sensitivity, this method is widely used due to its speed and convenience.

Isoelectric focusing is also used to characterize primary structure because it is sensitive to charge differences. Although once a cumbersome technique when performed in urea gels, it is now typically performed in ampholyte (a mixture of charged peptides) containing capillaries with laser detection. Single-site changes involving charge residues are easily seen. For example, the spectrotypes of immunoglobulins resulting from individual deamidation events and sialyation differences are well resolved [2]. Similarly, reversed-phase or ion-exchange HPLC is often sufficiently sensitive to detect small changes in sequence.

2 Secondary Structure

Circular dichroism (CD) is a method based on differences in optical absorption of left- and right-handed circularly polarized light [3]. It is particularly useful for characterization of both protein secondary and tertiary (see below) structure. During an absorption event, there is a change in the distribution of charge that can be described by a transition dipole moment. In the case of an asymmetric molecule, there is also an absorption-induced magnetic dipole moment, which is circular in nature. The interaction of these two components is through their dot product, which results in a helical circularization of charge. Thus, molecules which contain circularly averaged absorptive entities such as the peptide bonds in α-helices and to a lesser extent β-structure and various turns possess strong to weak circular dichroism.

In proteins, the interaction between the peptide bonds causes splitting of the CD signal that results in characteristic spectra (measured as the difference in the absorption of the two forms of circularly polarized light as a function of wavelength) for different secondary structure types in the 180–250 nm region. Spectra are normalized for mean residue molar concentration and can be deconvoluted to produce relative contents of the different secondary structure types based on reference spectra. No worry though, since this is automated in the software of modern spectropolarimeters. Changes in temperature, pH, and other variables of interest allow alterations that involve perturbation of secondary structure as a function of various forms of stress to be quantitatively analyzed. Plots of spectral changes (typically molar ellipticity for normalization purposes to permit comparison of spectra between different

proteins) can be used to detect conformational changes in terms of secondary structure alterations (Fig. 2a, b) [4]. Contributions of other chromophores such as aromatic side chains and disulfide bonds in the 290–220 nm region can sometimes complicate interpretation.

There are a wide range of conditions under which CD spectra in the far UV can be obtained but also distinct limitations. A common protein concentration used is 0.1–0.2 mg/mL with a 0.1 cm pathlength (PL) cell. Much higher concentrations can be examined with short PLs [5]. For example, spectra of proteins at concentrations of 180 mg/mL can be obtained with 3 μm cuvettes [6]. Conversely, low concentrations can be examined with long PL cells. The only problem here is that when concentrations below 10 μg/mL are tested, a significant portion of the sample may be adsorbed to the inner surface of the cuvette.

A common problem in obtaining CD spectra is absorption in the UV region by solutes. Most buffers and other solutes absorb in the far UV region. Thus, keeping the concentration of such agents low is highly desirable but may be limited by their stabilizing effects and actual interest in their effects on target proteins. Another problem is the phenomenon known as absorption flattening. When the size of investigated agents becomes larger, they begin to shadow one another, i.e., particles no longer see actual incident light. The effect is to reduce the intensity of the CD signal (it typically becomes less negative), and it shifts to the red. This is commonly seen when proteins aggregate. Thus, one should confirm a lack of aggregation by concentration independence of the observed CD signal. A less frequently encountered artifact is differential scattering of left and right circularly polarized light, but this is rarely seen. We should also mention that modern spectropolarimeters may also permit near UV CD, absorbance, scattering, and even fluorescence to be simultaneously monitored (see later). They may also possess multiple sampling capability and even be found in microtiter plate formats. Therefore, CD can in some cases be used for high-throughput screening.

A second method that is widely used for protein secondary structure analysis is Fourier transform infrared (FTIR) spectroscopy [7]. This technique is based primarily on changes in vibrations of the peptide bond. Originally, the use of dispersive instruments permitted only very high concentrations to be studied which produced low-resolution spectra. Furthermore, interference by water signals was a problem that had to be reduced by the unsatisfactory solution of the use of D_2O as the solvent. This has changed dramatically in the last 30 years with the advent of Fourier transform instruments and sampling methods such as an attenuated total reflectance (ATR) which has allowed high-resolution spectra to be measured at much lower concentration (e.g., 0.1 mg/mL). Due to the coupling of FTIR data to mathematical methods to deconvolute spectral bands, FTIR spectroscopy now rivals CD in its utility for protein secondary structure analysis.

Most studies focus on the amide I band from 1600 to 1700 cm^{-1}. This broad highly structured but poorly resolved peak arises primarily from CO stretching and to a lesser extent from CN stretching and CCN deformation. There are many additional amide bands (amide A and B and II–VII), but these are less frequently employed. The usual elements of secondary structure (e.g., α-helix, β-structure,

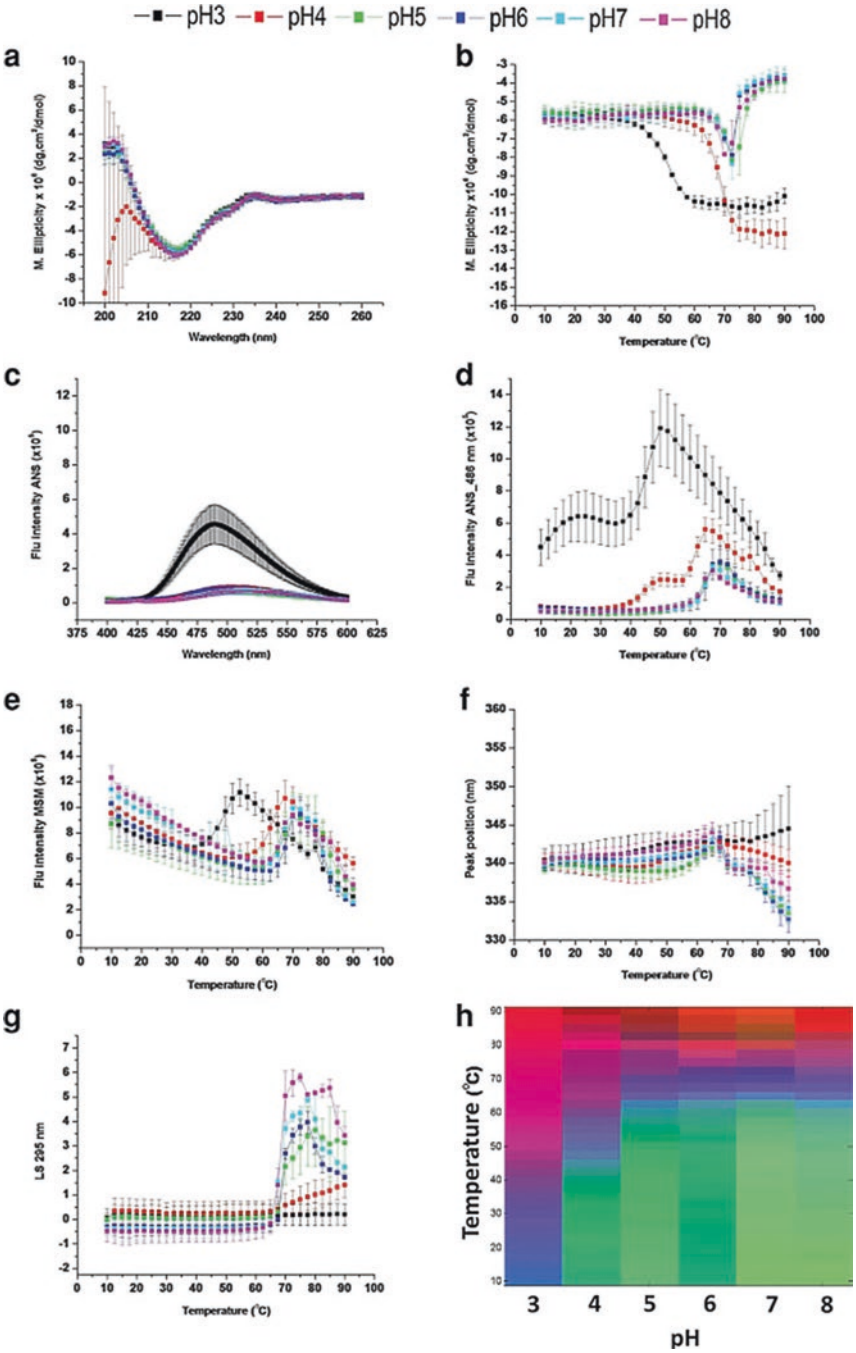

Fig. 2 Biophysical characterization of an IgG1 mAb as a function of temperature and pH. (**a**) CD spectra at 10 °C, (**b**) CD intensity change at 217 nm with temperature, (**c**) ANS spectra at 10 °C, (**d**) ANS melting curve at 486 nm, (**e**) fluorescence intensity versus temperature, (**f**) fluorescence peak position changes with temperatures, (**g**) static light-scattering intensity change with temperature, and (**h**) empirical phase diagram (EPD) analysis of this data. Data shown for $n = 3$ measurements. (See reference [4])

turns, loops, and disordered regions) all produce at least one and sometimes more distinct peaks that can usually be resolved by some combination of Fourier self-deconvolution, derivative analysis, and curve fitting [7]. Less common structured forms such as the 3_{10}-helix, poly (L-Pro) helix, and the left-handed α-helix can also be detected. A very strong signal is produced in the 1610–1620 cm^{-1} region by aggregated protein due to the cross-beta nature of the interactions between high associated materials. This signal may be of special importance to the pharmaceutical scientist due to the need to detect aggregated protein as a degradant in protein samples (Fig. 3) [8].

Although transmittance was initially used as the most common sampling geometry, its application has been reduced by the use of attenuated total reflectance geometries. The very high precision of such methods allow water signals to be quantitatively subtracted and subsequently quantitative secondary structural analysis to be performed. A number of amino acid side chains also absorb in the amide I region (Asn, Glu, Arg, Lys, His, Tyr, etc. with their contribution depending on their state of ionization). These signals are usually weak and therefore ignored, but an unusually high concentration of a particular side chain may require its subtraction before secondary structure analysis is performed. Values of the extinction coefficients of side chains are available for this purpose [9, 10].

Unlike CD, FTIR spectroscopy has been routinely used to examine proteins in the solid state. This is usually done either by grinding the protein solid with IR transparent KBr and compressing a pellet or by the use of diffuse reflectance or ATR. This has been especially useful to search for protein structural changes in lyophilized formulations. As with CD, interference by solutes needs to be considered. Any substances with bonds will produce IR spectra although spectral windows in amide regions can sometimes be found. One can unfold proteins by a salt such as LiBr, but many common chaotropic agents such as urea and guanidine HCl are usually of little use due to their strong IR absorbance. The usual solution variables of interest (temperature, pH, ionic strength) can all be easily studied by FTIR spectroscopy. FTIR instruments are often significantly cheaper than spectropolarimeters further increasing the attractiveness of this technique.

A second vibration-based technique that can be used to examine the secondary structure of proteins is Raman spectroscopy [11]. The difference in spectra between the infrared and Raman methods lies in their unique spectral selection rules. An infrared active transition requires a change in dipole moment, while Raman involves an alteration in polarizability. Vibrational transitions can be IR active, Raman active, or both. The utility of the Raman method was dramatically enhanced by the advent of lasers, which enhanced naturally weak Raman signals. Raman spectroscopy is actually a light-scattering method in which scattered light shifts both up and down

Fig. 3 (continued) a function of pH and temperature as monitored at 1637 (**b**), 1620 (**c**), and 1668 cm^{-1} (**d**). FTIR spectra were baseline corrected and vector normalized between 1590 and 1710 cm^{-1}. The peak at 1620 cm^{-1} reflects aggregation of the protein. Differential absorbance data at 1668 cm–1 was parallelly shifted by one or multiples of 0.002 units for clearer presentation of the data. Error bars represent 1 SD with $n = 2$. (See reference [8])

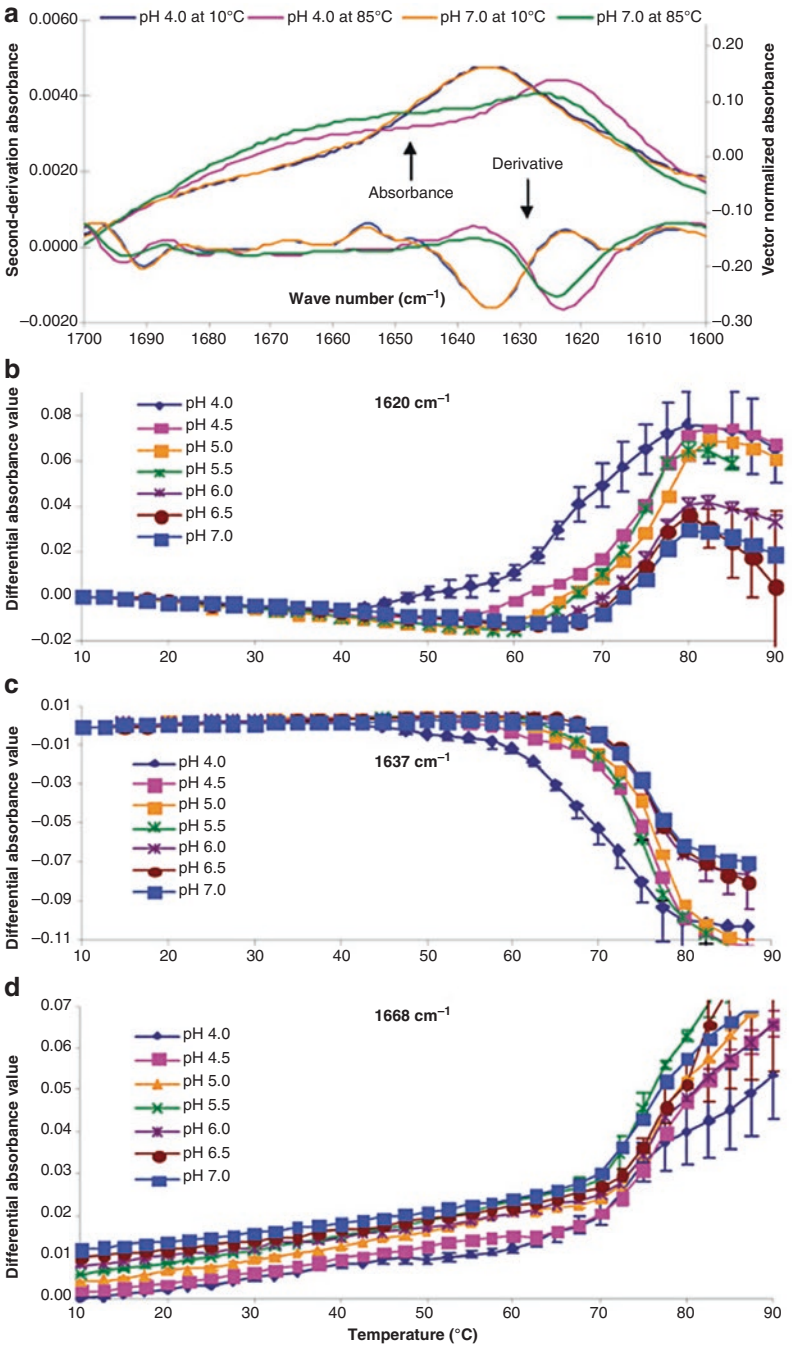

Fig. 3 Fourier transform infrared analysis of an IgG2 mAb as a function of pH and temperature. Representative FTIR absorbance and second-derivative spectra of the IgG2 mAb at indicated solution pH before and after heat treatment (**a**). FTIR differential absorbance of the IgG2 mAb as

in frequency due to interactions with the vibrational transitions of target molecule. The difference in frequency of the incident (laser) light with vibrational transitions constitutes the Raman spectrum. The weak signals produced even with laser excitation require relatively high protein concentration (5–10 mg/mL). Amide bands are present, but the amide III band is often preferred due to its structure and separation of the individual secondary structure element signals. Unlike FTIR spectroscopy, amino acid side chain signals are often easily resolved making the technique of potentially broader utility. Like FTIR spectroscopy, the Raman method can be applied in both the liquid (aqueous) and solid state. Water bands are much weaker eliminating their interference as a major problem. As indicated above, however, Raman signals are intrinsically quite weak. Two modified forms of Raman spectroscopy, however, overcome this problem. The first is resonance Raman spectroscopy (RRS). In this method, an ultraviolet (UV) laser excites the sample within an absorption band(s). With proteins, this most often involves the peptide band itself in the far UV region. The electronic absorption band transitions of the peptide bond become coupled to vibrational transitions resulting in a dramatic increase in the intensity of the vibrational signals and a much more intense Raman spectrum. The two major problems with this approach are the need for expensive UV lasers and the degradation of the sample due to the intense, focused UV light. The latter can be at least partially overcome by the use of a flow cell. As appropriate instruments become more readily available, we expect to see expanded use of this method.

A second technique is known as surface-enhanced Raman spectroscopy. Spectra can be enhanced by as much as 10^{11}–10^{12}. The sample is usually deposited on a metallic surface such as silver or gold, often in the form of a nano- or microparticle. The mechanism is thought to involve the electric field of the surfaces and the excitation of localized plasmons. Visible or near IR light is used to excite the surface-bonded molecules of interest. The extraordinary sensitivity of the method makes it very attractive, but the need for surface localization and the particular surfaces which display the necessary properties have so far limited its use in the biopharmaceutical world.

3 Tertiary Structure-Sensitive Methods

The precise tertiary structure of a protein can only be determined by X-ray crystallography, NMR, or cryo-electron microscopy. A potential 3D structure may also be estimated by homology modeling and energy minimization. None of these methods are usually directly applicable to protein formulation and stabilization work. Thus, lower resolution methods are again typically employed with an emphasis on changes in tertiary elements rather than the actual 3D structure itself. Here we will describe the most prevalent of these methods, their routine use, as well as their advantages and disadvantages.

The simplest but not the most widely used method to examine tertiary structure changes is UV absorbance [12]. This method has been widely used to measure

protein concentration but has been increasingly applied to gather additional information. Below 300 nm, proteins primarily display two peaks. One in the far UV arises from peptide bonds (this is the same peak used in far CD analysis) and a second from the side chains of the three aromatic amino acids with a weak contribution from disulfide bonds. In principle, the far UV peptide bond peak found between 180 and 200 nm could be used to analyze secondary structure, but it is poorly resolved, and interference in this region by oxygen and other agents is difficult to overcome with conventional spectrometers. Thus, its use has been superseded by CD analysis in the same spectral region. In contrast, the near UV aromatic region possesses great utility, which we will describe below. All three aromatic side chains feature structured spectra with the magnitude of their extinction coefficients in the order Trp > Tyr > Phe. Even a simple visual inspection of a protein near UV spectra often permits the presence of all three side chains to be confirmed if they are present in at least moderate concentration. The high information content of a protein's spectrum can be easily seen by calculating its second derivative, which will typically display 7–8 distinct negative peaks. Using a diode array instrument and curve fitting (splining works especially well), the positon of these peaks can often be established to a precision of ±0.02 nm. Since the Phe residues are usually buried, Tyr interfacial due to its hydroxyl group and Trp can potentially be found throughout the structure; the derivative UV spectrum of a protein can provide significant information about changes in the tertiary structure of a protein. Effects of temperature, pH, ionic strength, solutes, and long-term real-time and accelerated stability can all be probed (Fig. 4) [13]. Furthermore, if light scattering (turbidity) is present, this is usually

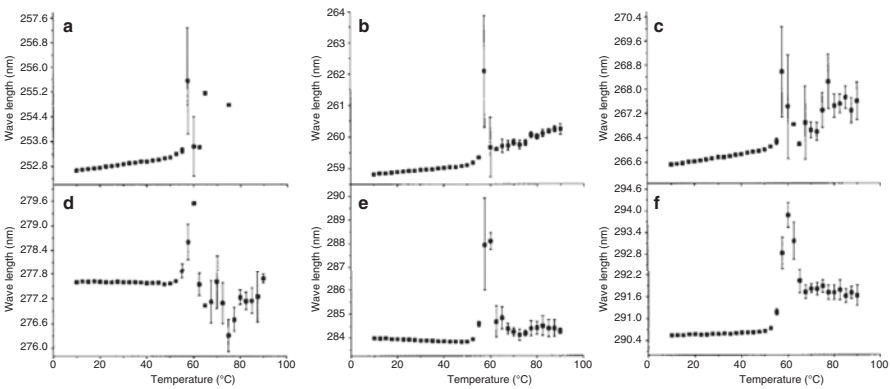

Fig. 4 Bovine granulocyte colony-stimulating factor (bGCSF) is a member of the four helix bundle family of proteins and has veterinary therapeutic potential, whereas its human analog is already an important drug. Derivative absorbance studies of bGCSF are shown here as a function of temperature at pH 5. The wavelength positions of six negative peaks were followed as a function of temperature: (**a**), Phe; (**b**), Phe; (**c**), Phe; (**d**), Tyr; (**e**), Tyr/Trp; and (**f**), Trp. Protein concentration was 5 μM in 10 mM citrate buffer. Spectra were collected at 2.5 °C intervals, with a 5-min temperature equilibration period included before data collection. All errors are reported as standard error ($n = 3$). (See reference [13])

manifested by optical density (OD = absorbance + turbidity) above 300 nm which is proportional to a high power (3–4) of the wavelength (due to Raleigh scattering). Note that absorption spectra can be corrected for the presence of light scattering by a variety of different methods including extrapolation of OD value above 300 nm into absorbing regions (which is nonlinear) and the use of derivatives [14]. Using temperature-dependent second derivative absorbance spectroscopy of aromatic amino acids [15] or by analysis of the effects of cation-π interactions on absorption [16], aspects of protein dynamics can also be probed.

Probably the most widely used class of methods employed to examine changes in the tertiary structure of proteins are those that involve fluorescence spectroscopy [17]. The origin of the fluorescence signal used in pharmaceutical protein development activities can be either intrinsic or extrinsic. The intrinsic fluorescence from proteins arises primarily from aromatic residues. If Trp is present, this will almost always dominate the emission spectrum since Tyr and Phe are weak emitters and can lose energy by energy transfer (see later). If no Trp is present, then the fluorescence of Tyr and to a lesser extent Phe can be seen. One exception occurs when the emission of the indole side chain is quenched, perhaps by a proximate positively charged amino or guanidine group. The effect of temperature on aromatic emission results in a smooth decrease due to thermal quenching of the excited state, but this can usually be differentiated from structure changes which produce an alteration in the continuous decrease or a change in inflection of the curvilinear thermally induced decrease in intensity. Another method to resolve structural changes is to measure peak position instead of spectral intensity. Peak position can be accurately determined using derivative analysis or by determining the mean spectral center of mass. The latter is usually more precise but is redshifted 8–14 nm relative to actual peak position because of the asymmetry of Trp fluorescence peak (Figs. 2e, f, and 5) [18].

The quenching of intrinsic fluorescence can be achieved by extrinsic agents [19]. The most common method employs small molecules such as acrylamide or heavy metal salts, which effectively quench some aromatic side chains. A neutral molecule-like acrylamide can actually diffuse through the dynamic protein matrix. Varying the concentration of the quencher and its effect on quenching can be used to probe the dynamic nature of a protein, although care must be taken to ensure the quenching probe does not alter the structure of the protein (especially with acrylamide). In contrast, a charged ion such as iodide or cesium can be used to selectively quench surface residues (usually Tyr).

Polarization measurements can also often be of utility in certain applications. When a protein is illuminated with polarized UV light, it will selectively excite those fluorophores whose absorption transition dipole moments are approximately parallel to that of the incident light. As a target fluorophore rotates, the polarization of the emitted light is decreased. From an analysis of such data, rotation correlation times of entire molecules can be determined. The rotational correlation time is the time required for the molecule to rotate 1/3 of its circumference. Since the rotational time is sensitive to the size and shape of a protein, the technique can be used in studies requiring such information. Furthermore, when two molecules interact, the

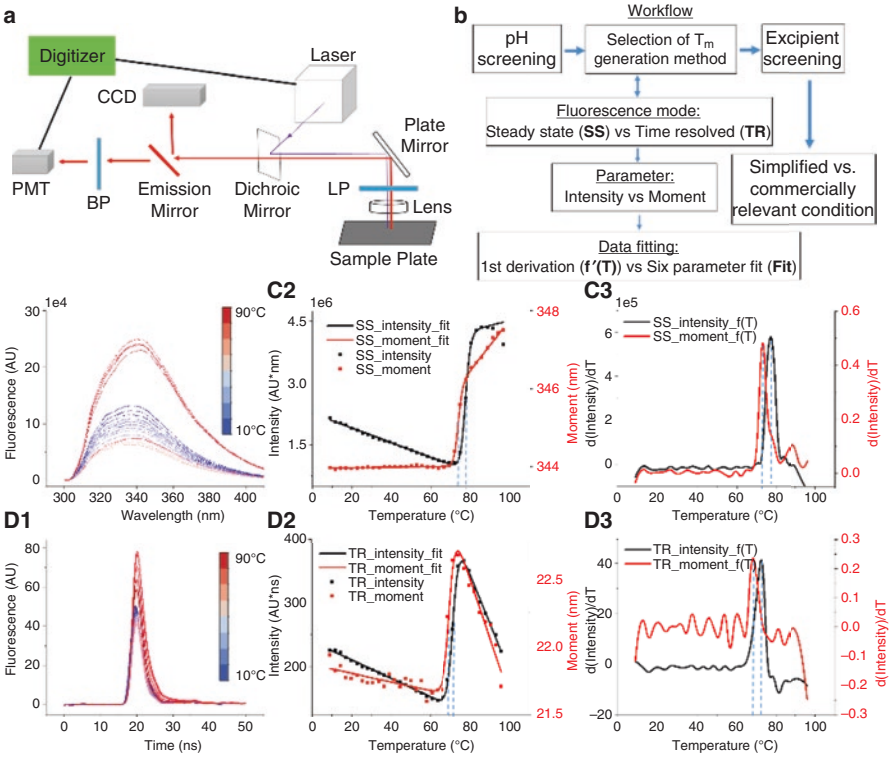

Fig. 5 A schematic diagram of a high-throughput fluorescence plate reader (**a**). This instrument is capable of recording both (time-resolved fluorescence) TRF and (steady-state fluorescence) SSF. An emission mirror is placed to direct the emission signal to a CCD detector for the measurement of SSF. Otherwise, the TRF signal is recorded by a PMT. A general workflow for the formulation optimization of proteins using this fluorescence plate reader (**b**). Thermal melting study of protein samples using intrinsic SSF (C1–3) or TRF (D1–3). Representative temperature-dependent raw fluorescence spectra (C1) of samples; the processed melting curves are monitored by moment (red dot) or intensity (black dot). Fitted melting curves using a six-parameter fitting method are shown as a continuous line (C2). Calculation of the melting temperature (T_m) using the first derivative (C3). Representative temperature-dependent raw waveform (D1) of samples; the processed melting curves are monitored by moment (red dot) or intensity (black dot). Fitted melting curves using the six-parameter fitting method are shown as a continuous line (D2). Further calculation of the T_m using the first derivative (D3). The dashed lines indicate T_m. (See reference [18])

rotation of the fluorophores is slowed, and the polarization is decreased along with the rotational correlation time. In fact, this method is infrequently used during the formulation and development of therapeutic proteins but is much more commonly used in a wide variety of binding assays. We should mention here that one is not limited to intensity and peak position measurement in fluorescence. The lifetime of the excited state can also be obtained with microtiter formats for such determination now available (Fig. 5).

There are, in fact, a remarkable number of experimental approaches that employ fluorescence emission. We will very briefly mention a few more of these although most are not commonly employed during the general pharmaceutical development of proteins. Fluorescence resonance energy transfer (FRET) is a method that takes advantage of the phenomenon in which excitation of a fluorophore (the "donor") whose emission peak overlaps the absorption peak of a second fluorophore (the "acceptor") can produce a resonant transfer of energy such the excitation of the donor results in a decrease of its emission and the sensitized emission of the acceptor. The efficiency of this process is dependent on the distance between the donor and acceptor and the angle between their dipoles and their spectra (in the form of a spectral overlap integral). In ideal cases, the distance between the donor and acceptor can be calculated. This usually involves the use of one or more extrinsic fluorophores covalently attached to known locations, a situation not ideal for the pharmaceutical scientist since it could significantly alter the size and solution behavior of a therapeutic protein. It is thought, however, that the energy transfer between Tyr and Trp residues is partially responsible for the weak emission of Tyr residues. The technique is, however, widely used in fluorescence microscopy, a method of great importance, which we will not consider here. There are a wide variety of other fluorescence-based methods not routinely encountered in pharmaceutical analysis. There include fluorescence photobleaching and recovery, fluorescence correlation spectroscopy, red-edge excitation, and single-molecule fluorescence which might have some utility under special circumstances but are more in the realm of the specialist. The use of extrinsic dyes, however, opens up a wide variety of methods that are of significant importance to the pharmaceutical scientist. We will focus on just a few of these applications here based on their relative importance in the analysis of therapeutic proteins.

Extrinsic fluorescence probes have several advantages over intrinsic fluorophores including usually greater fluorescence (quantum yield), sensitivity to their local environment, and the wide variety of such molecules with diverse properties that are available. One such use is the direct binding of probes (often called dyes for historical reasons) to particular regions of both native and conformationally altered proteins. Most commonly used probes have at least an element of apolar (hydrophobic) character. Thus, if a protein has a site or sites of an apolar nature, it is possible that a dye (depending on its structure) may bind to a protein. This is especially the case if a protein is partially unfolded or altered in such a way that binding is facilitated. For example, molten globular states of proteins which contain near-native secondary structure but a loosening of tertiary structure often bind a variety of dyes. Binding can be manifested by either an increase or decrease in the intensity of the dye's fluorescence and/or a shift in its emission wavelength optimum. Some dyes are strongly quenched by an aqueous environment, and their intensity becomes dramatically enhanced when they are located in an environment where their quenching is at least partially relieved. Therefore such dyes can be used to characterize the stability of a protein as a function of temperature, the most common perturbant. The dyes 8-anilinonaphthalene sulfonic acid (ANS) and bis-ANS are often used for this purpose (Figs. 2c, d, and 6) [4]. Other forms of stress (e.g., pH, a chaotropic agent, etc.)

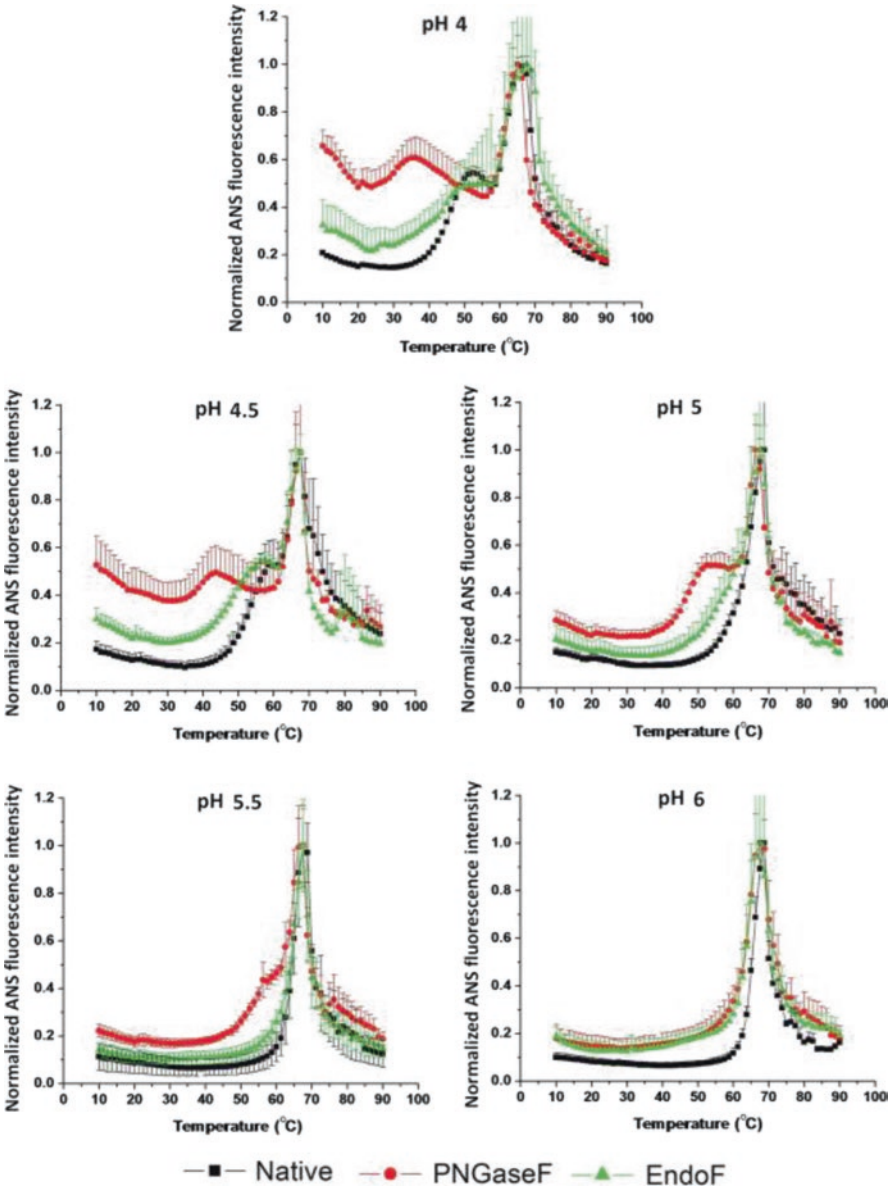

Fig. 6 Normalized ANS fluorescence intensity change as a function of temperature in the presence of an untreated (control) IgG1 (black line), partially deglycosylated IgG1 (green line), and fully deglycosylated IgG1 (red line) from pH 4 to 6. Normalized results were generated by fitting the data to be equal to one at the maxima for incorporation into EPDs and radar charts. Curves shown here are averages of three runs. (See reference [4])

can be problematic since they can alter the interaction of a dye with a protein independent of structural perturbation. In recent years, the availability of PCR instruments in many laboratories has led to their use for fluorescence melting studies employing dyes such as SYPRO orange. If detergents are present in formulations, there are a class of dyes known as molecular rotors, which appear to not bind to detergent and can therefore be used as probes [20]. It is also possible to conjugate fluorescent dyes to proteins where they can serve as conformational or environmental probes or as FRET donors and acceptors. In pharmaceutical formulation and development, however, this produces a significantly altered protein which cannot be considered an accurate representation of a drug substance.

Circular dichroism can also be used to examine tertiary structure changes. While the far UV region (180–250 nm) reflects peptide bond chirality and secondary structure, the near UV (250–320 nm) contains primarily signals from the aromatic groups and disulfide bonds. These spectral features are much weaker than those in the far UV and therefore require higher concentrations and longer pathlength. Typical conditions used might be 0.5–1 mg/mL protein and 0.5–1.0 cm pathlength. Spectra in this region are not well resolved, but the combined intrinsic and induced optical activity make aromatic rings and disulfide bonds quite sensitive to subtle changes in their immediate environment. Derivative analysis in the near UV CD region can be quite helpful in resolving spectral features, but it is generally difficult to make unambiguous assignments other than through the approximate locations of the spectral features. Both thermal studies and pH and chaotropic effects can all be explored in this region.

4 Quaternary Structure and Protein Aggregation

Proteins can self-associate into defined oligomers (where the individual proteins are known as subunits) or into larger, more random structures which we refer to as aggregates. These higher molecular weight entities can be characterized in terms of their size, size distribution, molecular weight, and shape among other properties. Such analyses have recently become especially important with the recognition of the presence of both submicron and larger particles in pharmaceutical formulations of therapeutic proteins. Such aggregates can be both immunogenic and display losses of their therapeutic activity requiring their identification and eventual removal from final formulations. As a consequence of their all-too-common presence, a wide variety of methods have been developed to facilitate their identification and characterization. We do not have sufficient space here to describe all such methods currently available, so we will briefly focus on the more commonly used techniques and their utility with regard to the formulation and development of proteins.

The most commonly used method to characterize the size of macromolecules, their oligomers, and aggregates is size exclusion (molecular sieve or gel filtration) chromatography. In this technique, the test solution is pumped through a bed of porous beads made of materials such as agarose, dextran, or polyacrylamide.

The flow of a protein or other macromolecule is impeded by its diffusion into the beads, which is dictated by the size of the channels in the beads and the dimension of the macromolecule. The result is a separation of the proteins based on their size, shape, and molecular weight with their appearance at the exit of the column detected by their optical absorbance, fluorescence (intrinsic or extrinsic), light scattering, refractive index, or some other property of the eluted protein (Fig. 7b) [21]. It is possible to estimate the molecular weight of the chromatographed material by its elution positon relative to MW standards, but a more accurate value of the hydrodynamic radius can be obtained by this method. Higher molecular weight material (e.g., aggregates) usually appears in the void volume of the column since they are unable to enter the bead pores and are not separated further. Although this is a very powerful method and is widely employed, it does suffer from a couple of potential problems. Proteins are diluted as they pass through the column and thus their oligomeric state may change. It is also possible that a protein may interact with the column matrix thereby distorting an interpretation of its behavior in terms of its size. This can sometimes be eliminated by the addition of high salt or a chaotropic agent. There is an alternative separation method that avoids the potential problems of interaction with the column material. This is known as field flow fractionation. Here a perpendicular field is applied to sample flowing through a narrow tube. It is especially effective for larger particles but can separate proteins, their aggregates, and complexes over a very wide range. Although this method has been available for some time, it is usually not as readily available as SEC.

Probably the second most common method to size proteins is dynamic light scattering (DLS) (also known as quasielastic or photon correlation light scattering) [22]. Samples are illuminated with a laser, and the scattered light is detected at one or more angles, with 90^0 the most common value. The fluctuations in intensity of the scattered light due to the Brownian motion of the macromolecule or its aggregates are analyzed in the form of an autocorrelation function. This can be interpreted in terms of diffusion coefficients and converted to hydrodynamic radii by the Stokes-Einstein equation. The usual size range that can be examined by this technique is over the range of 1–1000 nm. Although a specialist technique, only 20 years ago, the availability of commercial instruments has made DLS a routine laboratory method. Such instruments are now often employed as "black boxes," but care is required in their use and subsequent data analysis. Because all samples display some degree of heterogeneity, the values obtained reflect a range of sizes. Such data can be viewed in a number of different formats including in terms of intensity, mass, and number averages. An averaging method known as cumulant analysis can provide a "mean" diameter and a measure of polydispersity. Intensity deconvolution methods reflect primarily scattering from larger particles and make it appear samples are dominated by the larger entities. This can be useful for detecting the presence of aggregates but presents a distorted view of the actual particle size distribution in the solution. In contrast, the number average calculated distribution reflects the actual distribution and is usually recommended if the polydispersity is low. A problem of which to beware is an apparent decrease in size at higher concentration. An observation of this type is usually caused by multiple scattering. If a

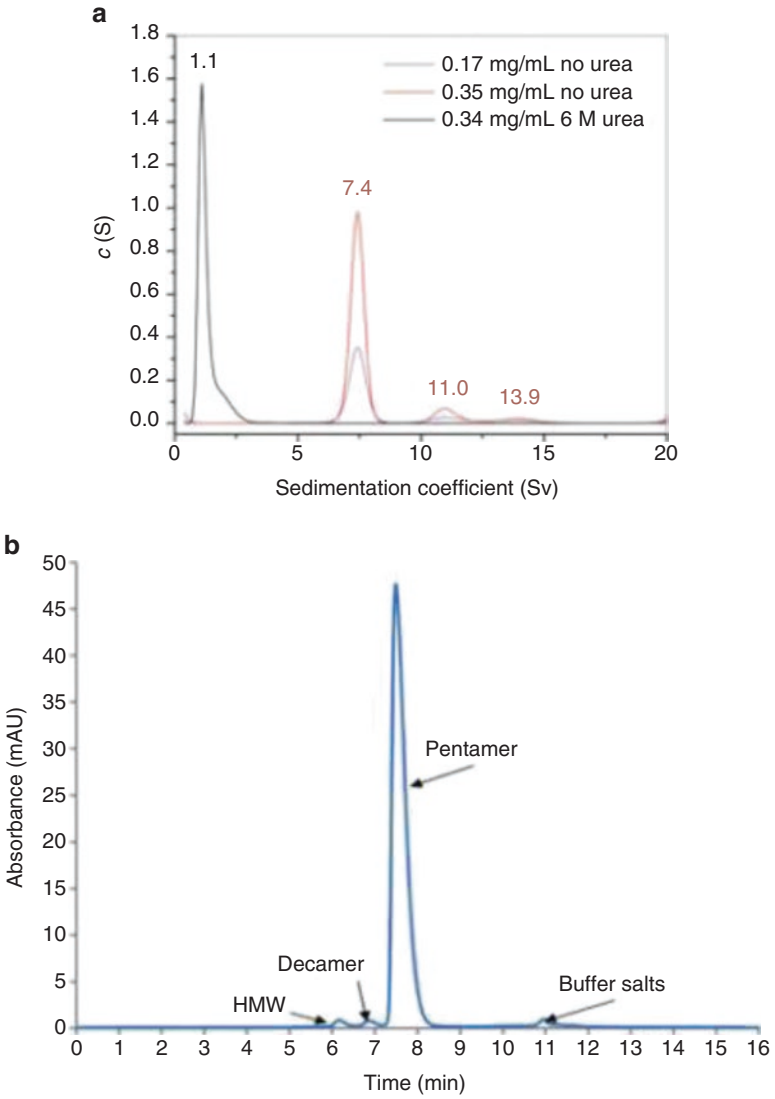

Fig. 7 The potential human therapeutic protein pentraxin (PTX-2) is a large, glycosylated plasma protein consisting of five monomers that self-associate noncovalently into a pentameric, ringlike structure. Determination of the size distribution of rh-PTX-2 in solution. (**a**) Distribution of sedimentation coefficients as determined by sedimentation velocity analytical ultracentrifugation for rhPTX-2 at 0.17 mg/mL (magenta) and 0.35 mg/mL (red) in PBS buffer and at 0.34 mg/mL in PBS buffer containing 6 M urea (black). (**b**) A SEC chromatogram for rhPTX-2 in 10 mM sodium phosphate pH 7.5 with 5% (w/v) sorbitol was generated by dilution into the SEC mobile phase buffer before analysis. Both methods detect the subunit structure of the protein. (See reference [21])

photon is scattered more than once, this adds a fast component to the autocorrelation function which can be mistaken for a decrease in size. This can be partially corrected for by the use of back-scattering angles so the radiation does not penetrate deeply into the solution, but this usually is only partially effective. DLS experiments can usually be performed as a function of temperature, and the absolute intensity can also be obtained simultaneously, adding to the utility of this method (Fig. 8) [23].

Static light scattering (SLS) can be considered of equal importance to the dynamic form [24]. This method has been employed in a variety of forms ranging from the simple to the complex. The most sophisticated approach involves measurements of scattering intensities at multiple angles and concentrations. The scattering determinations are often in the form of a chromatography detection on a SEC column and can provide detailed information about the molecular weight and radius of

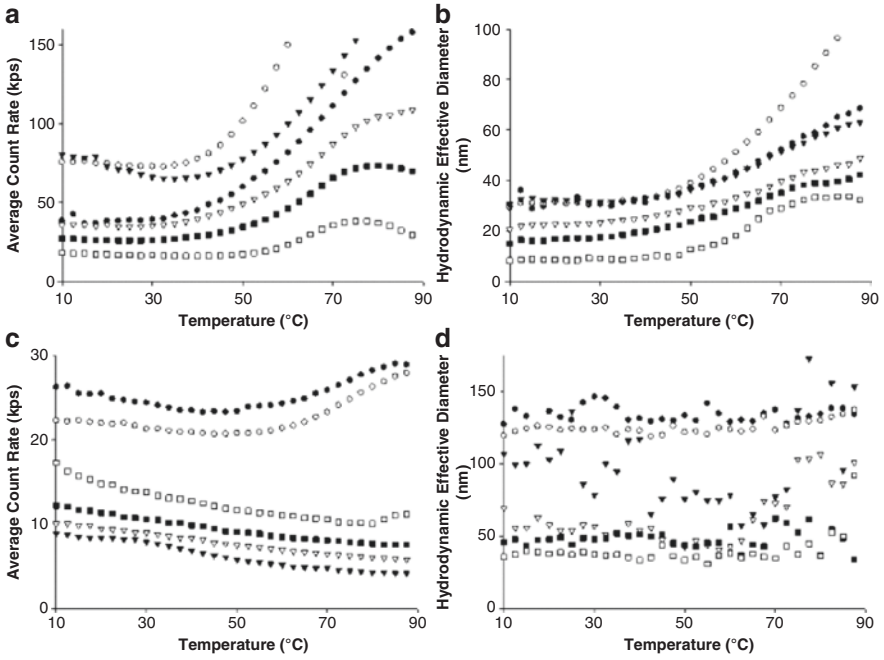

Fig. 8 The hepatitis C virus (HCV) envelope glycoprotein E1 has been employed as a potential vaccine antigen. A truncated form (amino acids 192–326) of the E1 protein (E1y) was expressed in the yeast *Hansenula polymorpha* and purified from the cell lysate. E1y forms protein particles in the absence of detergent and remains monomeric when detergent concentration is high. Dynamic light scattering (DLS) measurements of HCV E1y (1 mg/mL) in the presence of Empigen BB from 10 to 87.5 °C. The DLS intensity at 532 nm was measured at a 90° angle to the incident beam with a 30-s integration time. The light-scattering intensity is shown in panel (**a**) for pH 5 and panel (**c**) for pH 7. The effective hydrodynamic diameter is calculated using the cumulant method and presented in panel (**b**) for pH 5 and panel (**d**) for pH 7. The mean values of five measurements are shown for the following Empigen BB concentrations (w/v): ●, no detergent; ○, 0.01%; ▼, 0.1%; ▽, 0.5%; ■, 1%; and □, 2%. (See reference [23])

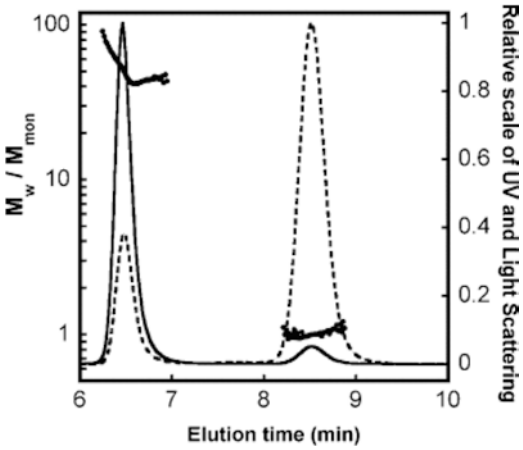

Fig. 9 Illustrative SEC-MALS chromatogram and weight-average molecular weight (M_w) profile for an aggregated bovine α-chymotrypsinogen A (aCgn) created by incubating an initially monomer sample (initial protein concentration, C0 = 1 mg/mL) at 65 °C for 10 min to achieve approximately 30% (by mass) loss of monomer. A Protein PAK 125 SEC column is used. The peak at ca. 6.5 min is high-molecular-weight aggregate; the peak at ca. 8.5 min is monomer. Solid and dotted lines, respectively, are relative light-scattering intensity (left vertical axis, only 90° scattering angle shown) and relative UV absorbance at 280 nm (right vertical axis). M_w values (scaled by theoretical monomer M_{mon} = 25.7 kDa) for each 1 s "slice" of the two peaks are given by the symbols (left-hand vertical axis). (See reference 25)

gyration of a protein using a Zimm-type analysis (Fig. 9) [25]. A setup to do this, however, can be somewhat expensive. Much simpler but less informative procedures can simply employ the scattered light at the excitation wavelength in a fluorescence experiment or the optical density (turbidity) from a simple spectrometer. These simple procedures are surprisingly powerful approaches which are usually available in a microtiter plate format making it especially useful for screening purposes. Whatever the instrument used to obtain such scattering data, it is usually used in a relative rather than absolute manner. In a common experiment, scattering intensity or turbidity is recorded as a function of time. These values are then analyzed as initial rates, delay times (reflecting nucleation events), or final values reached at longer time. When screening for inhibition of aggregation, one then looks for a decrease or complete elimination of one or all of these parameters. This approach is widely used since aggregation is often a key, undesired event in the degradation of therapeutic proteins. One should also not underestimate the utility of simple visual examination of a protein solution. This can be manifested as "cloudiness" or translucence. Recently, it has been recognized that actual colloidal phase separation can also occur. While at first this usually appears like the above, with time the solution will actually separate into two distinct phases, one much more concentrated in protein than the other. Another observed form of precipitation appears as particles or strands of material with a wide range of physical properties.

The preceding are probably the most common methods used to characterize the size, oligomeric state, and aggregation of pharmaceutical biomolecules. There has been a recent explosion of interest in alternative techniques with individual advantages. We will briefly consider four of these. The first is known as micro-flow imaging (MFI). This method directly images particles employing a digital camera and measures both counts and size and characterizes various aspects of the morphology of the observed entities. To a limited extent, it can differentiate different types of particles (proteins, air bubbles, aggregates, etc.) and provide an accurate picture of the distribution of sub-visible particle sizes. Depending on the instrument, it can measure particle size in the range of 1–2 μm to 300 μm. Such instruments have become increasingly widely used to characterize protein formulations and their aggregates (Fig. 10) [26].

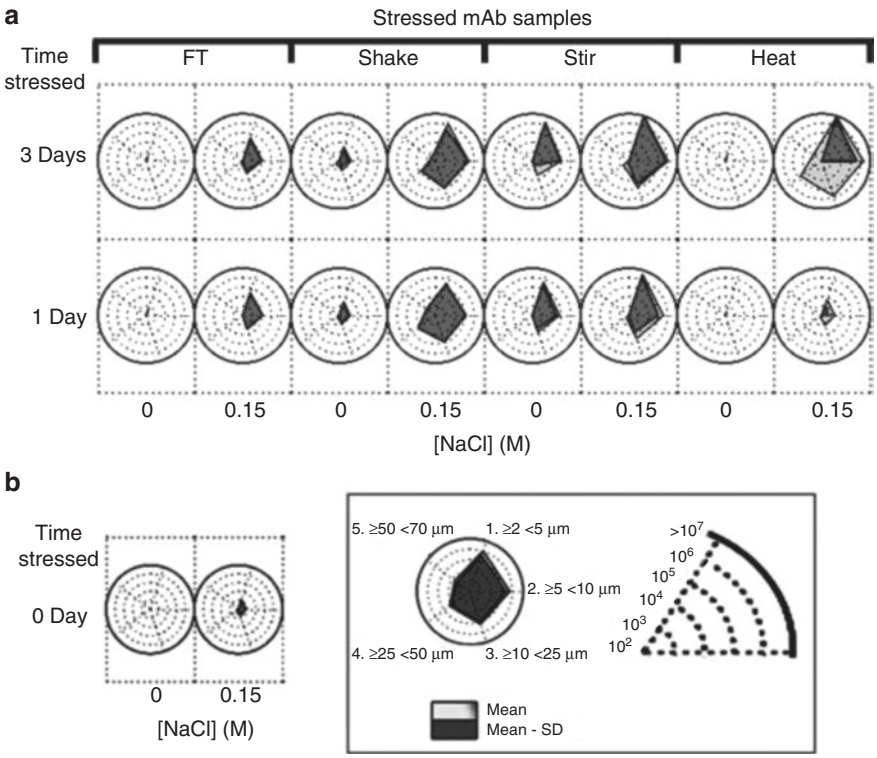

Fig. 10 Radar plots for visualizing formation of sub-visible particles (concentration and size distributions) in IgG1 mAb solutions exposed to different stresses as measured by MFI. Radar plots show MFI particle concentration and size data distributions as generated by four indicated stresses when applied to a 1 mg/mL antibody solution in 10 mM sodium acetate, pH 5 with and without 150 mM NaCl. See reference [26] for details of radar plot analysis. The data shown are the average of three separate experiments ($n = 3$), and the error represents one standard deviation. The relative sizes determined by MFI are shown as the magnitude of the axes in the radar diagrams (see box). (See reference [26])

A second instrument employs a method known as nanoparticle tracking analysis (NTA). This method is effective at determining smaller sizes (10–2000 nm) and functions by measuring the diffusion of individual particles, in contrast to DLS. Particles are individually observed undergoing Brownian motion. Using a camera (e.g., a CCD), the motion of the particles are tracked on a frame by frame basis with the Stokes-Einstein equation used to calculate a hydrodynamic radius. An accurate particle distribution can be obtained. A third method is known as resonant mass measurement. This method employs a micro electromechanical system and is usable over the particle size range of approximately 50 nm–5 μm. It measures both particle size and number, but in addition it can be used to determine particle surface areas, density, and dry and buoyant mass among other parameters. There are additional methods such as cell-sorting procedures available in addition to the three described above, but in an ideal situation, a combination of the methods described above can be used to build an accurate picture of a protein, its oligomers and aggregates, and the distribution of the various components and their sizes.

An older approach which is both high resolution and information rich involves the use of the analytical ultracentrifuge [27]. Two different methods are available both with their advantages: sedimentation velocity and equilibrium analysis. The instrument employed is a typical ultracentrifuge, but it is equipped with an optical device that allows one to directly monitor the behavior of a protein or other macromolecule in the presence of a centrifugal force produced by the spinning of a centrifuge's rotor. Special cells are used that permit multiple solutions to be monitored as a function of centrifugation time.

In a sedimentation velocity experiment, the rate at which a macromolecule is sedimented down a sector-shape cell is measured. This rate in the form of a sedimentation coefficient (s) is measured. This velocity normalized to the centrifugal field strength is directly proportional to the molecular weight of the particle after correction for buoyancy and the friction coefficient of the protein, both measurable parameters. Such measurements can resolve individual particles to a high resolution (Fig. 7a) [21].

In a sedimentation equilibrium study, the sample is spun at a lower speed until an equilibrium concentration gradient is created within the cell. This gradient can be analyzed to yield a molecular weight for a homogenous sample. In the case where an equilibrium between species is present, the data can be fit to a variety of association models to yield a description of any association which is occurring. Both methods are extremely powerful, but sedimentation velocity is the more commonly used method in pharmaceutical analysis. Analytical ultracentrifugation is generally not amenable to high-throughput work, and the instrument is expensive, so it is primarily used as a research tool rather than directly in formulation development.

There are a number of other methods that are commonly used in the development of protein pharmaceuticals. Of particular importance are those involving calorimetry. Differential scanning calorimetry (DSC) is the most widely applied form and is of great importance in establishing the thermal stability of proteins. In DSC experiments, the difference in energy (heat capacity) required to maintain a sample and reference at the same temperature as the overall temperature is varied and measured (Fig. 11) [4]. When a molecule undergoes a structural transition, an

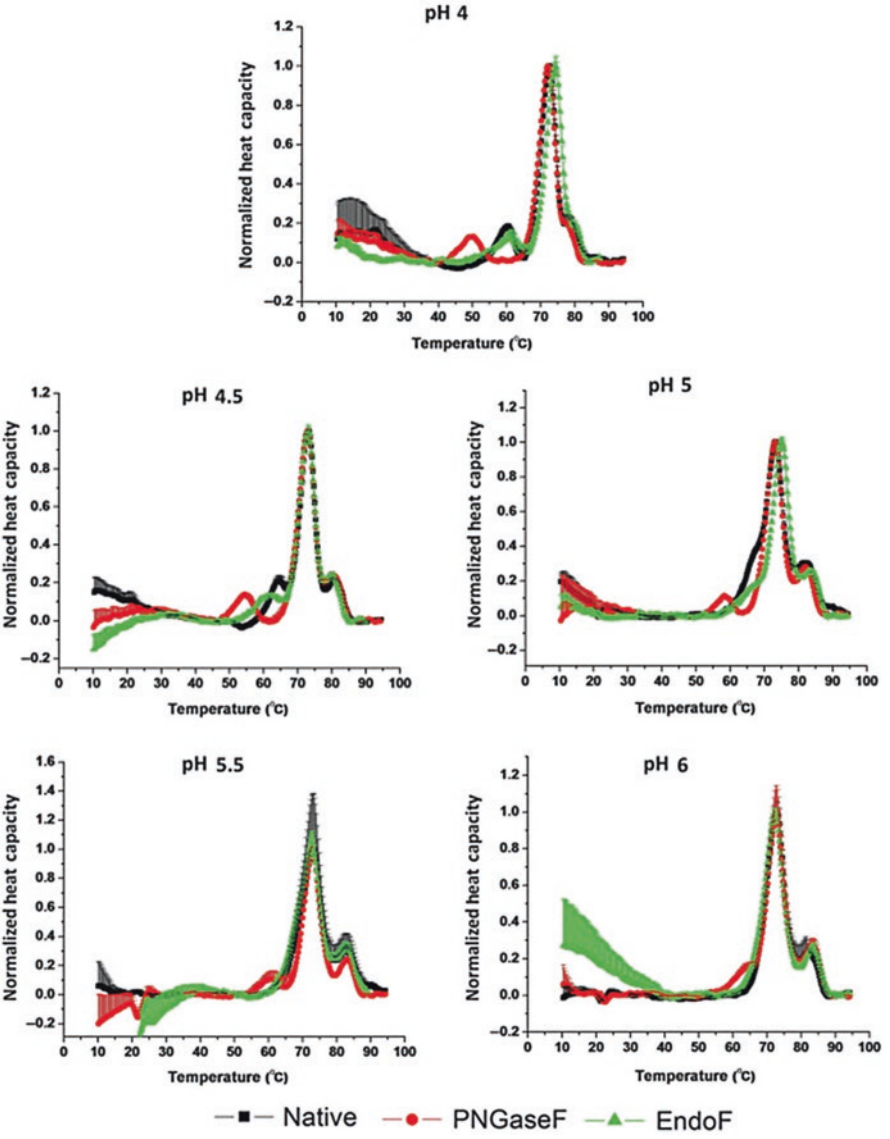

Fig. 11 The state of glycosylation of therapeutic proteins (in this case an IgG1) is known to have a significant effect on their structure, action, and pharmacokinetics. Shown here is a differential scanning calorimetry analysis of untreated (control) IgG1 (black line), partially deglycosylated IgG1 (green line), and fully deglycosylated IgG1 (red line) from pH 4 to 6. Normalized heat capacity changes were generated by fitting the data to be equal to one at the maxima and to zero at the minima for incorporation into the EPDs and radar charts. Curves shown here are averages of three runs. (See reference [4])

endo- or exothermic peak is seen. If the transition is reversible, the area under the peak corresponds to the enthalpy of the transition. In some cases, multiple peaks may be observed corresponding to individual subunits or structural domains within individual proteins. If aggregation is occurring, a peak in the opposition direction of a structural disrupting peak may be seen, but the design of modern calorimetry cells has reduced or eliminated their magnitude. Variation in protein concentration and scan rate can also be used to probe protein-protein interactions. High-sensitivity DSC instruments are relatively expensive but are available with autosamplers making them higher throughput than previous scanning calorimeters.

Another type of calorimeter is the isothermal titration calorimeter. In these instruments, one solution is titrated into another. The most common type of experiment involves the titration of a smaller molecule (a "ligand") into a protein (a "receptor") solution. The heat absorbed or released in the titration steps (via a syringe) can be used to obtain the enthalpy, entropy, and stoichiometry of binding based on fitting to various binding models. In a similar manner, the binding of one macromolecule to another can be characterized and both the thermodynamics and number of binding sites obtained. This is an especially nice method to examine excipient/protein interactions if they are reasonably strong. There are other calorimetric methods, but one that is occasionally useful to the pharmaceutical scientist is the dilution calorimeter in which the dissociation of oligomeric systems can be studied. It should be especially noted that lower sensitivity DSC and thermal gravimetric analysis are important to do in the study of protein solids.

A method that has just come into its own in the last few years is hydrogen/deuterium exchange (HDX). A protein is exposed to D_2O for various periods of time, and the partially exchanged forms are analyzed by enzymatic peptide mapping and mass spectrometry. The rate of appearance of labeled peptides serves as a measure of their exposure to solvent and provides a picture of the dynamics of a protein. Localized regions of the protein manifesting different exchange rates provide peptide level resolution which can be further enhanced to a residue level if additional information such as crystallographic temperature factors are available. Thus, the binding sites of excipients and "hot spots" of protein association can be localized by this method (Fig. 12) [28]. Although the instrumentation to perform such studies is expensive due to the presence of a mass spectrometer, measurements are so information rich that this is becoming an important method for the pharmaceutical scientist. For example, it can be used to localize excipient binding sites as well as those of protein/protein interactions [29].

We will conclude with comments concerning three additional methods that are perhaps less commonly used but can be important in a number of specific situations. With the advent of high-concentration protein formulations (especially those of monoclonal antibodies), the need for viscosity measurement has become increasingly important. There are a variety of instruments available to measure solution viscosity. A traditional method used in protein chemistry employs a U-shaped hollow tube. These are also known as Ostwald capillary viscometers. The temperature of the test solution is tightly controlled, and the time for the test liquid to pass through a fixed volume is determined. These instruments are not highly accurate

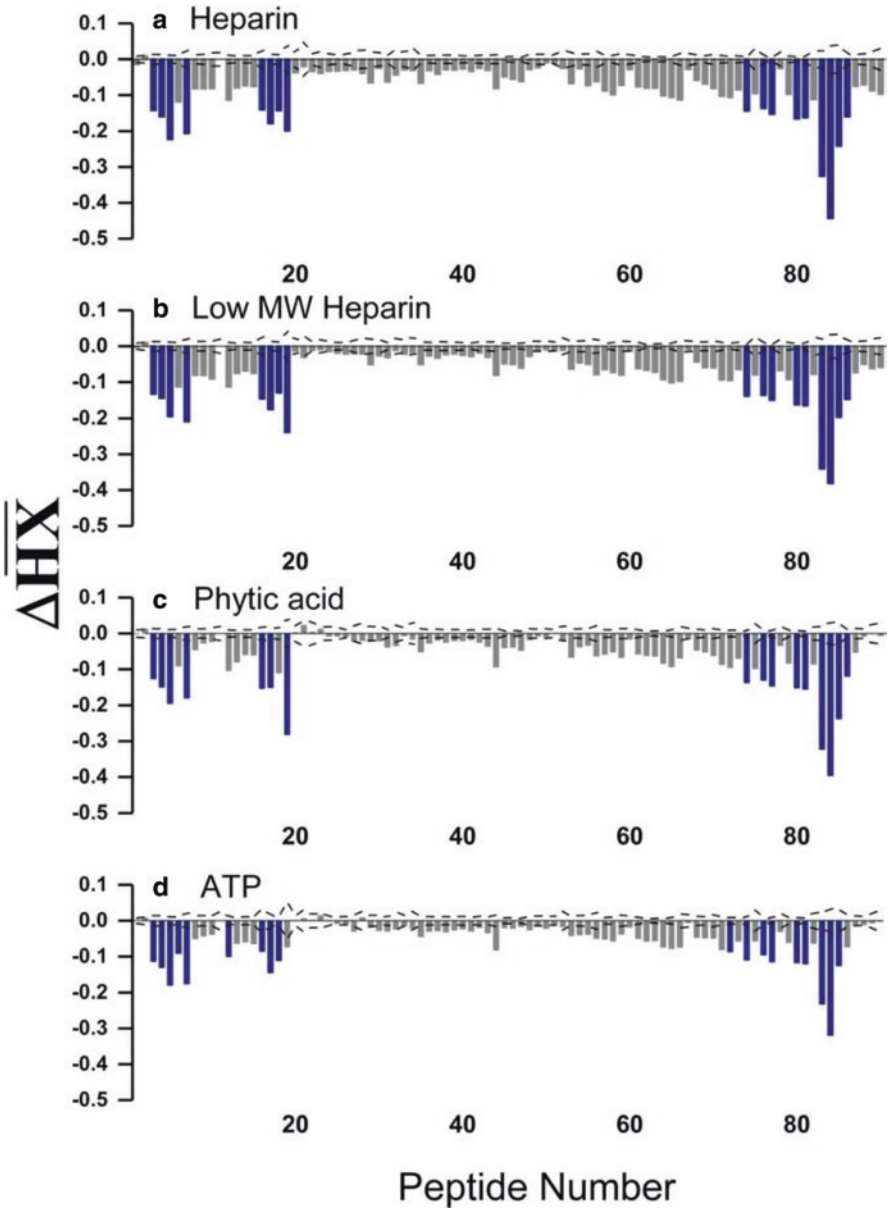

Fig. 12 Fibroblast growth factor-1 (FGF-1) is a protein of significant clinical utility. It is dramatically stabilized by polyanions. Here isotope exchange was used to establish their binding site, and it is clearly seen that it is the same for each polyanion and its location identified. Relative protection of FGF-1 peptides by heparin, low MW heparin, phytic acid, and ATP: HX-MS was performed with FGF-1 in the presence of (**a**) heparin, (**b**) low MW heparin, (**c**) phytic acid, and (**d**) ATP. The ΔHX values for each FGF-1 peptide are shown and colored according to their k-means categorization: strong protection, deep blue; intermediate protection/insignificant, gray. (See reference [28])

and are rarely used today. In a falling sphere viscometer, solid balls (usually steel) are allowed to fall through the medium of interest, and the time to reach terminal velocity is measured. Other types include vibrational, oscillating and falling piston, Stabinger, and rotational viscometers. Recently a number of new methods have come into increased use. The quartz viscometer uses an oscillating quartz crystal. The vibration of the sensor causes shearing of the fluid which is monitored by an electric signal. A recent favorite is the rectangular-slit viscometer usually employing microelectromechanical and microfluidic systems. In this method, the pressure drop in the test solution is monitored by an array of sensors. The instruments use very small volumes (microliters), can measure very high viscosities, and are relatively high throughput (Fig. 13) [30]. There are many other types of viscometers which may be appropriate for certain applications, but the slit types seem to be the current device of choice for high-concentration protein solutions.

As mentioned several times above, the measurement of the density of protein solutions is a critical parameter. It is possible to calculate approximate protein densities (and their reciprocal, the partial specific volume) from amino acid compositions, but actual measurements are to be preferred. Traditionally, hydrometers have been used which are based on the buoyancy of a floating glass body and the depth it sinks in a supporting liquid. Pycnometers simply measure the weight of the sample solution in a device of fixed volume. Both methods are simple and inexpensive but possess a number of undesirable properties. From the perspective of pharmaceutical protein therapeutics, hydrostatic balances are relatively accurate but involve an expensive apparatus and a large, complex air-conditioning system. In this method, a sinker is placed in the sample, and the apparent weight loss of the sinker is determined.

In pharmaceutical applications, none of these methods are currently used with any frequency. They have been replacing by digital density meters. Like several of the methods already described, a density meter employs the U-tube principle. A hollow tube is filled with the sample liquid. The U-tube is set in a counter mass block and can be set into oscillation. As the mass of the liquid increases, the frequency slows allowing the density of the particles in the liquid as well their partial specific volume to be determined. With care, this technique is extremely accurate and is now usually the preferred method.

Osmometry is also often used by the pharmaceutical chemist. Once upon a time, this method was used to determine molecular weight but has been superseded by many of the methods described above, including mass spectrometry. The osmotic pressure of a protein solution is, however, an extremely important property of a pharmaceutical formulation for obvious reasons. Thus measurement of this parameter is extremely important. There are three common techniques used to measure the osmotic strength of a solution. These are vapor pressure depression, freezing point depression, and membrane osmometers. All of these work well for determining the total concentration of dissolved salt and sugar among other compounds in pharmaceutical formulations, which are typically desired to be in the physiological range (300 mOsm).

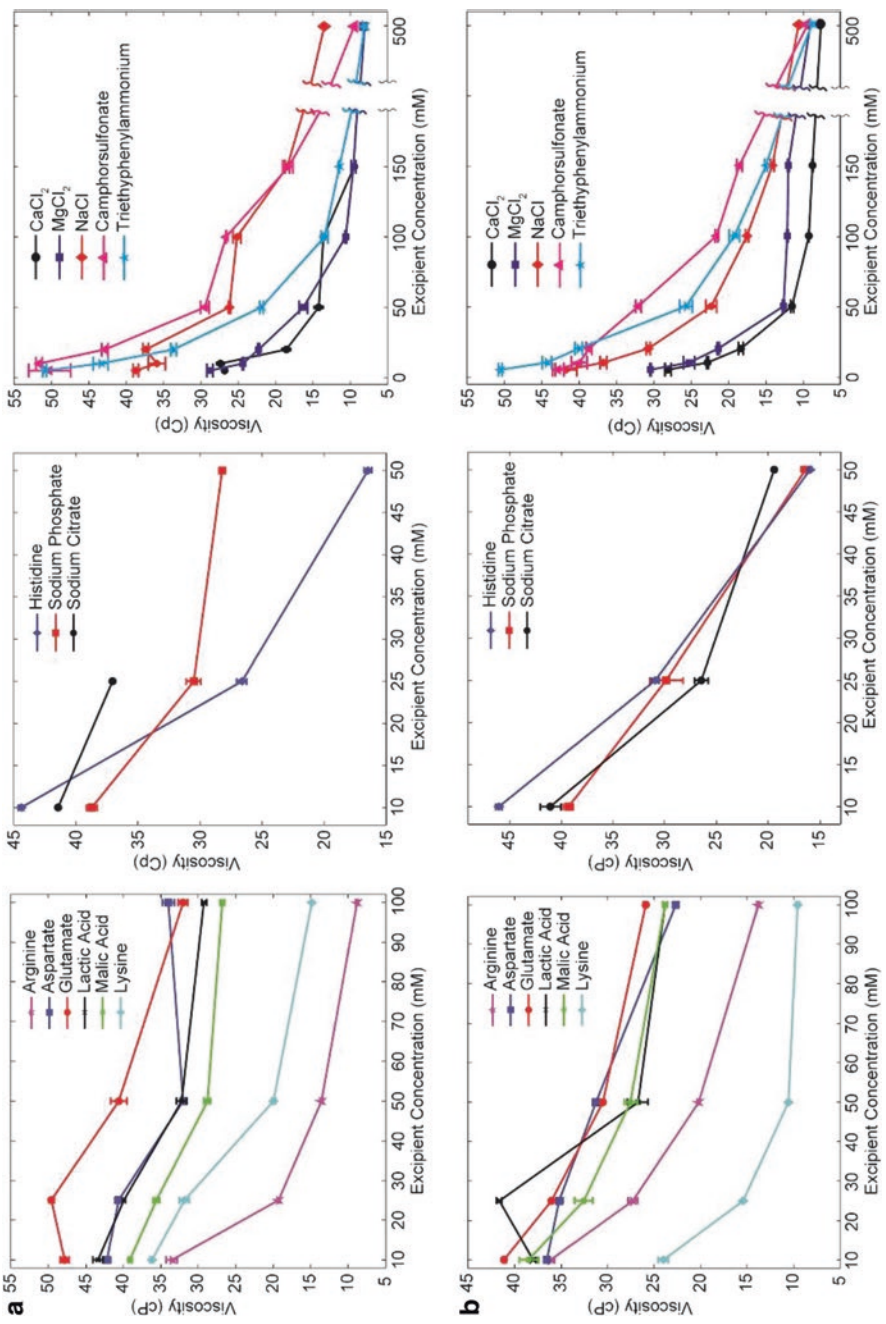

Fig. 13 With the advent of high-concentration mAb solution, the need to lower their viscosity has become apparent. The effect of the concentration of selected excipients on the solution viscosity of 175 mg/mL solutions of (**a**) mAb A and (**b**) mAb C containing 10 mM histidine at pH 5.75 is shown here. MAb A precipitated in formulations containing sodium citrate concentrations of about 25 mM and higher. Values are average and SD from triplicate measurements. (See reference [30])

In this brief discussion, we have described many of the major methods (with an emphasis on the biophysical) that are used by pharmaceutical scientists to characterize and formulate therapeutic proteins. Similar or identical techniques can be used for the development of nucleic acid-based pharmaceuticals and many vaccines. It is possible to combine the data obtained from such methods to paint a detailed picture of target proteins using methods such as empirical phase and radar diagrams. A variety of stresses can be used including temperature, pH, buffer identity, agitation, and freeze thaw. Such approaches are described in another chapter of this volume. Furthermore, as mentioned previously, multiple methods are often available in single instruments. For example, CD spectropolarimeters can combine near and far UV CD, fluorescence, absorbance, and light scattering. Certain fluorometer can monitor intrinsic and extrinsic fluorescence, lifetime, and scattering data. Thus secondary, tertiary, and quaternary (including aggregation) structure can be simultaneously characterized. This can be also done in a microtiter plate format allowing the high-throughput acquisition of data. All of this together suggests it has become possible to develop and formulate therapeutic proteins with increased speed and efficacy.

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Chapter 9
Leveraging the Multi-attribute Method (MAM) to Improve Biotherapeutic Development

Lisa Connell-Crowley, Bruce Kerwin, Randal Ketchem, Jeff McGrew, and Richard S. Rogers

1 Introduction

The biopharmaceutical industry is producing an increasing number of therapies for multiple indications. These new molecules are not only monoclonal antibodies but include gene therapies, siRNA, chimeric antigen T-cell receptors (CARTs), and bispecific T-cell engagers (BiTEs). New therapies can have added complexity with co-formulations of multiple molecules. The immense structural complexity of these therapies presents a characterization challenge. At many biopharmaceutical companies, analytical methods have been specifically developed for each individual molecule. The traditional assays would typically monitor attributes in an indirect manner (e.g., cation-exchange (CEX) monitoring charge heterogeneity). These assays would subsequently require fractionation and characterization by complementary assays, typically utilizing mass spectrometry. This stepwise process development characterization is not efficient and expensive.

Mass spectrometry, specifically high resolution/accurate mass (HRAM), has enabled the analytical labs in the biopharmaceutical industry to better characterize the molecules at both the intact and molecular level [1–7]. HRAM MS has been instrumental in the identification and monitoring of critical quality attributes (CQAs). Effective monitoring of CQAs is essential for ensuring the safety and efficacy of the complex molecules developed by the biopharmaceutical industry. Leveraging HRAM MS at every stage of process development aligns the biopharmaceutical industry with the quality by design concept. An attribute focused quan-

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titative analytical tool could be used from molecular optimization to commercial release of the molecule from the QC lab.

The current analytical characterization approach, for biotherapeutics, involves multiple assays [8]. These assays detect aggregation, degradation, charge variants, glycosylation, and other posttranslational modifications (PTMs) and impurities. The assays used for degradation, charge variants, and purity are typically reduced capillary electrophoresis (r-CE), cation-exchange chromatography (CEX), and/or capillary isoelectric focusing (cIEF). r-CE, CEX, and cIEF each require development to determine if the assay is stability indicating and fit for purpose for the biotherapeutic. Co-purifying host cell proteins (HCP) and leached protein A (pA) ELISAs are also used to assess the purity of the biotherapeutic. There are commercial kits available for the HCP and pA ELISAs, but ELISA results do not identify the HCP present in a sample. Many biotherapeutics are glycoproteins. Some biotherapeutics need specific glycan profiles to be present to be effective as well as similar in the case of biosimilars. If glycosylation needs to be monitored, a release glycan assay is typically used to assess the glycan profile. Lastly, peptide mapping with mass spectrometry is used to identify PTMs. Peptide mapping is the only method that provides the location of the attribute on the biotherapeutic. r-CE, CEX, cIEF, and HCP profiling require orthogonal attribute assays (OAA) like mass spectrometry to identify the constituents of a peak or the cause of a signal.

Multi-attribute method (MAM) is a mass spectrometry-based method that can be used to monitor known attributes and detect impurities [9, 10]. MAM can be used as an identity test and a purity test. The attribute analytics and purity components of MAM make it possible to potentially replace r-CE, CEX/cIEF, released glycan assays, ID ELISA, HCP ELISA, and pA ELISA from the QC labs. Throughout the process development, MAM is used to characterize the product quality attributes (PQAs) on a biotherapeutic. Specific HCP and leached pA levels can be monitored with MAM during development. MAM accomplishes this because the attribute analytics component of the MAM is peptide based. Product quality attributes and process impurities such as HCP and pA are all made up of peptides. These peptides are included in a processing method that can be used in an automated fashion to monitor PQAs in every cell culture production screen, bioreactor DOE, and engineering run during process development. MAM data is instrumental in designing the optimal amino acid sequence, media conditions, downstream unit operations, and formulation. The purity component of MAM is accomplished by comparing the biotherapeutic that was produced from an experiment to a reference standard that has been previously characterized. The workflow for the purity component of the MAM consists of alignment of the chromatograms from the test sample and the reference standard. Peak detection and differential analysis follow the alignment. Finally, the software identifies the presence or absence of new peaks or significant changes in existing peaks. Implementation of MAM in the process development lab significantly increases the understanding of a biotherapeutic because it provides a direct measure of CQAs at a molecular level.

This chapter will present an MS-based multi-attribute method for the characterization and monitoring of attributes and impurities of biotherapeutics during process development. MAM leverages HRAM mass spectrometry for automated data analysis for reporting molecular attributes and impurities. MAM is utilized at every stage of process development, from molecular optimization to formulation. Specific examples of how MAM was used for molecular optimization, clone screening, upstream process optimization, downstream optimization, and formulation will be described.

2 Biotherapeutic Design

Many of the challenges associated with the development of biotherapeutics stem from the fact that they are derived from an in vivo system, e.g., antibodies, derived through human B-cell isolation and sequencing techniques or other species. B-cells are designed to produce and deliver the antibodies at the target site in a specific environment, without the need for ex vivo, large-scale production, viral inactivation, purification, long-term storage, or development-induced PTMs. While antibody design and engineering for improved stability could provide a positive impact for yield, aggregation, and other biophysical properties manifested during production and processing, PTMs may be detected and targeted for engineering using methods such as the MAM. PTMs could consist of deamidation, isomerization, glycation, methionine oxidation, tryptophan oxidation, N-linked glycosylation, clipping, ragged signal peptide cleavage, cysteine conjugations, and others [11–13]. MAM is the perfect tool to aid the detection of PTMs that could occur under development stresses such as exposure to light or oxidizing agents, subjection to a range of pHs, interactions with purification resins, variations in temperature, freeze/thaw cycles, exposure to media, interaction with expression system cellular components and proteases, agitation, variations in buffer systems, and long-term storage in a formulation environment.

Biotherapeutic development is often done using initially discovered molecules without optimization, frequently resulting in issues during the therapeutic development processes. Early in silico biotherapeutic sequence and structure model evaluation to drive early optimization design are highly beneficial to the success of a biotherapeutic during early-stage development, enabling fast development through a manufacturing platform, and during late-stage development. Without early optimization, sequence issues could arise such as inadequate titer, low stability resulting in multiple pathways of aggregation, high viscosity, fast in vivo clearance, and, the focus here, sequence-based PTMs. Evaluation and engineering of the antibody sequence, in concert with structure, at the early stage of development enables faster development of biotherapeutics with improved developability. Purely in silico methods to predict and evaluate potential PTMs, however, lead to the detection of multiple potential sites, many of which will not actually be realized as a PTM. Attempting to

engineer a larger number of potential in silico predicted PTMs in a combinatorial fashion quickly leads to an untenable number of potential variants to produce and test. Alternatively, to reduce the number of combinatorial variants, multiple rounds of engineering and variants could be performed, but this could still waste valuable development time on PTMs that do not affect product quality. Further, actual PTMs could be missed via in silico screening, leading to sites missed during the engineering stages of development. If such sites prove detrimental to the development or function of the antibody, this could lead to a return to engineering or selection of a different lead, resulting in added time and expense to the development cycle, or to the unfortunate outcome of being forced to go forward with a lead biotherapeutic containing realized, detrimental PTMs. Therefore, along with in silico sequence- and structure-based screening methods to detect potential PTMs, the use of a fast, low consumptive method such as MAM to experimentally detect PTMs and impurities greatly benefits early lead selection and subsequent engineering efforts to enable the development of an optimized biotherapeutic.

A comparison of in silico PTM predictions to the MAM results illustrates the reduction of potential combinatorial variants required to explore the resulting optimization design space. As shown in Table 1 for a sample panel of three distinct antibodies, the number of variable domain hot spots differs substantially between in silico prediction and MAM detection, showing that MAM detection on actual samples leads to a reduction in the required number of designed variants for optimization designs of PTM developability. Variant design utilizing the in silico predictions in the worst case would require the production of either 2047 variants at large expense and complication or multiple rounds of engineering leading to a significant development time increase for multiple variant productions and evaluations. MAM analysis offers the ability to only target the PTMs that are real sites of modification rather than mutating many putative problematic sites.

On the other side, failure to repair a site, because it was either missed by the in silico analysis or deemed safe during the in silico analysis, which is later proven by MAM or downstream evaluation to be problematic, is potentially an even larger issue for biotherapeutic development since the molecule is less likely able to be repaired as it moves further along in development, thereby leaving in place PTM developability issues. This issue is illustrated in mAb2, highlighted in Fig. 1, in which one of the asparagine residues in the heavy chain is followed by a proline, would not have been flagged during the in silico evaluation as a potential deamidation site, but was found by MAM to deamidate at nearly 20%. While the presence of such a large amount of deamidation at this site does not impact the antibody's function, this risk could have been eliminated during the engineering phase with routine application of MAM.

Antibody design is the first step in developing a biotherapeutic. The use of MAM along with in silico modeling for molecular optimization can significantly speed up process development. MAM can dramatically reduce the number of variants that need to be screened and identify hot spots for PTMs that were not predicted with in silico modeling. The data shown in Table 1 serves as an example of the MAM data which could be utilized in predictive modeling. Coupling the positional-specific

Table 1 Illustration of in silico predicted versus MAM-determined PTMs for three different antibodies, showing the disparity between them and number of variant design differences

	mAb1			mAb2			mAb3		
	PTM (in silico)	PTM (MAM > = 1%)	MAM range %	PTM (in silico)	PTM (MAM > = 1%)	MAM range %	PTM (in silico)	PTM (MAM > = 1%)	MAM range %
Deamidation	1	0	0.85%	2	3	0–19.78%	0	0	0.00%
Glycation	0	0	0.05–0.33%	0	0	0.00%	0	0	0.00%
Isomerization	3	0	0–0.22%	2	0	0–0.39%	4	1	0–1.7%
Met oxidation	1	1	1.14%	0	1	0.58–1.7%	1	0	0.00%
N-Link Glycosylation	1	1	88.70%	0	0	0.00%	1	0	0.00%
Trp oxidation	5	0	0–0.61%	2	0	0.00%	4	0	0.00%
Total	11	2		6	4		10	1	
Combinatorial variants	2047	3		63	15		1023	1	
Combinatorial variants less oxidation	31	1		15	7		31	1	

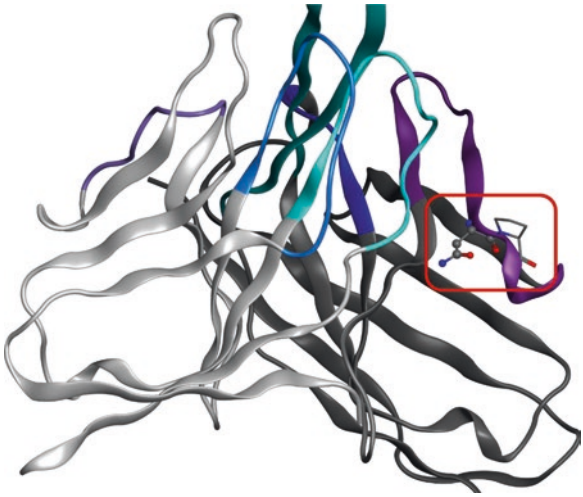

Fig. 1 mAb2 showing the NP deamidation site with the asparagine shown as ball-and-stick, with its C-terminal proline shown as stick

MAM data with antibody structure features would serve to focus the data sets, provide detailed PTM data, and would benefit the development of predictive models for PTM realization. Leveraging MAM during the antibody design stage shortens the overall development timeline and enables upstream cell line development to begin sooner than expected.

3 Upstream Development

Producing cell lines for the production of clinical and commercial biotherapeutics involves the selection and screening of clonal cell lines that have to express the recombinant biotherapeutic at high levels and have appropriate product quality attributes. MAM is a powerful tool to help identify those cell lines producing biotherapeutics with desired product quality. In addition to identifying cell lines with desired PTMs, MAM can identify cell lines bearing mutations in the recombinant protein and mis-incorporated amino acids due to depletion of amino acids in cell culture media (Rogers 2015).

One application where MAM is a particularly useful tool is selecting cell lines for biosimilar development. MAM was utilized during the isolation of cell lines for two Fc-fusion biosimilar molecules (Fc-A and Fc-B). Fc-A has four N-linked glycosylation sites, and the degree of sialyation was determined to be a critical quality attribute. The different Chinese hamster ovary (CHO) host cell lines were predicted to produce Fc-A with different product quality characteristics. The host cell lines were stably transfected with an expression vector encoding Fc-A, and the Fc-A protein was purified from fed-batch cultures of these transfected pools. As shown in Fig. 2, the three host cell lines yielded transfected cell pools with different glycan distributions. The % sialic acid (% SA), % afucosylation (% Afuco), % beta-galactosylation

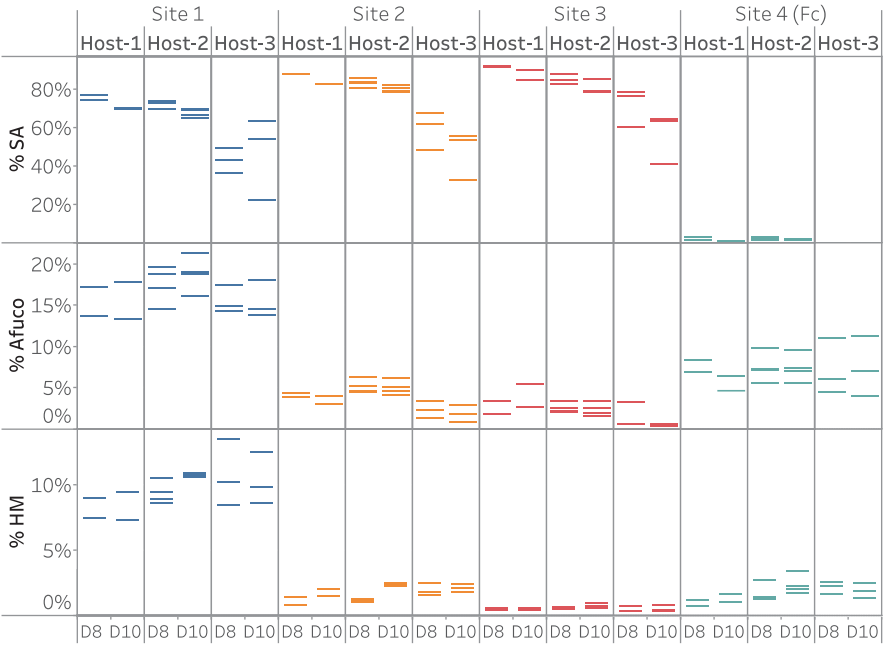

Fig. 2 Variation in glycan profile of Fc-A produced from different CHO host cell lines. Individual lines represent glycans from different pools. Afuco is % afucosylated glycan; % SA is percent sialic acid; and %HM is percent total high mannose. This Fc-fusion has four glycsosylation sites with site four present on the Fc portion of the molecule. Samples were taken on day 8 (D8) and day 10 (D10)

(%B-Gal), and the % high mannose (%HM) are shown in Fig. 2 and show considerable variation depending on the pool, host, and day of culture. Transfected pools derived from host-1 tend to have the highest levels of sialyation, whereas host-3 had distinctly lower levels of sialylation. The levels of sialic acid also tended to be lower on day 10 compared to day 8. These data identify harvest day as a potential process lever to help match innovator product quality. Site 4 on the Fc portion of the molecule had low levels of sialyation as is typically seen on molecules produced from CHO cells [14]. The % afucosylation of the glycan on the Fc portion of Fc-A was also considered a critical quality attribute. Figure 2 shows that the amount of % afucosylation showed more pool to pool variation than host to host variation. High mannose levels varied as well and there was a slight tendency for high mannose to increase from days 8 to 10 in culture. Clones derived from host-2 were explored in more detail. The glycan variation from clone to clone was less than that observed from pool to pool, but these variations allowed identification of clones with characteristics very close to the innovator (data not shown).

Once a clonal cell line is identified with characteristics similar to the innovator, MAM can then be utilized to monitor the impact of process changes on CQAs.

These data can be used to identify processes conditions that produce attributes that match the innovator. In the next example, MAM data were collected for an Fc-fusion molecule (Fc-B) that was produced in bioreactors where pH was varied. Fc-B has five N-linked glycosylation sites. It is essential for a biosimilar to match the glycosylation pattern of an innovator to ensure similar activity. The goal of this experiment was to identify operating conditions where consistent product quality would result even under potential fluctuations in process performance. TS6 was material produced using an initial version of the upstream cell culture process. The data shown in Fig. 3 demonstrates that the updated cell culture processes produce material similar to innovator that is relatively insensitive to pH when compared to TS6. In this example, MAM data showed process impacts on glycosylation. However, MAM has the capability to monitor other product quality characteristics that are sensitive to process conditions such as oxidation, glycation, and clips.

Cell line development is the second stage of process development that can utilize MAM. The experiments above demonstrate how MAM was able to provide timely data on CQAs. MAM data enabled the upstream scientists to choose clones that not only had excellent viability and titer but also had the desired glycan profile needed for proper activity. Culture conditions can also impact other product quality attributes. MAM data can identify cell culture levers needed to produce similar product quality for biosimilar programs. Leveraging MAM to optimize product attributes at the cell line development stage can make it easier to develop the downstream unit operations.

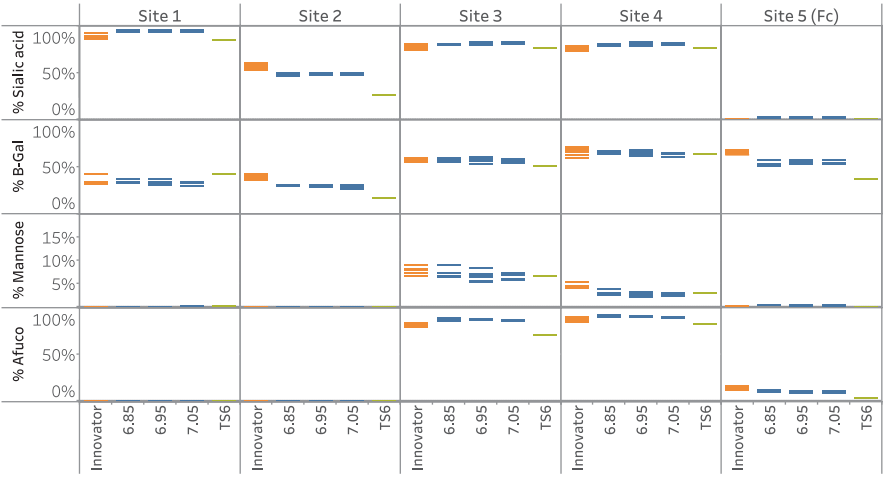

Fig. 3 Impact of pH on Fc-B glycans. The innovator molecule is compared to Fc-B biosimilar run in bioreactors run at different pH and a previous process (TS6). % sialic acid is NANA; %B-gal is % exposed beta-galactose; % Mannose is % of all high mannose species; and % Afuco is % afucosylated species

4 Downstream Process Development

MAM can be useful during downstream process development because it can provide specific information about the product and impurities associated with the product. Two examples are presented here: identification and monitoring of host cell protein contaminants during downstream development and evaluation of intermediate pool hold stability.

One application for MAM during downstream development is to identify specific host cell protein (HCP) contaminants that may be difficult to remove. Here we present an example of highly glycosylated recombinant Fc-fusion proteins that had high HCP levels, measured by ELISA, that were not cleared using typical downstream chromatography operations. MAM was used to identify and quantify specific host cell proteins that were associated with the product. Several proteins were identified, but the most abundant HCP was galectin-3, a 32-kD galactose-binding protein.

Additional downstream development was performed to identify chromatography conditions that could selectively remove galectin-3 from the product. One set of experiments involved screening various protein A chromatography wash conditions and using MAM analysis to track galectin-3 levels (Fig. 4a). The data show that wash A was able to remove galectin-3 to undetectable levels, resulting in more than two logs of clearance. Wash B also removed more than 2 logs of galectin-3 and is relatively unaffected by pH; however the product yields declined at pH 6.5 and 7.0. Wash C removed less galectin-3 and seemed to perform better at lower pH. These results were used to select a wash for the protein A step to remove galectin while retaining high yields.

A second set of experiments were performed to track galectin-3 levels during a CEX chromatography step operated in bind and elute mode. Figure 4b shows the levels of product, HCP by ELISA, and galectin-3 by MAM in elution fractions collected across the NaCl elution gradient. The data show that both the galectin and total HCPs were retained on the column during the bulk of product elution and then began to elute around 500 mM sodium. These results were used to determine peak-cutting criteria for the elution in order to remove significant levels of HCP and galectin-3 while retaining a high yield.

Another application of MAM for downstream development is the evaluation of pool hold stability. Once the downstream process has been developed, the stability of intermediate product pools is used to define acceptable hold conditions for manufacturing. A typical hold stability study examines the effects of parameters such as hold duration, temperature, and light conditions on product aggregation and degradation. Aggregation is relatively straightforward to quantitate by size-exclusion chromatography. In contrast, product degradation such as clipping and amino acid modifications, such as oxidation, deamidation, and isomerization, are typically evaluated indirectly, and thus the actual degradation site is not identified. For example, clipping is monitored using gel separation techniques, such as SDS-PAGE or capillary electrophoresis, which can provide the size of the clipped product but not

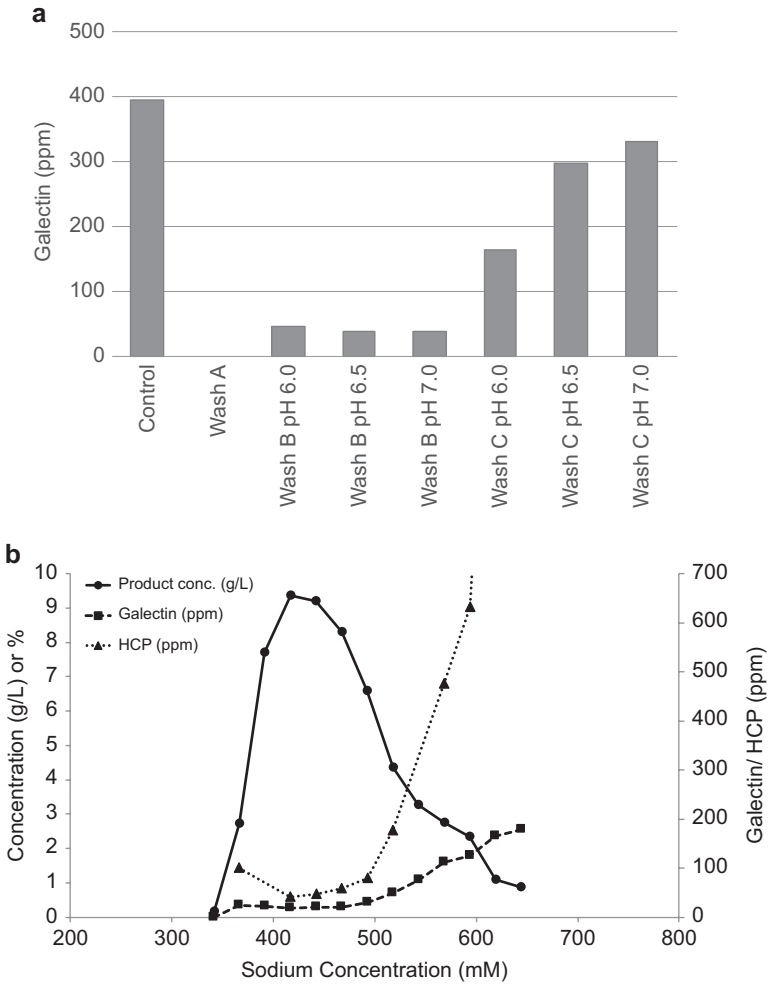

Fig. 4 Evaluating galectin-3 removal by different chromatography steps using MAM. (**a**) Levels of galectin-3 observed in a protein A chromatography wash screen. (**b**) Levels of galectin-3 and HCP observed in NaCl gradient elution fractions for a CEX chromatography step

the actual site of cleavage. Similarly, amino acid modifications can be monitored using charge-based assays such as cIEF, analytical CEX chromatography, or capillary zone electrophoresis (CZE), as peak profiles change to more acidic or basic species. However, the actual site and type of modification is not determined.

The use of MAM allows for the monitoring of clipping and amino acid modifications directly. Figure 5 shows an example of data from a hold stability study of two different chromatography pools held for up to 9 days at room temperature or 2–8 °C. For the room temperature samples, storage in room light or full darkness was also evaluated. The data show changes in the % oxidation of four methionine

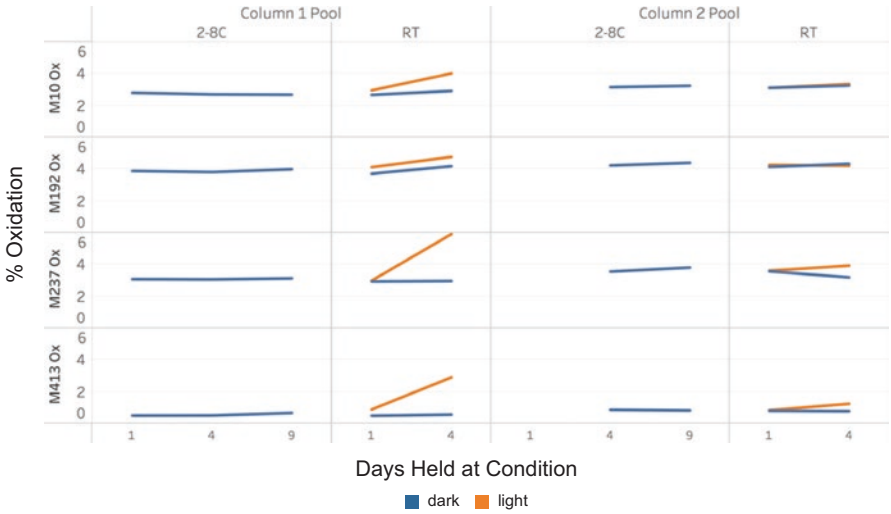

Fig. 5 Evaluation of oxidation percentages at four different methionine residues under different hold conditions. Ox: oxidation, RT: room temperature

residues over time. No changes are observed over 4 days at 2–8 °C for both column pools. Significant oxidation is observed at room temperature in the column 1 pool in the presence of light, but not the column 2 pool, with M237 and M413 showing more susceptibility to oxidation than the other two methionine residues. These data indicate that the column 2 pool is stable under all of the conditions tested, while the column 1 pool should be protected from light at room temperature.

Downstream process development is the third stage of process development that can utilize MAM. The experiments above demonstrate how MAM was able to provide data to guide the optimization of downstream unit operations to achieve the desired product profile. MAM data revealed optimal wash conditions for the protein A purification step and where to stop collecting fractions during CEX chromatography to effectively reduce impurities like HCP. MAM also provided important information on hold times and conditions for different chromatography pools. Understanding the optimal hold times and conditions for chromatography pools ensures that the biotherapeutic does not become modified unnecessarily before the biotherapeutic is formulated.

5 Formulation

Since the initiation of the biotechnology revolution in the 1980s, drug product developers have struggled to provide ever more precise definitions of the posttranslational modifications and degradation products encountered during long-term stability [15–20]. Biologics including monoclonal antibodies, cytokines, enzymes,

and many other protein-based drugs are inherently unstable and must be stored either frozen for drug substance or at either 2–8 °C or lyophilized for the final drug product. Each of these strategies is designed to reduce modifications due to inherent instability of the protein such as oxidation, deamidation, isomerization, backbone hydrolysis, and aggregation and those due to instability of the excipients including oxidation and glycation which can lead to aggregation, loss of potency [21], increased immunogenicity [22, 23], increased clearance [24], and particle formation [18, 25]. Traditionally, posttranslational modifications are detected and quantified using separation techniques such as high-pressure size exclusion chromatography, ion-exchange chromatography, reversed-phase chromatography, capillary zone electrophoresis, and SDS-PAGE, which is slowly being replaced by SDS-capillary electrophoresis. While these methods have been refined over time, they suffer from being blunt methods which provide information on the overall molecule without providing the precise location-specific information required for a well-characterized protein. For example, reduced SDS-PAGE provides quantitation of clips but does not provide information on the exact site(s) of backbone cleavage or the cleavage products. The same is true for ion-exchange chromatography and isoelectric focusing which can demonstrate the change in the overall charge of the protein, i.e., acidic vs basic species, but does not provide information on the species leading to the changes such as deamidation, isomerization, oxidation, N-terminal cyclization, or glycation. These changes can be obscured by other modifications such as aggregation or backbone cleavage. For highly glycosylated molecules such as erythropoietin, charge-based methods can report on the changes to the sialylation of the molecule, but are not precise enough to define when other charge-based modifications can occur. More precise methods defining what and where the modifications occur on a molecule are needed in order to understand potential stability issues and possible correlations to immunogenicity or off-target binding over time.

Elucidation of PTMs leading to changes observed by the more common analytical techniques has been accomplished using a combination of peptide mapping and mass spectrometry. Expanding the use of mass spectrometry into characterizing degradants formed during stability is important since the position of chromatographic peaks such as acidic and basic forms of antibodies by CEX may remain constant while the degraded forms of the proteins within the peak can change dramatically. Eng et al. (1997) [26] characterized multiple PTMs of recombinant human nerve growth factor that appear as a single peak by RP-HPLC following stress studies but contained mono-oxidized methionine, dioxidized methionine, deamidated asparagine, and isomerized aspartate. Gandhi et al. (2012) [27] showed the formation of an acidic pre-peak during storage of an IgG1 at elevated temperature during characterization by cation-exchange chromatography. While the pre-peak was stable at 2–8 °C, a variety of PTMs contributed to the CEX pre-peak including differences in sialic acid, N-terminal glutamine cyclization, and glycation. The same CEX pre-peak increased at 25 °C and higher and was caused by additive degradation pathways of deamidation, related isomerization, and clipping which were clearly different from the original PTM antibody forms within the peak.

Finally, Kim et al. (2010) [28] investigated the formation of two main peaks during CEX method development for a mAb following forced degradation of the protein. Results of the characterization studies showed the peaks were due to isoAsp and Asp formation from a single Asn deamidation demonstrating that quantification of any one peak would not account for the total amount of degraded mAb.

The prior examples, and many others from the literature, demonstrate the importance of fully characterizing degradants which form during storage and stress studies, an area that is lagging behind in formulation development. The need for this type of analysis is especially important as the analytical tools for defining the sites of degradation become ever more sophisticated. Recent reports have used this approach to define the criticality of deamidation and oxidation occurring in the mAbs during storage. Liu et al. (2009) [29] showed that deamidation of Asn-384 of a mAb occurs naturally in the serum at a rate such that exposure due to in vivo deamidation would far outweigh that due to storage induced degradation. Similar conclusions were reached for studies on Trastuzumab by Schmid et al. (2018) [30] in which degradation of solvent exposed residues of the CDR occurred faster in vivo (within days) compared to the levels observed for bio-process and real-time storage conditions.

In recent formulation studies, MAM analysis was used to delineate specific amino acid residues, their degradation products, and the rates of degradation occurring during formulation development studies providing a much more detailed view of the antibody degradation profile. This was an especially important technique during co-formulation studies of the anti-HIV antibodies 3BNC117 and PGT121 in which the two antibodies were mixed together to produce the final drug product [31]. Although both antibodies were IgG1s, the distinct sequences of the CDRs allowed us to identify and quantify residues for each antibody susceptible to degradation. While isomerization of the Asp-108 within the hinge region was common to both antibodies, sites specific to each antibody were identified showing that heavy chain Met-82 of 3BNC117 oxidized while heavy chain Asp-83 isomerized. Other residues specific to each antibody were also identified showing additional oxidation, isomerization, deamidation, and glycation during stress storage studies of the combined mixture. More recently we've used MAM analysis to identify PTMs of a formulated antibody stored at 40 °C in an acetate/sucrose/polysorbate buffer. MAM analysis of the samples during stability identified 40 distinct PTMs including Asp isomerization, Asn deamidation, Met oxidation, glycation, and peptide backbone hydrolysis. Samples showing a consistent increase in a particular PTM are shown in Table 2. As can be seen, the analysis was able to distinguish the changes occurring at 40 °C, while little to no change was observed at 4 °C. Additionally, we were able to detect differences in rates occurring in differing portions of the molecule for similar PTMs providing a much more detailed analysis of the degradation products than would not have been detected by techniques such as ion-exchange and capillary electrophoresis allowing the formulation scientist to make a much better informed decision concerning which PTMs are critical quality attributes.

Table 2 Selection of representative data collected by MAM for a mAb. Modifications include clipping, oxidation, isomerization, glycation, and deamidation.

PTM	$T = 0$	12 weeks, 4 °C	4 weeks, 40 °C	8 weeks, 40 °C	12 weeks, 40 °C
Fc-N:47 to Fc-N:48 clip	0.01	0.04	0.06	0.23	0.33
Fc-N:48 to Fc-N:49 clip	0.01	0.01	0.22	0.40	0.41
Fc-N:87 to Fc-N:88 clip	0.01	0.01	0.09	0.16	0.17
Fc-N:51 isomerization	0.11	0.11	1.24	2.17	3.08
Fc-N35 isomerization	0.01	0.01	0.25	0.71	1.19
HV:94 deamidation	0.31	0.10	0.36	0.25	0.31
HCnst-Ig:49 deamidation	0.29	0.17	0.36	0.26	0.30
Glycation Fc-N:95	0.02	0.03	0.05	0.11	0.22
Fc-N:93 NH3 loss	0.94	1.03	1.02	1.21	1.00
Fc-N:93 deamidation	0.51	0.27	0.65	0.60	0.71
Fc-C:25 deamidation	0.41	0.23	0.57	0.42	0.45
Fc-C:51 deamidation	0.94	0.93	1.01	1.39	1.55
Fc-C:58 deamidation	1.35	1.53	1.98	2.13	2.89
Fc-C:112 deamidation	0.38	0.20	0.46	0.41	0.57
Fc-C:95 deamidation	0.73	0.47	1.40	1.44	2.34
Fc-C:104 oxidation	3.36	2.98	4.20	4.11	4.49
KV:4 oxidation	0.37	0.21	0.41	0.20	0.31
KV:27 deamidation	0.38	0.33	0.38	0.38	0.57
KV:29 deamidation	0.48	0.23	0.71	0.38	0.54
KV:40 oxidation	0.22	0.21	0.24	0.20	0.18
HV:40 oxidation	0.50	0.52	0.67	0.73	0.91
HV:80 oxidation	0.51	0.37	0.55	0.42	0.55
HV:103 oxidation	0.79	0.77	1.24	1.09	1.40
Fc-N:22 oxidation	6.78	6.12	7.49	9.19	9.60
KCnst-Ig:58 deamidation	1.12	0.62	1.49	0.89	1.09
KCnst-Ig:67 isomerization	0.18	0.20	0.92	1.40	1.59
LmdV:109 Glycation	0.15	0.13	0.26	0.70	0.86
KV:109 to KV:110 clip,	0.27	0.84	0.45	0.52	1.05
KV:105 to KV:106 clip,	0.11	0.31	0.14	0.17	0.34
KV:94 to KV:95 clip,	0.31	0.61	0.40	0.37	0.55
KV:91 to KV:92 clip,	0.05	0.10	0.06	0.07	0.08
KV:137 to KV:138 clip,	5.00	8.71	6.35	0.77	8.80
KCnst-Ig:87 Glycation	0.22	0.23	0.38	0.96	1.72
KCnst-Ig:44 Glycation	0.22	0.21	0.30	0.60	0.90
Hinge:109 Glycation	0.06	0.06	0.08	0.19	0.32
Hinge:108 to Hinge:109 clip	0.03	0.03	0.29	0.52	0.70
Hinge:111 to Hinge:112 clip	0.00	0.01	0.02	0.04	0.07
Fc-N:106 Glycation	0.09	0.14	0.18	0.24	0.52
HV:53 isomerization	2.83	3.25	16.59	29.05	38.15
HV:58 oxidation	0.57	0.39	0.45	0.30	0.32

6 Conclusion

MAM is a mass spectrometry-based multi-attribute method. MAM can simultaneously monitor multiple product quality attributes and test the purity of a biotherapeutic. MAM outputs the specific site of modification, degradation, or identity of an impurity. MAM can accomplish these outputs because the assay is peptide based. The detected peptides (or peaks) are identified by searching the fragmented peptide data against amino acid sequences that may be present in a biotherapeutic. The purity component of MAM elevates it above conventional purity tests because of the ability to directly identify the impurity. MAM has been deployed throughout the biopharma industry at every stage of process development. During molecular design, MAM is streamlining the variant design process by highlighting only the truly modified amino acids on a biotherapeutic. Upstream development has been improved by MAM. MAM provides essential information for clone selection when PTMs are critical for activity or biosimilarity. MAM then guides the optimization of the cell culture conditions to ensure consistent product quality throughout biotherapeutic production. Downstream development benefits from MAM in multiple ways. HCP identification and pool hold stability were highlighted. HCP identification enabled the downstream scientists to optimize the wash conditions and peak cutting for two downstream unit operations. Chromatographic pool hold conditions were implemented as a result of MAM data. Lastly, MAM is an essential tool for formulation development. MAM enables formulation scientists to monitor CQAs in the variable regions of co-formulated molecules and understand the rate of modification or degradation of a molecule over time at different formulations. MAM is truly a universal analytical tool for process development. The biopharma industry has the opportunity to improve the product quality of a biotherapeutic and potentially develop it faster by leveraging MAM at every stage of process development.

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Chapter 10
Analytical Methods for Antibody Drug Conjugate Characterization

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1 Introduction

Monoclonal antibodies (mAbs) have been on the market for decades, evolving into very successful medications for a broad variety of different indications like oncology, infectious diseases, immunology, and neurology. Over time, more advanced constructs such as bi- or tri-specific antibodies, single-chain fragments, or antibody-drug conjugates (ADCs) have been developed to leverage combined biological functions. The mechanism of ADCs is to deliver a highly potent drug preferentially to disease-associated cells by a safe transportation system where the drug is chemically bound to an antibody. After the ADC is bound to a target cell expressing the antigen, the ADC will be internalized, and the highly potent small molecule drug will be released through one of a variety of mechanisms previously reported. This concept has been evaluated extensively in oncology but is also under investigation for immunological diseases where an ADC can enhance the therapeutic effect of a stand-alone monoclonal antibody therapy or minimize side effects of highly potent small molecule drugs. Hence, the development of ADCs is a very interesting and potentially groundbreaking product platform.

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ADCs may be classified by their conjugation chemistry, which impacts important physicochemical and pharmacological properties like drug-to-antibody ratio (DAR), hydrophobicity, and metabolism. In general, drug-linkers are conjugated to either lysine or cysteine residues on the antibody using succinimide ester or maleimide chemistries, respectively. Additionally, within the cysteine-conjugated class of ADCs, the use of mild reduction native cysteines before conjugation is now being supplanted by the use of engineered antibodies with unpaired thiol groups that add specificity to the conjugation process. The engineered amino acid variant has the advantage of ensuring dedicated chemical linkage of the drug-linker to defined positions in the antibody sequence. Despite the risk of introducing immunogenic sequence via engineered amino acid sites, these newer ADC constructs lower manufacturing costs and risks associated with the broad-spectrum of conjugation variants. The development of these next-generation biologics, including new formats, creates unique analytical challenges due to their increased complexity that needs to be properly characterized. This chapter will therefore discuss the methods most commonly used for quality control analysis during release and shelf life analysis and also those applied for extended characterization/elucidation of structure of these molecules.

2 Protein Content

Advances in instrumentation have introduced slope spectroscopy as an alternate approach to the traditional UV measurement. With the slope spectroscopy method, the protein sample is measured neat at various path lengths, generating a slope from an absorbance versus path length plot. Calculation of the protein concentration is then accomplished by dividing the slope by the extinction coefficient.

ADCs may present a specific challenge when measuring the protein content of a sample as the drug-linker may also absorb at 280 nm. A typical approach to overcome this challenge is to determine a correction factor for the drug-linker to accurately calculate the amount of protein present in the sample. There are two ways to determine the correction factor. The first approach is to calculate the ratio of the drug linker's absorbance to the mAb's absorbance at a second wavelength. This approach requires separate measurements of the drug-linker and mAb over a range of molar equivalent concentrations at the second wavelength. After a linear regression analysis of the resultant datasets, the correction factor is calculated as a ratio of the slopes of the drug-linker to the mAb. The ADC's absorbance at 280 nm is then corrected for absorbance contribution from the drug-linker at the second wavelength by using eq. 1. Finally, the protein concentration is calculated using the equation (2):

$$\text{Corrected A280} = \text{Abs280} - \left(\text{Correction factor} \times \text{Wavelength 2}\right) \quad (1)$$

$$\text{Protein Concentration}\left(\frac{\text{mg}}{\text{mL}}\right) = \frac{\text{Corrected A280} \times \text{Dilution Factor}}{\text{Extinction Coefficient}} \quad (2)$$

The second approach is to determine a DAR-specific value based on the drug-linker's absorbance at 280 nm. Again, separate measurements of the drug-linker and the mAb at 280 nm are made over a range of molar equivalent concentrations for a DAR ratio of 1. The resulting ratio of the drug-linker slope to the mAb slope is then adjusted based on the expected DAR ratio of the ADC. Then the protein concentration is calculated by equation (3):

$$\text{Protein Concentration}\left(\frac{\text{mg}}{\text{mL}}\right) = \frac{\text{A280} \times \text{Dilution Factor}}{\text{Ext.Coefficient} \times \text{Correction Factor}} \quad (3)$$

3 Biological Potency

Currently there is no specific regulatory guidance to industry on ADC development, but it is a general expectation that potency assays ideally reflect the biological mode of action (MOA) of the drug molecule. Antibody-drug conjugates contain in principle two functional attributes: an antibody directed against the target antigen and a linked drug like a cytotoxin, an inhibitor, or a steroid. Both attributes have to be covered and characterized by potency assays. Furthermore Fc effector functions (e.g., antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)) need to be addressed even when they are not part of the MOA.

In the preclinical phase of development where a suitable cell line may not be available, a quantitative ELISA will be used as an antigen-binding potency assay [1]. If the same binding assay can be used for the mAb intermediate (unconjugated mAb) as for the ADC drug substance (DS)/drug product (DP), it can be demonstrated that the conjugation chemistry does not impact target binding (adapted from Miller et al. [2]). In case of a drug-linked toxin, the biological activity of the ADC (DS/DP) in Phase 1 is mostly determined by a cell killing (cytotoxicity) assay as functional antigen binding and toxin activity are determined concurrently. It is also decent to implement this bioassay as the only potency method, i.e., no additional binding assay is introduced for the ADC. Beside cytotoxicity assays, reporter gene assays are of growing interest especially if the MOA of the linked drug is more complex than just cell killing (Fig. 1). After binding and internalization of the ADC, the released drug will bind and activate the response element promotor resulting in the synthesis of the reporter protein (e.g., luciferase). The conversion of an added substrate leads to the release of light energy (RLU, relative light units). The emitted light energy is proportional to the reporter protein expression.

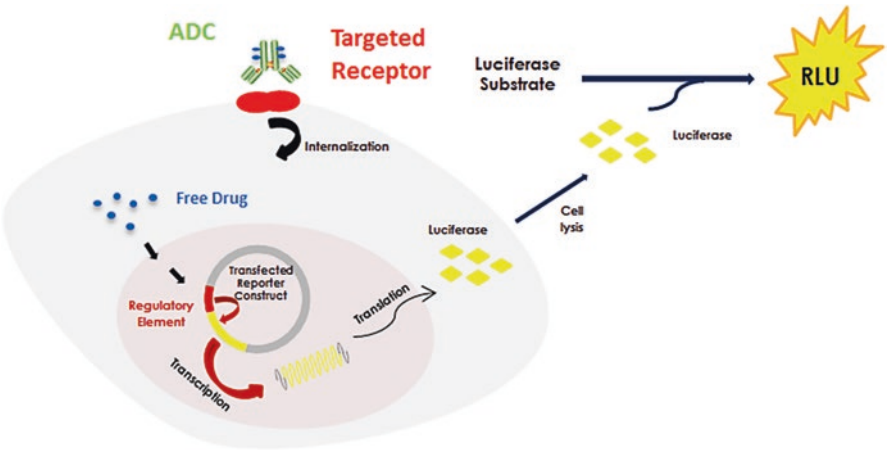

Fig. 1 Example design of a reporter gene assay. The assay principle comprises several steps starting with binding of the ADC to the target at the cell surface, internalization of the bound ADC, release of the drug, released drug-related secondary reaction (generation of luciferase), and finally conversion of added substrate and release of light energy

Table 1 Example of a potency assay control strategy

Assay	Naked mAb	Ref Std ADC	Phase 1 DS	Phase 1 DP	Phase 2 DS	Phase 2 DP	Phase 3 DS	Phase 3 DP
ELISA (mAb)[a]	XY							
ELISA (ADC)		X	XY	XY	XY	XY	XZ[b]	XZ[b]
Cytotoxicity assay		X	XY	XY	XY	XY	XY	XY
ADCC functional assay[c]				Z		Z		

X = performed as part of release testing; Y = performed as part of stability testing; Z = performed as part of characterization

[a]If possible, use same ELISA for mAb, ADC drug substance (DS), and ADC drug product (DP)

[b]Data supporting use of cytotoxicity assay (instead of ELISA) for release of DS/DP could be presented at a regulatory meeting (e.g., end of Phase 2 meeting) to readdress DS/DP control strategy

[c]If detectable, but not relevant for mode of action (MOA)

In progression toward Phase 3, the expectation is that the functional cell-based assay will be the primary method of potency determination supported by an ELISA, to demonstrate consistency of antigen binding and for toxin identity (Wilson et al.; see Table 1 for an example).

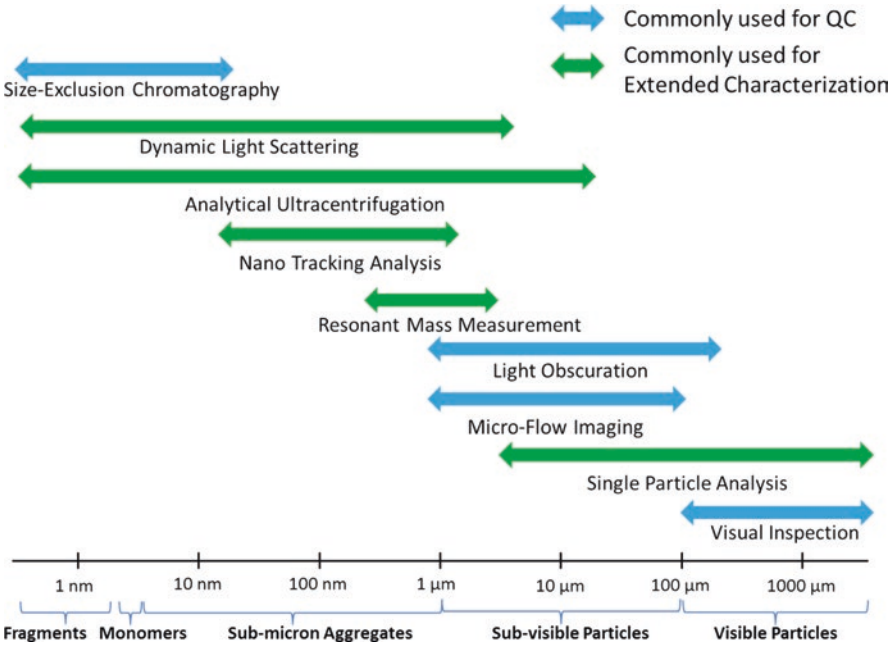

Fig. 2 Typical size ranges for techniques used in HMW variant and particle assessment. Techniques commonly employed for routine quality control testing are displayed in blue, whereas nonroutine characterization methods are displayed in green. Values on the X-axis are on a logarithmic scale, and the position of the arrows are meant to approximate the accessible size range for the associated technique

Appropriate acceptance criteria for the ADC potency assays have to be established. For the ELISA 50–150% is a common range for the early phase (Phase 1–2). For the functional cell-based assay, values of, for example, 60–140% or 70–143%, are accepted.

4 Purity and Impurities

4.1 Characterization and Control of Size Variants

Characterization and monitoring of size variants in ADC products is a priority for drug development groups due to the prevalence of size-related impurities and the potential effects of size variants on product quality. Size variants may include small fragments of the target molecule caused by chemical degradation or improper assembly (low molecular weight or LMW variants), submicron aggregates of the target molecule (high molecular weight or HMW variants), or larger particles in the subvisible (>1 µm) and visible (>100 µm) size ranges. Due to the range of molecular weights and physicochemical properties of potential size variants, multiple experi-

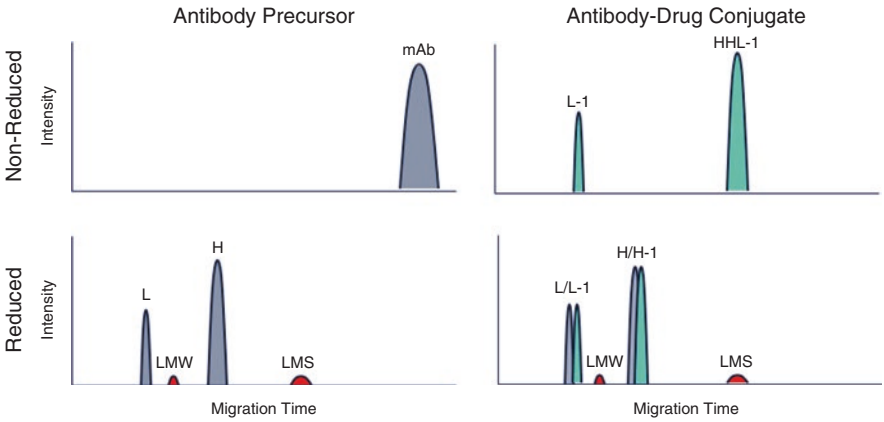

Fig. 3 Typical CE-SDS electropherograms for mAb precursors and thiol-conjugated ADCs. Peaks with gray coloring represent unconjugated antibody chains or disulfide-bonded components. Green peaks represent antibody chains or disulfide-bonded components with thiol-conjugated drug-linker. Red peaks represent impurities which may be associated with the mAb intermediate or the ADC

mental methods, as shown in Fig. 2, are necessary for a comprehensive analytical characterization and control strategy.

Control and Characterization of Low Molecular Weight Species (LMW Species)

LMW size variants are caused by degradation of the target molecule or by improper assembly, and as such there may be an ensemble of molecular species present. Although SEC methods can in some cases detect LMW species, it is difficult to ensure separation of all size variants from the monomeric base peak. Of typical concern with ADC products is the disulfide bonding pattern and DAR of the molecule, as many ADCs are produced by partial reduction of disulfide bonds followed by conjugation with thiol-reactive drug-linkers. Thus, capillary electrophoresis with sodium dodecyl sulfate (CE-SDS) is an attractive choice for control of LMW species, as operation in reduced and non-reduced mode allows for monitoring of chemical degradants and disulfide bonding patterns, respectively. Figure 3 shows example electropherograms for an antibody precursor and an antibody-drug conjugate with conjugation sites on heavy and light-chain cysteine residues. In this ideal case, the ADC achieves a DAR of 2 by conjugation of the residues which formed the disulfide bond between the light and heavy chain on one side of the antibody. Although the achieved resolution between conjugated and unconjugated chains varies widely depending on the drug-linker utilized and the DAR of the molecule, the configuration can be deduced by the presence of L/L-1 and HHL/HHL-1 as the prominent non-reduced species. Other non-reduced species such as HH-2, HL-2,

and H-3 can emerge in ADCs with a DAR >2 due to additional cysteine sites being occupied by drug-linker rather than in the disulfide form. If reduction/conjugation is not achieved for all antibody molecules, a non-fragmented IgG peak may also be present to a certain degree. In reduced mode, it is confirmed that approximately 50% of all light and heavy chains are conjugated; however, this information may not be accessible with poor resolution between L and L-1 or other conjugated species. The LMW species observed in the reduced electropherograms can be deduced to originate from fragmentation of the heavy chain, as the peak migrate between the light- and heavy-chain species and appear in both the mAb and ADC datasets. The late migrating species (LMS) in these datasets could result from covalent cross-linking in the mAb or ADC, e.g., by trisulfide bond formation. For characterization purposes, reverse-phase LC-MS in reduced and non-reduced modes provides an orthogonal separation method for the denatured species observed in CE-SDS, with mass spectrometric identification capabilities.

Characterization and Control of Submicron Aggregates (HMW Species)

Traditionally, ADC development programs have relied primarily on size exclusion chromatography (SEC) with UV detection for monitoring submicron variants in release and stability testing. Currently implemented SEC methods allow robust separation of monomeric and oligomeric variants of most IgG-based candidates, allowing for validation of methods reporting the relative abundance of HMW variants. Although the relative abundance of submicron aggregates may be readily controlled with SEC, larger aggregates at low relative abundance in the tetramer-pentamer range may be undetected in SEC workflows due the limited porosity of the column and/or frit. For this reason, it is usually necessary to employ an orthogonal, non-chromatographic method for submicron aggregates such as analytical ultracentrifugation (AUC), asymmetric flow field-flow fractionation-MALS, nano-tracking analysis, or resonant mass measurement. One or more of these methods should be employed for elucidation of structure/comparability studies; however, they are not generally necessary for ongoing drug performance monitoring. AUC, the most widely used of these methods, is capable of detecting aggregates across a broad size range; however, a method-inherent limitation of AUC is the need for dilution to protein concentrations of 1–2 mg/mL. Hence, dilution dependent, reversible aggregates may not be captured. A more detailed discussion of methods for submicron aggregate determination methods and their advantages and drawbacks can be found elsewhere [3, 4]. Additionally, ADC aggregates may differ in chemical composition and/or drug load compared to the monomeric fraction, and it may be necessary to employ chemical methods to determine the mechanism or biological impact of these species. Where possible, molecular weight determination of HMW species using native mass spectrometry is desired for characterization, as it can be coupled directly with SEC (usually with little or no changes to the method) and provides enough mass resolution to calculate both the oligomeric state and DAR distribution for small oligomers in the range accessible by SEC.

Characterization and Control of Particulates >1 μm

Characterization of particulate matter is also a necessary component of the ADC control strategy, although the technological considerations are highly similar to current mAb products which have been discussed previously. Traditionally, subvisible particle quantities have been monitored using light obscuration technology, which allows particles to be quantified with specific cutoffs as outlined in the USP. Although light obscuration remains the standard method for monitoring of subvisible particles, microflow imaging (MFI) is emerging as a quantitative technology that requires lower sample volumes and also provides qualitative information that may be used to classify particles into extrinsic, intrinsic, or inherent categories with proper method development. Moreover, MFI technology is better suited for detection of translucent particles and can differentiate silicone oil droplets from protein particle by application of commonly used S-factor calculations [5]. Many other methodologies are also available for characterization of particulate matter, referred to, and discussed elsewhere [6].

Visible particles, those above 100 μm, are in most cases still monitored by visual inspection. Multiple technologies are available for aiding in this visual inspection, including camera and automation systems. Whenever visible particles are present in a sample, it is usually necessary to determine the source of the particle, whether proteinaceous, product-related, or extrinsic. For this purpose, sample preparation coupling filtering or other isolation technology with spectroscopic or microscopic particle identification may be required.

4.2 Characterization and Control of Charge Variants

The charge profile of an ADC product is often very different from its parent mAb, as drug-linker conjugation chemistries often result in shifts to more acidic isoelectric point (pI) through loss of basic functional groups or creation of carboxylic acids. Additionally, degradation products of both the mAb and drug-linker may contribute to charge shifts which may impact clinical efficacy; thus charge variant control is imperative for analytical development programs.

Chromatographic methods such as cation exchange chromatography that have often been used for mAb analysis face challenges with ADCs due to preferential access of the hydrophobic drug-linker to the stationary phase, resulting in loss of information about protein-level modifications. Electrophoretic methods have proven more useful, as they can also directly measure the isoelectric point distribution of the sample. Chromatographic separation techniques are well established in the field for characterization studies, as they allow for fractionation of differentially charged species for subsequent identification by MS. However, online capillary electrophoresis-mass spectrometry methods are rapidly becoming commonplace due to new commercial instrumentation and open the door to identification of more charge variants in ADC samples. Among those hyphenated electrophoresis-mass

spectrometry combinations, free-flow electrophoresis (FFE) bears a couple of advantages as the separation can be done in buffer(water)-based systems omitting any stationary phase or the addition of a solid phase matrix. Consequently, recovery of all species to be separated is improved.

icIEF

Imaged capillary isoelectric focusing (icIEF) has become the industry standard for control of charge variants, as it offers high resolution and quantitative reproducibility. In icIEF, the ampholytes in the buffer electrolyte generate a pH gradient when an electric field is applied. Analyte molecules migrate in this field depending on their intrinsic charge. In reaching the isoelectric point, where the molecule net charge is zero, migration stops and distinct peaks are detectable. In imaged cIEF, pictures of the whole capillary are taken, and the emerging peak pattern is photographed repeatedly during the focusing process. For cIEF, the analyte needs to pass a UV detector for visualization. As migration stops once focusing is completed, a chemical mobilization step is applied afterward.

CZE

The emerging alternative to icIEF, capillary zone electrophoresis (CZE), offers more options in method optimization and often allows for faster assays but needs additional peak identification if used as charge heterogeneity assay, and it cannot be used for determination of the isoelectric point. In capillary zone electrophoresis, proteins close to their native state are separated according to their mobility in a continuous electrolyte. In addition to a system-specific intrinsic mobility toward one electrode ("electroosmotic flow", EOF), the mobility of analyte molecules in an electric field depends on their charge and size. Charged analytes intrinsically migrate toward the complementary electrodes, with small or highly charged molecules moving the fastest. Uncharged molecules are dragged along by the EOF. Control of the EOF (e.g., by pH of the electrolyte) allows for the migration of all analytes toward one electrode, thus enabling detection of all charge variants. The method can routinely be used for identity testing as the CZE peak pattern differentiates mAbs/ADCs with small changes in sequence or drug-linker structure. An ADC and its corresponding unconjugated mAb can however not necessarily be differentiated by their peak patterns, depending on the nature of the drug-linker. Peak identi-

Table 2 Major mechanistic differences between (i) cIEF and CZE

	icIEF	CZE
Buffer	Ampholytes forming a pH gradient	Continuous electrolyte
Analyte mobility	No more migration at isoelectric point	Consistent
Separation by	Charge	Charge and size

fication can be done by coupling with MS. The peak identification also allows a quantitative evaluation like purity, as the method separates main species from its acid (acidic region) and basic species or regions (basic region). The major differences between icIEF and CZE are summarized in Table 2 below.

Monitoring Posttranslational Modifications Using Charge Variant Analysis

The charge variant analyses such as icIEF and CZE capture the overall charge heterogeneity in the ADC molecule. For an ADC, the heterogeneity can be due to chemical changes on both the antibody and the drug-linker. Changes in the antibody structure like unprocessed lysine, deamidation, glycation, and sialyation can introduce additional positive or negative charges to the molecule. For ADCs there is the additional complexity of the drug-linker leading to more potential modifications. For example, maleimide-based chemistry is often used to attach the drug-linker to the antibody. However, depending on the subsequent linker chemistry, the maleimide ring can undergo hydrolysis, which creates a carboxyl group that increases acidic species distribution of the ADC.

4.3 Characterization and Control of Conjugation Variants

The conjugated small molecule is an integral part of the efficacy of ADCs and therefore must be carefully monitored during manufacturing and stability. Significant heterogeneity can be generated during the conjugation process, yielding product with a wide mixture of drug loads. The drug load is often expressed in terms of drug-to-antibody ratio, or DAR. Two metrics for the drug load are often quantified: the average DAR value of the product and the distribution pattern of the DAR. The two parameters give complementary information about the overall potency of the compound and whether the desired manufacturing profile was achieved.

Separation of DAR Heterogeneity

As the small molecule component is often the most hydrophobic part of an ADC, separation via hydrophobicity-based chromatography should yield distinct DAR species peaks as illustrated in Fig. 4. Depending on the manner of conjugation and whether further analysis of the peaks is needed, a salt-based hydrophobic interaction (HIC) column or a reverse-phase (RP) column can be used. HIC is a non-denaturing method that helps maintain the non-covalent interactions and the biological activities of the molecule. In cases where conjugation occurs at native cysteine locations, thereby breaking the covalent disulfide bond interaction, HIC can keep the non-covalently associated components of the ADC together. In addition, if purification and further studies of each DAR peak (i.e., assessing the

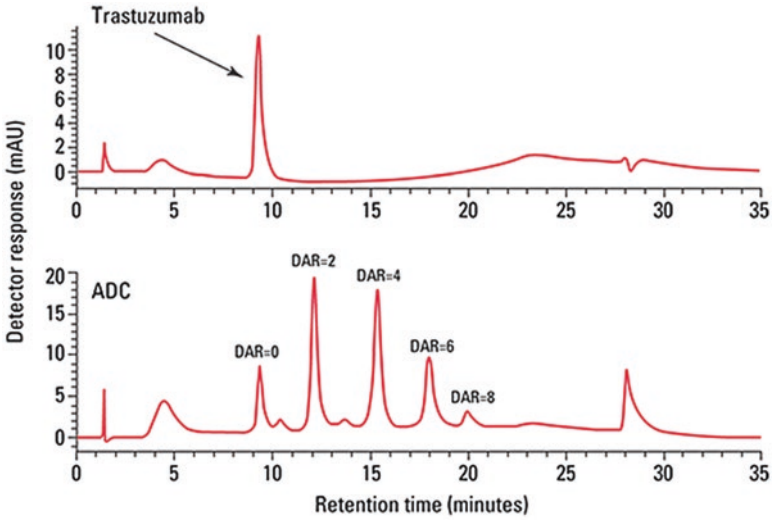

Fig. 4 Separation of unconjugated vs. conjugated mAb by HIC chromatography. Comparison of unconjugated vs. conjugated Trastuzumab HIC chromatogram shows that the heterogeneity in drug-to-antibody ratio results in well-resolved peaks with different retention times than the unconjugated antibody. The higher the drug load, the more hydrophobic is the overall compound and therefore elutes at later retention times. (*Reprint with permission from Tosoh Bioscience (as appeared in LCGC, Volume 30, Issue 1, pg 170))*

potency) is needed, separation by HIC is necessary to preserve the structure and function of the molecule. Reverse-phase columns can be used in cases where the conjugation does not disrupt the native interactions of the antibody, i.e., engineered cysteine residues. An advantage of reverse phase is the mobile phase compatibility with mass spectrometry, which means the separation can be connected directly to the MS detector for mass confirmation of the DAR.

Structural Elucidation of DAR Peaks by Mass Spectrometry (MS)

In order to calculate the average DAR or DAR distribution for a particular chromatogram, the identity of each peak must be known. If the conjugation occurs at native cysteine locations, the identity of the DAR needs to be confirmed using native MS. Native MS desalts the samples with a size exclusion column using volatile salts such as ammonium acetate before injection onto the MS, preserving the structural integrity of the non-covalently associated molecule. The detected mass is then matched to theoretical masses of antibody with various level of conjugation. If the conjugation occurs at engineered locations that do not disrupt native covalent bonds in the molecule, a direct injection of the sample onto a reverse-phase column connected to the MS is sufficient.

As a project progresses through the pipeline, further structural elucidation to assess the conjugation configuration and the exact conjugation location can be per-

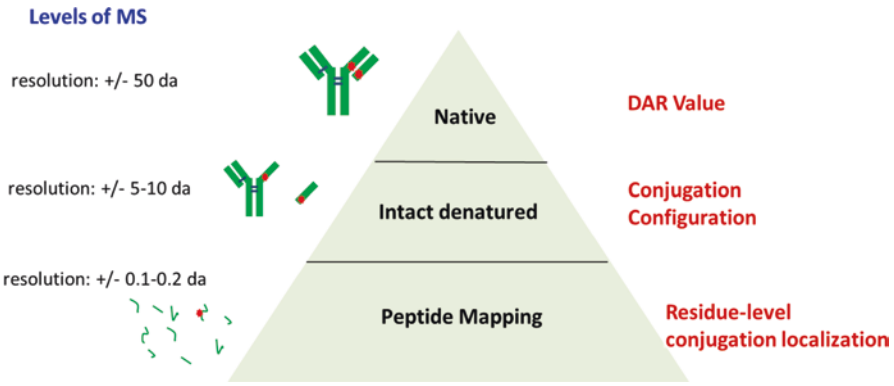

Fig. 5 Different mass spectrometry techniques for analysis of conjugation heterogeneity. Native, intact denatured, and peptide mapping mass spectrometry techniques need to be utilized together to gain a holistic picture of the structure heterogeneity of the ADC. Native MS is performed to assess the drug loads that exist in the sample; it is especially utilized in cases where the conjugation disrupts covalent interactions holding the molecule together. The intact denatured MS helps determine the covalently linked subunit components of the ADC, elucidating the disulfide status of the molecule which is important for understanding the conjugation configuration. Peptide mapping gives residue-level localization for where the conjugations are actually occurring

formed using other MS techniques. These studies will require the fractionation of individual peaks using HIC-based chromatography in order to facilitate further sample processing and analytical interrogation. If the conjugation is at native cysteine locations, it will be informative to determine which disulfides are preferentially broken to give the conjugation configuration, and a neat injection of the HIC fraction into a RP-MS system will elucidate all the non-reduced, covalently bonded components in the mixture. Furthermore, residue-level conjugation localization can be obtained by digesting and running peptide mapping on the fractions. In addition to structural elucidation, other characterization information (i.e., potency) about individual DAR peaks is also collected in order to determine their impact to other critical quality attributes. These additional characterizations are important for setting the correct specifications for individual peaks. Summary of the MS techniques and how they are used to characterize the conjugation heterogeneity is depicted in Fig. 5.

5 Process-Related Impurities

5.1 Small Molecule Impurities

Process-related small molecule impurities are often controlled by reverse-phase chromatography (RP-HPLC) combined with UV detection. The conjugation of drug-linker to the antibody can comprise several chemical reaction steps. After successful

conjugation the resulting reaction mixture needs to be processed through several steps for separation of a dedicated DAR species (desired API) and for depletion of remainder educts (sometimes added to the mixture in excess) and other process-related impurities. One of the most prominent process-related small molecule impurities are residual concentrations of the potent drug-linker, quenched drug-linker, and degradation products of the same. The most challenging step during method development is the precipitation of protein during sample preparation ensuring proper recovery of all relevant chemical structures. Here, proper control by mass spectrometry needs to be on-boarded until adequate recovery of all species is ensured.

5.2 Residual Solvents

Residual solvents in pharmaceuticals are defined as organic volatile chemicals used in the manufacture of ADC drug substances or as excipients or in the preparation of drug products. As analytical methods to control residual solvents, oftentimes gas chromatography is employed due to the volatile nature of the analytes. For detection FID (flame ionization detection) is a very common technique enabling adequate sensitivity to control maximum concentration allowed by guidelines. Besides, reversed phase or HILIC-based HPLC combined with different detection techniques can be used.

5.3 Elemental Impurities

Sometimes catalysts are used for certain chemical reactions especially when generating potent small molecule drugs. Hence, if such small molecule drugs are linked afterwards to an antibody, residual elemental impurities need to be controlled in the final drug product. However, in general, due to the ubiquitous nature of arsenic, cadmium, lead, and mercury, they (at the minimum) can occur in any excipient used for drug product compounding and manufacturing and therefore must be considered and need to be controlled by suitable technologies. Inductively coupled plasma (ICP) mass spectrometry (MS) is an analytical tool to quantify metals and metalloids. The sample is desolvated, atomized, and ionized in the plasma. Then, the ions are separated in a quadrupole mass filter and subsequently detected. Qualitative and quantitative investigations are possible. Microwave-assisted digestion is a more sophisticated sample preparation applied for more complex pharmaceutical materials used in tablets and capsules. ADCs, however, are commonly formulated as lyophilizates or liquids, and therefore simple dilution of protein solutions to 1 mg/mL in 0.5% HCl solution is sufficient.

6 Physicochemical Characterization

6.1 Sequence, Chemical Modification, and Disulfide Bonding Characterization by LC-MS

In addition to understanding the intact and subunit primary structures of an ADC using MS, the amino acid sequence of an ADC is critical for insuring proper structure and function. The preferred method for confirmation of the sequence is peptide mapping LC-MS, where the product is digested using a specific protease of choice and reduced before the resulting peptides are chromatographically separated and identified by tandem mass spectrometry. Typically, 90–100% sequence coverage can be obtained using this method, and sequence variants and amino acid modifications can be identified. In some cases, posttranslational modifications such as oxidation, deamidation, and drug-linker conjugation are quantified using peptide mapping, enabling detailed monitoring of multiple attributes in a single experiment. Similarly, the disulfide bonding pattern of an ADC can also be characterized by peptide mapping in the non-reduced mode or with differential labeling of free thiol groups before and after reduction. One important chemical modification not discussed here is the glycosylation state of the antibody. Typically, the glycans present in the antibody are characterized at release of the antibody intermediate and are expected not to change during the conjugation and drug product manufacture of the ADC. For this reason, we direct the reader to more focused reviews of glycan characterization in antibody therapeutics for more information.

6.2 Characterization of Higher-Order Structure

Circular Dichroism (CD)

Proper folding is critical for effective function and serum half-life of antibodies and subsequent constructs like ADCs. Ultraviolet (UV) CD spectroscopy is used for elucidation of secondary and tertiary structure of proteins. Proteins are comprised of optically active chiral units in their backbone that will adsorb at corresponding individual bands of circular polarized light, and different secondary structures have distinctive CD spectra in the far ultraviolet region. Alpha helix structures show strong positive bands at approximately 190 nm and negative bands at approximately 184 and 260 nm. Characteristic CD spectra of beta-sheet structures show a positive band at approximately 200 nm and a negative signal at 216 nm. Random coils have a negative band at approximately 195 nm and a positive band at about 220 nm. The overlay of far-UV CD spectra for a monoclonal Ab and the corresponding ADC are shown in Fig. 6 [8], indicating that secondary structure remains unchanged through the conjugation process. Near-UV spectra is depicted as expanded view in the insert of Fig. 6 indicating minor differences in tertiary structure between mAb and ADC. The ADC compared to mAb in Fig. 6 reveals a different pattern for near UV

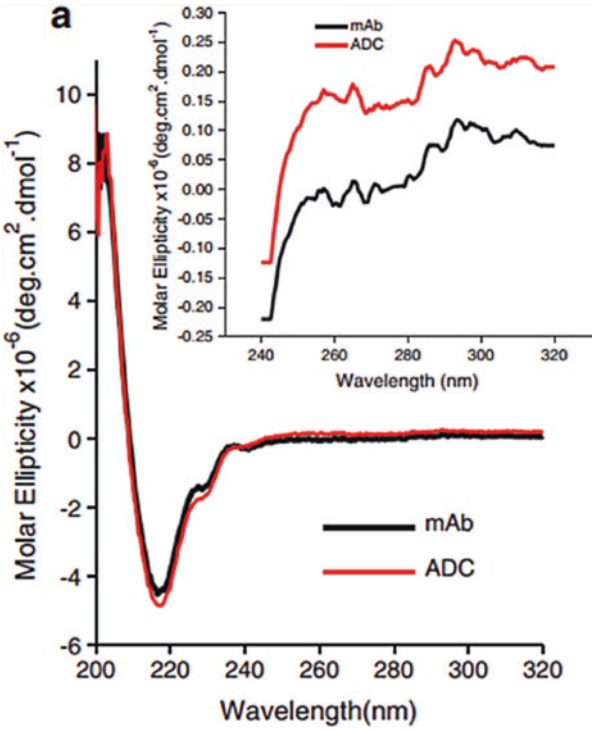

Fig. 6 Example CD spectra comparison between a mAb and an ADC. Scanning the mAb and ADC reveal comparable but not identical pattern, i.e., for far UV spectrum, the negative absorption band at 220 nm is a bit more pronounced than for mAb, however, still indicating comparable secondary structure. Near-UV spectrum (expanded view insert) shows several differences of distinct absorption bands at 250–310 nm and different absorption intensities for mAb and ADC indicating differences in tertiary structure

with less expressed absorption bands, i.e., at 250 and 270 nm a couple of distinct bands are missing for the ADC construct.

Useful information on the tertiary structure of proteins can be obtained by scanning other spectral regions. The near-UV region (240–320 nm) is widely used in comparability studies, for example, where the side chains of amino acids, especially cysteine and aromatic residues such as tryptophan, provide a characteristic structural "fingerprint" that is sensitive to changes in the integrity of proteins.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) characterizes the stability of a biomolecule by measuring the energy (expressed as heat capacity Cp) required to provoke a state transition such as protein unfolding as the sample is heated at a constant rate. The midpoints of these thermal transitions are expressed as melting temperatures (Tm). A stable protein requires more energy intake corresponding to a higher temperature

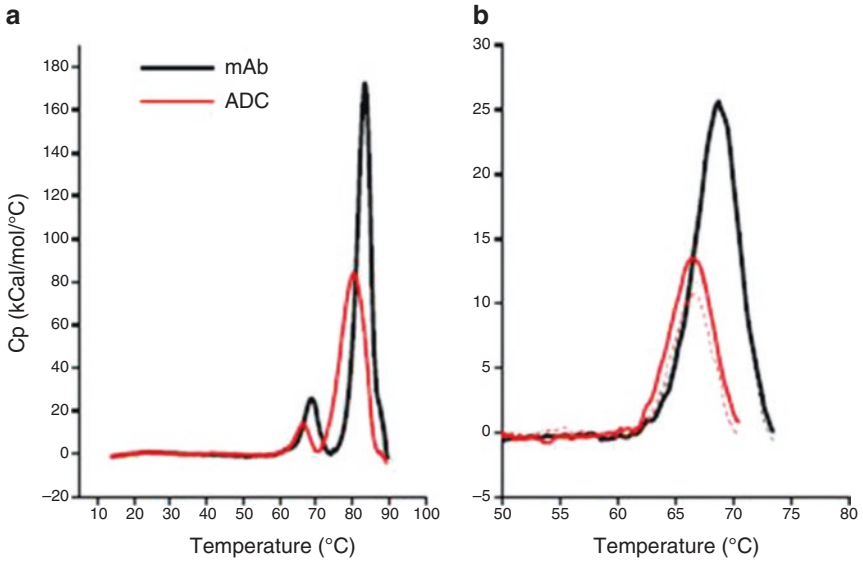

Fig. 7 DSC thermograms of mAb and ADC. Temperature scanning of defolding states for mAb and ADC show that ADC consumes less heat capacity (Cp) for defolding (red line) compared to mAb (black line) (**a**). Calorimetric reversibility for unfolded to native state is only given for mAb (dotted and solid black line are superimposed) versus ADC shows less heat capacity consumption for second temperature ramping (dotted red line) than first temperature scanning (solid red line) (**b**) indicating that unfolding of ADC is partly irreversible

to change from a folded, native state to an unfolded, denatured state. In addition to the Tm, the calorimetric reversibility of transitions can also be assessed. The calorimetric reversibility evaluates whether the transition from the native to the unfolded state is reversible and that the unfolded state will return to the native state upon cooling. This can be determined by rescanning the cooled sample again applying the same temperature ramping rate and checking for superimposability of the resulting scan (Fig. 7). Oftentimes, an ADC is compromised in overall Cp (Fig. 7a) and superimposability of rescans when compared to mAb (first scan depicted as solid line and rescan shown as dotted line in Fig. 7b).

Hydrogen/Deuterium Exchange-MS

Hydrogen/deuterium exchange-MS (HDX-MS) is a specialized tool for interrogating the solvent accessibility and dynamics of proteins, including antibodies and ADCs. In a typical experiment, the analyte is diluted into D20 for a specified amount of time, or several timepoints, before being quenched and analyzed by peptide mapping-MS to measure the uptake of deuterium at specific sites in the protein. The experiment is complicated by the fact that during sample clean up, digestion, and chromatographic separation, deuterons may back exchange with protons which ultimately decreases the sensitivity of the measurement. Nevertheless, automated HDX-MS systems are commercially available and in regular use in many pharmaceutical set-

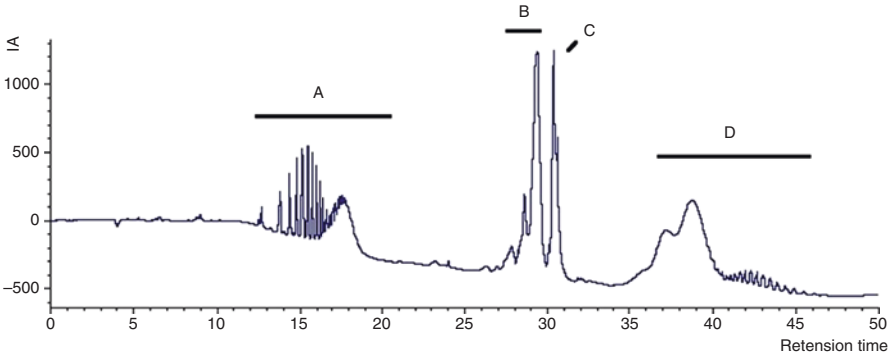

Fig. 8 Representative liquid chromatographic profile for polysorbate 80 using a USP L1 column. The profile illustrates the typical isomers found in polysorbates: (a) nonesterified, (b) sorbitan monoesters, (c) isosorbide monoester, and (d) polyesters of sorbitan and isosorbide

tings. The experiment is useful for comparing the higher-order structure of antibodies/ADCs across batches or to establish that the conjugation process did not significantly impact the higher-order structure of an ADC compared to its antibody precursor. However, the method is only sensitive to changes in deuterium uptake greater than about 15%.

7 Surfactant Characterization

Polysorbate 20 (Tween® 20) and polysorbate 80 (Tween® 80) are nonionic surfactants commonly used in many biologics formulations to shield the therapeutic protein from adsorption to surfaces. Most biopharmaceutical products containing peptides, proteins, antibodies, and vaccines are formulated with polysorbates (PS) – about 80% of the commercial mAbs contain PS20 or PS80. Polysorbates enhance protein stability either by (1) competitive adsorption to the hydrophobic interfaces or (2) direct binding to the protein. The use of polysorbates for protein stabilization in antibody formulations is well accepted and approved by regulatory agencies for parental administration. However, alternative surfactants, such as poloxamers, sodium dodecyl sulfate, Solutol HS 15, Cremophor, lecithin, and /alkylsaccharides can be used in parental applications as well.

Polysorbate concentrations in protein formulations tend to range from 0.001% (ReoPro®) to 0.1% (HUMIRA®). A typical profile for Tween 80 is illustrated in Fig. 8. The profile illustrates the typical isomers found in polysorbates (a) nonesterified, (b) sorbitan monoesters, (c) isosorbide monoester, and (d) polyesters of sorbitan and isosorbide.

In addition to general structural heterogeneity, polysorbates themselves can degrade in pharmaceutical formulations through chemically or enzymatically induced oxidation and hydrolysis. Chemical hydrolysis of PS catalyzed by basic or acidic conditions is almost negligible under pharmaceutically relevant conditions and was found mainly during forced degradation studies. In protein formulations derived from cell cultures,

hydrolysis initiated by residual host cell enzymes tends to be more significant in polysorbate degradation and tends to occur at the fatty ester bond.

Oxidation can occur in the polyoxyethylene (POE) region, at the ester bond, and/or the unsaturated regions of the alkyl chains. PS80 preferentially oxidizes at the double bond of the fatty acid chain, while PS20 preferentially oxidizes at the α-carbons of the POE. Tween 80 typically has more unsaturated alkyl side chains than Tween 20, making it more prone to oxidation. Oxidation mechanism in polysorbates can lead to peroxide formation which not only affects the surfactant content, but potentially can degrade the protein as well. In addition, polysorbates in general can bear significant levels of impurities themselves. For this reason, low peroxide grade materials are highly recommended. Handling and storage conditions are also typically controlled to minimize the risks of degradation.

Besides chemical degradation in the matrix, polysorbate degradation can also yield particles in the formulation which are characterized as "inherent particles." These particle impurities, typically from free fatty acids and fatty esters, have low aqueous solubility and can be detected in protein formulations. In case of high abundancies, this can lead to elevated levels of subvisible particle counts or even visible particle occurrences (Sect. 5). Due to the inherent risk of PS degradation, agencies request more and more the implementation of appropriate methods for monitoring of PS content and potential subsequent degradation products over time. Future expectations may comprise having an overall control strategy for polysorbate use in parenteral formulations.

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Chapter 11
Particles in Biopharmaceuticals: Causes, Characterization, and Strategy

Steven Messick, Miguel Saggu, and Anacelia Ríos Quiroz

1 Overview

Biopharmaceutical companies are required to control subvisible and visible particles in their products to ensure a consistent manufacturing process, assess product quality, as well as address potential safety concerns. Subvisible particles cover the size range between 1 and 100 μm, while particles >100 μm are generally considered to be visible [1]. According to USP guidelines, particles are classified into three different categories, namely, extrinsic, intrinsic, and inherent particles [1]. Extrinsic particles are defined as foreign particles unrelated to the manufacturing process, while intrinsic particles arise from the manufacturing process or primary packaging. Inherent particles can result from drug product degradation and can contain proteinaceous and/or other formulation components [2]. These three particle types are associated with different risk profiles, and an appropriate risk and safety assessment must be performed in order to set up an appropriate control strategy. In general, occurrence of extrinsic particles should be eliminated, and intrinsic particle types must be monitored/controlled to minimize their occurrence, while potential inherent particles must be well characterized and their presence justified and monitored/controlled over the product shelf life [3]. Thus, unless

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otherwise stated, hereafter the main focus will be given to the inherent particle type. In the last few years, more occurrences of inherent particles such as proteinaceous or fatty acid particles have prompted companies to develop more complex and risk-based control systems to control levels of these specific particle types. For this, general safety assessments based on prior knowledge and clinical experience with such inherent particles are required to demonstrate patient safety and guarantee product quality.

Particle counts of subvisible particles must be controlled for the same reasons mentioned above. Pharmacopoeia chapters specify limits for levels of subvisible particles ≥10 µm, ≥25 µm, and ≥50 µm (ocular only), while particle counts ≥2 µm and ≥5 µm should be monitored according to USP<788>/EP 2.9.19 [2, 4]. According to USP <788>, the preferred method for the determination of subvisible particles in biopharmaceutical formulations is light obscuration (Method 1), and in the case of samples with high viscosity or reduced clarity, membrane microscopy (Method 2) may be used (see Table 1). Over the last few years, concerns have been raised that proteinaceous subvisible particles may trigger immunogenic responses, but the roles of chemical composition and structure of the particles in generating an immune response are under debate as these attributes are particularly difficult to characterize [3]. Although some efforts have been published [5], it is noted that all biotherapeutics contain subvisible particles, most of which are well within USP specifications [6] and with no direct link to immune reactions under therapeutic conditions.

In general, for particle-containing products, identification/characterization of particles is key in assessing potential root cause and mechanism of formation. In addition, the impact on product quality and patient safety needs to be carefully assessed, and particle occurrences should be controlled within justified ranges during the product's shelf life.

Table 1 Summary of particle compendial specifications as per Ph. Eur. and USP

Size range				Subvisible				Visible	
Method				LO		Microscopy		White and black double chamber	
Compendial guide				Ph. Eur.	USP	Ph. Eur.	USP	Ph. Eur.	USP
				2.9.19	788/789	2.9.19	788/789	2.9.20	790
Sample nominal volume	>100 mL [ppc]	**Particle size [µm]**	≥10 ≥25	25 3		12 2		2000 lux and 3750 lux 5 seconds in front of each background	
	≤100 mL [ppmL]		≥10 ≥25	6000 600		6000 600	3000 300		
	Ocular [ppmL]		≥10 ≥25 ≥50	50 5 2		50 5 2			

2 Causes

2.1 Drug Product (DP) Degradation

Visible and subvisible particle formation during storage can be triggered by numerous factors, e.g., interaction of the active pharmaceutical ingredient with excipients/leachables or chemical degradation such as oxidation or hydrolysis, elevated temperature, or interfacial stress affecting the excipients itself. Recently, inherent particles arising from DP degradation, e.g., polysorbate degradation, are detected more frequently. We will discuss the most commonly found inherent particle types below.

2.2 Interfacial Stress

Most biopharmaceutical formulations contain surfactant to protect the protein against interfacial stress that can occur during storage and transportation or in clinics when diluted into Intravenous therapy bags (IV bags) [7]. Interfacial stress can occur at all interfaces, e.g., at liquid-liquid interfaces in samples that contain silicone oil as lubricant such as pre-filled syringes where the protein can aggregate on the surface of the oil droplets [8]. Protein aggregation at liquid-air interfaces can occur in IV bags at the interface between solution and headspace, in particular because the surfactant is diluted too. Other types of interfacial stress, e.g., at solid-liquid interfaces, can occur during storage or sample freeze-thaw.

2.3 Polysorbate Degradation

As described above surfactant is usually added to formulations to protect proteins against interfacial stress. The most commonly used surfactants include polysorbates 20 and 80 as well as poloxamer 188. Polysorbates are nonionic surfactants, comprised of a hydrophilic polyoxyethylene sorbitan head group and a hydrophobic fatty acid side group. The composition of polysorbates is highly heterogeneous due to a variety of different fatty acids present as side chains. It is known that polysorbates can degrade via hydrolytic and/or oxidative degradation causing different insoluble degradation products that can form particles [8, 9]. During hydrolytic degradation, the fatty acid ester bond is cleaved resulting in the formation of free fatty acids, while oxidative degradation is believed to cleave the polyoxyethylene chain at the ethylene oxide subunits resulting in fatty acid esters as well as smaller amounts of fatty acids. During long-term storage, released free fatty acids or ester levels can exceed the solubility limit resulting in subvisible as well as visible particles. It is worth noting that these particles are usually not associated with immunogenic responses as they normally dissolve during dilution in IV bags or when administered

to patients. More recently, a clear hydrolytic degradation pathway based on enzymatic cleavage by residual lipases has been proposed. However, analytical identification of the involved enzymes is hampered by their very low abundance [4]. If present, a thorough characterization of these particles is required to identify the degradation pathway (oxidative or hydrolytic), assess the impact on product quality and safety, and develop a proper control strategy.

3 Characterization

3.1 Subvisible Particles

The subvisible size range for particles is commonly defined to be between 1 and 100 µm. Specifications of compendial methods cover particles ≥10 µm, ≥25 µm, and ≥50 µm (the latter one only for ocular products). These specifications are for the well-established light obscuration and microscopic methods and are summarized in Table 1.

Additionally, there is increased scrutiny by health authorities to characterize smaller particle sizes due to the potential immunogenic risks associated with proteinaceous particles as described recently [10–12]. Although more complex factors might play a role on the development of immunogenic reactions [5], companies are required to establish safety profiles for their drugs. In this context, the number of newly available analytical instruments in this area increased significantly over the past recent years [13].

Among the most popular emerging techniques (see Fig. 1), Flow Imaging and Electro Zone Sensing are the most commonly used orthogonal techniques to the

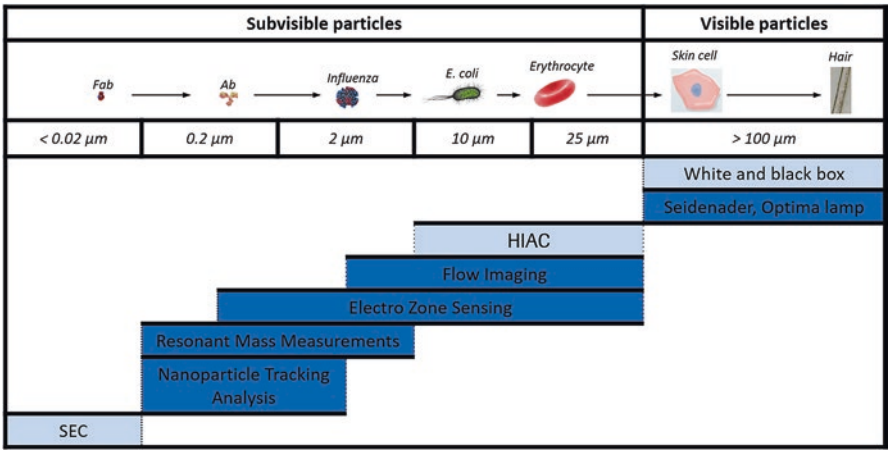

Fig. 1 Size ranges of common analytical techniques

compendial methods in the pharma industry [14, 15]. The Electro Zone Sensing (or Coulter Counter) principle is based on an electrical measurement that utilizes a glass aperture with an orifice of known size in which particles are being detected. The measurement requires that the samples have sufficient conductivity. Whenever a particle passes through the orifice, a drop in voltage between the solution outside and inside of the tube is detected. The drop in voltage is directly proportional to the volume of the displaced electrolyte and can be used to accurately size and count particles. The required conductivity depends on the size of the used orifice, and more conductivity is required for smaller orifices as less volume is being displaced by the smaller particles. When apertures ≥100 μm are being used, buffers that are typically used in biopharmaceutical formulations provide sufficient conductivity [15].

In the flow imaging techniques, a camera is located at a 90-degree angle with respect to a liquid flow cell and collects images of particles as the sample fluid passes through the cell. The particles are being detected by image analysis after a proper background image has been subtracted. The instrument provides size, counts, and morphological descriptors of each individual particle. Additional statistical analysis can be applied to the data set allowing for classification of different particle populations based on differences in morphologies, e.g., proteinaceous particles, silicone oil, or fibers [16].

Due to increased regulatory expectations over the past years, more attention has been given to the submicron size range. Analytical techniques in this size range are constantly emerging but currently lack robust analytical performance as only small sample volumes are being analyzed, e.g., Nanoparticle Track Analysis [17] and Resonant Mass Measuremet [18]. Although emerging pharmaceutical modalities [19] might benefit from such analytical toolboxes, at the moment these techniques are mostly implemented in Research and Development (R&D) environments [20–22].

3.2 Visible Particles

In general, visible particles are considered to be those of size >100 μm [23, 24]. However, the limit of visibility depends on many factors besides a single size descriptor. For example, particles might be visible to the unaided eye even if they are <100 μm provided that they have high refractive index as compared to the matrix on which they are suspended or provided that they are present at high concentration on which case the turbidity can be high enough to reach visibility. Furthermore, the type of illumination applied might also set different thresholds for particles to appear to the naked eye. Other factors to consider are, for example, color, shape, transparency, reflectivity, as well as the analyst's training.

Typically, one important and challenging step in the characterization of visible particles is their isolation. In cases in which the particles are fragile or at the border of visibility, instead of picking out the particle manually utilizing tweezers, micro-

Table 2 Classification and tracking of particulates

Descriptor	Class	Examples/definition
Type	Extrinsic	Particles from outside the process
	Inherent	Particles that are naturally present in protein therapeutics and may be acceptable with the appropriate control strategy; this includes protein aggregates
	Intrinsic	Particles generated within the manufacturing process and may include silicone oil, rubber, glass, or stainless steel
Source	Primary packaging	Vials, syringe barrels/plungers
	Fill/finish process	Silicone tubing, stainless steel
	Environment	Clean wipes, depyrogenated material
	Product related	Protein, excipients
Frequency	Random	Particles can be clearly assigned to a specific unexpected event
	Systematic	Particles within controlled ranges are established as unavoidable and systematically linked to the product

capillaries, or fine probe, it is better to filtrate the entire sample container. Either isolation method should be followed by removal of interferences (e.g., soluble excipients by sample rinsing with water) and microscopic characterization methods and/or digital imaging for collection of physical descriptors.

The chemical identification commonly involves spectroscopy techniques like Fourier-Transform Infrared (FTIR) and Scanning Electron Microscopy/E Raman spectroscopy, which are suitable for the identification of organic materials like elastomers, fibers, and product-related particulates (protein, excipients). If inorganic materials like metals, salts, or glass are present, Scanning Electron Microscopy/Energy-Dispersive X-ray (SEM/EDX) can be used for identification.

Together, physical and chemical identification will provide information on the type, source, and frequency of the finding (see Table 2). This will help the determination of the particulate origin, route of introduction, and most importantly preventive/corrective actions.

4 Strategy

4.1 Regulatory Expectations and Current Landscape

Health authorities, worldwide pharmacopeias, as well as ICH guidelines have increasingly reflected stricter considerations in relation to particulates in biopharmaceutical formulations [6, 25, 26]. Particulate control usually starts at the subvisible range [27]. Control of smaller particle sizes (i.e., $<1\ \mu m$) is not a compendial requirement as such submicron particles have not proved to be in relation to any other size range, and there is currently no robust technology to analyze them in a quality control environment.

For subvisible particles, the gold standard is the light obscuration method. Briefly, in this technique particles are injected by a syringe pump into a sensor with a constant light illumination. As particles present in the sample flow through the cell, the light is then obscured and a drop in voltage is registered. This change is proportional to the size of the particles (internal calibration curve using polystyrene beads). Knowing the volume of the analyzed sample, the instrument provides a number of particles per milliliter of sample.

The light obscuration method is not suitable for those samples on which a basal color of the solution might interfere with a clear blank measurement. For such cases or cases on which light obscuration method has failed the acceptance criteria, the second method of choice is the microscopic membrane method. In this case, sample is filtered through a gridded cellulose membrane, and with the aid of a microscope, particulates are manually quantified.

Several orthogonal methods to these two techniques are available in a R&D setting. More detailed discussion about their pros and cons is presented below.

For visible particles (i.e., particles larger than 100 μm), release analysis involves 100% inspection of all produced containers [28, 29]. This is a manual analysis (but can also include additional automatic or semiautomatic procedures) in compliance with compendial method (see Table 1 and [30]) which relies on trained analyst's capabilities, at defined illumination intensities and observation time, to be able to identify defects in close containers by contrast against black and white background colors. Although this method is probabilistic due to a number of factors associated with the "visibility" of a particle (e.g., size, number, refractive index, etc.): providing a consistent and sound training program for the analysts generally gives confidence in an acceptable level of particle detectability of >70% PoD (Probability of Detections), (System bug does not allow comment in the specific position) PoD (see Knapp's methodology in [31]). After 100% inspection and followed by the definition of an appropriate sampling plan, an AQL (Acceptable Quality Level) test is performed. Only then and if no test failure occurs, the different batches can be released by quality control functions.

Besides the best efforts to control particulates, several commercial biotherapeutic products currently on the market filed their products reporting with the likelihood that the product "might contain" certain type of visible particulate matter (see Table 3 and [29]). Also, studies among various pharmaceutical companies have shown that commercialized products can contain elevated levels of subvisible particles without any associated patient risk or reported adverse effect [6]. Presently, no indication of immunogenicity linked to any size range has been established [5, 10].

4.2 Strategy: Control and Mitigation

Particulates in pharmaceutical products have always caught a lot of attention by regulatory agencies. Scientific research and risk/complain reports have shown that if this quality attribute is not controlled, particulates can impose safety and efficacy risks for patients [3]. Thus, pharmaceutical companies should strive in the case of

Table 3 Examples of marketed products with declared reference to presence of particles

Antibody	Trade name	Company	Reason
Cetuximab	Erbitux®	Bristol-Myers Squibb	May contain a small amount of easily visible, white, amorphous, cetuximab particulates
Panitumumab	Vectibix®	Amgen	May contain visible translucent to white, amorphous, proteinaceous particles
Nivolumab	Opdivo	BMS	May contain few bright particles
Golimumab	Simponi	Essex Pharma	May contain few small transparent or white protein particles
Reslizumab	Cinqaero (EU) Cinqair (USA)	Teva Pharmaceutical Industries	May contain proteinaceous particles
Obiltoxaximab	ANTHIM	Elusys Therapeutics, Inc.	May contain few translucent to white proteinaceous particulates
Ustekinumab	STELARA	Janssen-Cilag AG	May contain few small transparent or white protein particles

visible particles for products practically free from particles and in the case of subvisible particles for low-content products in line with defined compendial requirements. In order to achieve this goal, it is important to acknowledge that more knowledge about a product allows for a better understanding of the particle occurrence or the mechanisms of particle formation.

The control strategy has to be suited to each of the different stages of the product. While for an early-stage product the efforts might be mainly exploratory and include knowledge building, a late-stage project might involve the development of acceptance criteria limits at the top of those specified by the compendial pharmacopeias. This might include both wider or tighter specifications according to what historical knowledge on the products is available. Even more, control strategy is also dependent on the size range at which the particle phenomenon appears and whether or not a filter to reduce the particle load is used in clinics. Some of these cases are described in the following sections.

In general, a minimum control strategy for inherent particles should include the following aspects all inscribed into a safety/toxicological assessment that proves the particles to be of no biologic adverse impact (see Fig. 2):

1. Detection: the triggering method of the particulate investigation
2. Isolation: imaging of the closed container where a particle was observed followed by imaging of the isolated material
3. Identification: spectroscopic chemical component analysis to define the nature of the particle
4. Quantification: degree of the particle phenomenon observation

The following sections briefly describe some additional aspects to consider in a phase-dependent approach (see Table 4).

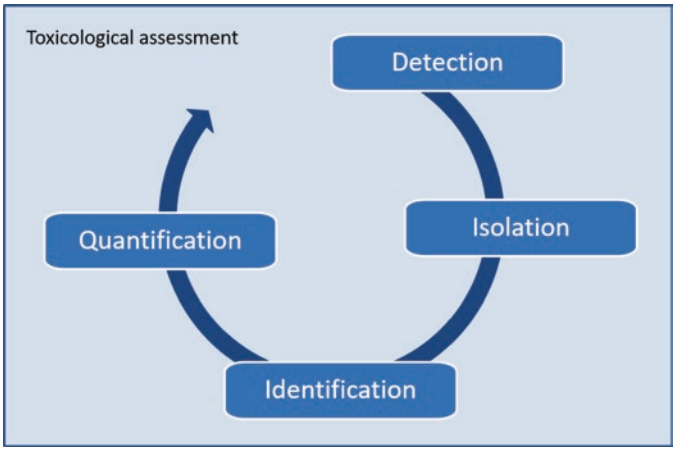

Fig. 2 Minimum components of a particle control strategy for biopharmaceuticals

Table 4 Exemplary analytical control toolbox for particulates at different development phases

Phase		SbVP	Visible
Knowledge building	Early-stage development	Dynamic light scattering Light obscuration Flow imaging microscopy	Seidenader Optima lamp Keyence/APK White and black box
	Late-stage development	Light obscuration [For extended characterization: Flow imaging microscopy]	White and black box [For extended characterization: IR Raman SEM/EDX Quantitative assessment]
Control strategy	Production (release)		
	Stability (clinical/commercial)		
Monitoring	Post marketing		

4.3 Control Strategy at Development Phase

During development, main efforts should focus on identifying the most robust and stable formulation. For this, different studies including accelerated (at 25 °C and 40 °C) and long-term stability programs together with statistical models that allow the projection of these data sets are of the highest relevance.

Particles might originate not only directly from the protein inherent tendency to aggregate due to external stress factors such as increased temperature or light. Another important factor to consider is the role of the primary containers. For instance, in order to increase the manufacturability of stoppers, they are covered with a layer of silicone oil that avoids that they stick in machine surfaces or among them, but instead the constant flow of all manufactured pieces is ensured. Another example

is the siliconization that pre-filled syringes have in the inner part of the barrel. This is meant to facilitate an acceptable plunger movement during administration. In these two examples, it is unavoidable that silicone oil droplets will end up in the solution adding up to the particle population. This happens mainly in the subvisible range and below, but if formation of complexes with other type of particles occurs, this effect might also be seen in the visible range. Thus, extensive studies on the compatibility with primary packaging material as well as with in-use material, e.g., bags for infusion, are highly recommended.

Especially when a certain level of sensibilization already exists due to preexisting knowledge on product, primary packaging, or excipient tendency to generate particles, it is important to closely monitor even early phases of the development. However, provided that other analytics are also in place, main focus is often given to the final nominated formulations (late-stage projects) and especially to those batches that will be submitted in communications with health authorities.

It should also be considered that the different analytical methods in place will set different thresholds at which a particle phenomenon can be identified. Thus, it is important to understand the differences and frame of each particle analytical result in the correct magnitude, and main priority should be given to the fulfillment of the compendial requirements.

In this regard, several orthogonal techniques can be included to expand the analytical toolbox. For example, although visual inspection against white and black background is the compendial method, other more powerful tools like enhanced visual inspection using higher illumination intensity and/or magnification, e.g., Seidenader or APK, might help in detecting particle onset at earlier time points. These techniques are particularly useful as they are noninvasive. Preventive and root cause analysis actions should be stablished in a proactive way. This might include the chemical identification of every particle finding allowing the compilation of a sound historical analytical data set. Finally, a descriptor about the magnitude or intensity of the phenomena in terms of particles per container will also help the monitoring of the particle phenomenon. While compendial methods define quantification limits for particles >10 μm and >25 μm, collecting information about the quantification ranges for particles in the visible range is also recommended, and some qualitative scales had been proposed for that purpose.

Very importantly, toxicological assessments should be initiated as soon as particulate findings are consistently and repetitively found among various batches. Until an effective mitigation strategy is available, toxicological assessments might help the continuation of the development phases.

4.4 Control Strategy After Filling Phase

Right after production, all units within a batch should undergo 100% inspection. This scrutiny aims to find any cosmetic defect including, e.g., missing stoppers or sealing defects in the metal ring. Related to particulates, given the high-quality

standards that the pharmaceutical industry has reached, particulates from the category *extrinsic* (not related to the production process like human or animal hair) are less often found. On the other hand, *inherent* particles (the ones protein- or excipient-related that have a clear link with the product nature) are normally stability indicative, and they generate over time. Thus, at production level, particle control strategies are in general considered as indicative of the manufacturing process, and often times they detect failures in the filling or assembling lines like glass breakages or oil leakages (*intrinsic* particles). During 100% inspection all defective units are discarded, and the remaining ones are further subjected to a second inspection with a statistical approach. On this second inspection, an AQL is set to define the worst tolerable level of defective units with a certain type of defect (critical, major, or minor) that are allowed in a batch sample of a defined size. AQL tests are generally very strict and after 100% inspection should give clear confidence that the batch is practically free from visible particles.

4.5 Control Strategy at Stability Studies

Most inherent particle phenomena arise over stability. This is the phase on which all learnings collected during development phases might be applied for the control strategy of the already commercialized batches or those dedicated to clinical studies. It should be clear that the larger the collected data set, the easier will it be to understand and map particulate trends for a specific product.

A control strategy might include – at the top of the compendial requirements previously described – a video recording and imaging of the particle findings ideally both in the closed container and after particle isolation. Although morphology is not always a strong descriptor of the particle phenomena, over time and after several time points of data collection, it provides a good picture of the type of findings that can be considered as non-atypical enriching the knowledge of the particles. Followed particle's morphology documentation, the next and most important stage within and holistic particle control strategy is the chemical characterization of the particle. This guides the root cause analysis and set precedent on the type of particles that are typical for the product under investigation. This chemical characterization step involves spectroscopic methods like FTIR, Raman, or SEM/EDX. The first two are particularly important because of its sensitivity toward organic compounds, whereas EDX offers insight on inorganic components, if any, on the particulate matter [32].

4.6 Control Strategy at Post Marketing

A continuous monitoring of the product after launching is important to maintain quality standards as those defined during development phases. Importantly, any introduction of changes in the manufacturing process (e.g., formulation change due

to product registration in new countries, primary container improvements, etc.) should be thoroughly assessed on its particle formation impact. Furthermore, in cases on which a particle phenomenon was described during development phases, this should be closely observed in order to detect any possible deviation to the predefined descriptors.

4.7 Mitigation Strategies

Particulates in biotherapeutics have the following main sources: primary containers [33], degradation of excipients, and interaction of inherent protein particles with any of the former. Thus, strategies aiming to reduce the risk of particle formation should explore alternatives on those pharmaceutical development components.

Related to primary containers, device development efforts might include the evaluation of stoppers, vials, and cartridges with low or zero silicone oil load. Technical advances on the siliconization coating process have shown that cross-linked or baked-on silicone oil has a reduced tendency to migrate into solution, thus reducing the risk of aggregation at the water/oil interface.

The most commonly used surfactants are polysorbates and poloxamers. While polysorbates are extracted from palm oil, poloxamers are synthetic block copolymers. Although both are proved to be successful for their intended use, it has been reported that polysorbates can undergo several different types of degradation pathways producing Free Fatty Acid (FFA) particles and that poloxamers might not be competitive enough to prevent interaction between protein aggregates and other hydrophobic surfaces as silicone oil. In this regard, efforts on the downstream and purification process should be explored together with detailed scrutiny of excipient screening studies. Additionally, super-refined polysorbates have been also explored to reduce the probabilities of FFA formation.

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Chapter 12
Strategies in the Development of Formulations for Antibody-Based Therapeutics

Feroz Jameel, Ehab M. Moussa, Brittney J. Mills, and Peter M. Ihnat

1 Introduction

Antibodies and antibody-based therapeutics are a fast-growing class of drugs that possess versatile biological functions that are employed to treat a wide variety of diseases spanning a number of immunological and neurological disorders, different types of cancers, as well as several metabolic and cardiovascular diseases. Being large biomacromolecules, antibodies are highly complex in structure and are, thereby, vulnerable to several degradation pathways during manufacturing, storage, and administration to patients. Accordingly, significant amount of work is typically required throughout the different phases of development in order to bring the right candidates to the clinic and then to successfully advance them forward to the market. In order to minimize these efforts and speed the development process while minimizing the risk of failure, rationale formulation development based on scientific reasoning and prior knowledge is very critical to the timely success of the programs.

In this chapter, we aim to provide strategies for the development of antibodies and antibody-based therapeutics from the candidate selection pre-formulation phase through late-stage commercial presentation development. The basic principles of protein structure, stability, and biophysical characterization are briefly discussed here but are not the main focus of this chapter. The interested reader is referred to

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several published works on these topics in the literature [1–4]. The chapter is divided into two main sections that are concerned with pre-formulation and formulation development. The formulation section is further divided into two subsections focusing on early and late-stage development. Throughout the chapter, recommended systematic studies at each stage and the rationale behind them are presented.

2 Pre-formulation Development: Moving the Right Molecule Forward

Biopharmaceutical companies are constantly looking to select the right biologic to move forward, decrease attrition rate in late-stage development, and, thereby, bring down costs and shorten timelines. Drug product development of biologics typically begins with a pre-formulation assessment, which involves forced degradation and physicochemical characterization of several candidates with the overall aim of selecting the right one to move forward with a minimized risk of failure [5, 6].

By definition, a forced degradation study is the intentional degradation of the drug molecule to an appropriate extent using different stress conditions such as high temperature, extreme pH, light exposure, oxidizing agents, freezing and thawing, and/or mechanical stress in order to elucidate its potential degradation pathways. These studies also help define the critical quality attributes (CQAs) of the product and develop and validate stability-indicating assays.

Forced degradation studies are typically applied early on during the screening of the molecules in order to rank order, nominate, and select the best molecule from a pool of candidates to ensure that it has suitable drug-like properties (e.g., acceptable degradation profile, high solubility, and low viscosity). In this regard, molecules can be evaluated under minimal formulation conditions in order to select the most stable candidates based on their primary sequences.

In this section, we describe a "fit-for-purpose" application of forced degradation and biophysical characterization to the molecular assessment and candidate selection during the late discovery phase along with recommended workflows [5]. In the screening stage of the candidates, several pharmaceutically relevant stress factors such as elevated temperatures, UV light, extreme pH, repeated freezing and thawing cycles, agitation, and/or oxidizing agents should be applied to identify the candidates that are most resistant to such stresses in addition to stability under target storage conditions.

2.1 Overview of the Analytical Methods and Stress Conditions Used in Pre-formulation Development

Throughout the life cycle of the development of antibody-based therapeutics, several analytical techniques are employed to characterize the molecules. In this subsection, the key properties and the methods used to characterize them are briefly

discussed as it pertains to the pre-formulation screening of these molecules. These properties include plasma stability, conformational stability, colloidal stability (aggregation, protein-protein interactions, solubility), charge heterogeneity/chemical liabilities, and glycosylation. As will become clear through the discussion, no single method, assay, or physicochemical property can be solely used to rank order candidates. As such, the data from the different stressed conditions and the various assays used should be considered in its entirety in order to be able to select the most intrinsically robust candidate [7, 8].

Plasma Stability

The first thing that must be considered during early-stage assessments is the plasma stability of the molecule. Even before accelerated stability screening, molecules are tested for their in vivo activity. Therefore, they must remain active even within the complex plasma matrix. Typically, different types of plasma (mouse, rat, human, and monkey) are tested as these species are relevant in preclinical assessments. Upon incubating the molecule within the appropriate plasma matrix for a relevant time period (37 °C for 1–5 days), assays should be performed to confirm antigen binding is not affected [9], as well as to pinpoint if any physicochemical modifications have occurred within the molecule (aggregation, chemical modifications, DAR loss for ADCs).

Conformational Stability

Conformational or thermodynamic stability of proteins is another important property that has been related to specific solution interactions and is a useful tool to rank order candidates. The melting point (T_m) of the protein upon exposure to heat is typically used as a measure of its conformational stability [10]. T_m is defined as the temperature at which half of the protein population in the sample are unfolded. The melting profile of antibodies and many of the related modalities usually involve different transitions due to the presence of different domains.

Unfolding can be determined through high-throughput methods such as differential scanning fluorimetry (DSF) or differential scanning calorimetry (DSC). DSF requires minimal sample, which makes it widely used when there are material limitations. Two methods of DSF exist: label free, which is based upon the intrinsic fluorescence of the aromatic amino acids, or labeling methods, which require the addition of a dye to bind to the unfolded form leading to a change in fluorescence. On the other hand, DSC, which requires more sample and instrument time, may be more sensitive to unfolding events not observed via the DSF methods. Due to the high sensitivity of DSC, formulation conditions can alter the observed T_m, particularly polysorbates [11]. While useful for determining the onset of unfolding, conformational stability is not always predictive of storage stability due to the mechanistic differences that exist between the two types of stability [12, 13].

Colloidal Stability

The interactions between two adjacent molecules highly influences multiple drug-like property attributes that are important to assess at the pre-formulation stage. At this stage of development, solubility assessments are difficult to complete due to the limited amount of material available. Use of small-scale ultracentrifugation allows for the determination of rough estimates or solubility minimums (i.e., at least 70 mg/mL), when there isn't enough material to test the absolute solubility. Oftentimes, solubility is governed by the surface properties of the molecule and the likelihood for interaction. The presence of hydrophobic groups (amino acids or toxins in terms of ADCs) or regions of opposite charge on the surface can promote interaction between the molecules (protein-protein interactions, PPI). While chromatographic methods can be used to evaluate PPIs [14–18], a more commonly used approach to quantitate interactions is the determination of the second virial osmotic coefficient (B_{22}). This parameter indicates the net attractive or repulsive forces between the protein molecules at specific solution conditions. B_{22} can be measured using static light scattering (SLS) or can be estimated from the interaction parameter (K_D) measured using DLS. In both methods, relatively high amount of material is required to run the experiment, which limits their usability for high-throughput screening (HTS). As is the case for conformational stability, a more positive B_{22} value (i.e., more repulsive interactions) does not always result in less aggregation. However, in the experience of the authors, candidates and/or formulations with both positive B_{22} and high T_m will typically have low aggregation profiles.

In addition to influencing solubility, PPIs can also lead to aggregation. At the pre-formulation stage, assessment of aggregation will allow for an early "flag" of aggregation-prone molecules to either promote the selection of a more suitable candidate or provide early notice for required formulation design and development. Size-exclusion chromatography (SEC) is a commonly used tool to assess aggregation. In SEC, molecules are applied to a porous resin, which leads to separation based upon size. This allows for separation between monomeric species and aggregates or fragments. As traditional SEC typically utilizes solely UV detection, it is difficult to differentiate the different type of aggregate species (dimers, trimers, etc.). For this reason, SEC has been coupled with multi-angle light scattering (SEC-MALS) to get a more accurate assessment of the distribution of aggregate species in solution [19]. As aggregates can be both reversible and nonreversible in nature, stressed samples should be assessed in both undiluted and diluted conditions, to determine the reversible nature of the observed aggregates. To avoid column interaction and dilution effects that can skew the SEC results, dynamic light scattering (DLS) can be used as an alternative method. In DLS, an undiluted sample is assessed based upon fluctuations in scattered light to gain information about species size with regard to mass and intensity. As the relative size of an antibody is known, the presence of aggregates can be determined. But, formulation components such as polysorbates may interfere with the measurement, so one must consider how the formulation composition could impact the observations.

pI and Posttranslational Modifications

The isoelectric point (pI) is an important property to consider in the design of a suitable formulation as the chosen pH will be a key factor in determining the overall surface properties of the molecule. Additionally, the pI may impact the pharmacokinetics profile [20], as molecules with more acidic pI values have been associated with decreased tissue uptake and blood clearance. The standard technique for pI determination is imaging capillary isoelectric focusing (icIEF). In addition to pI, icIEF will also provide the charge distribution profile, which allows for an evaluation of the acidic or basic species that exist. Subjecting the molecule to stressed conditions can lead to chemical modifications such as deamidation, succinimide formation, and oxidation, to name a few. As icIEF only allows for monitoring of the percentage of each species, additional techniques such as mass spectrometry will have to be employed to identify the type of chemical modification that is present.

Mass spectrometry coupled to capillary zone electrophoresis-laser-induced fluorescence detection (CZE-LIF) also serves as a useful technique for evaluating the glycosylation profile within antibodies [21, 22]. Glycosylation can impact the physicochemical properties, biological activity, and immune effector functions of the molecule [23–26]. Early-stage antibody production usually occurs in HEK cells, but upon progression of the candidate to later stages, CHO cells are typically used, which can lead to differences in the glycan profile between the early-stage molecule and the one progressing into development [27]. Therefore, glycan profiling should only be completed upon production of the candidate in the final cell line.

2.2 Developability and Candidate Selection of Antibodies

In recent years, significant advances have been made in developing platform technologies that enable discovering monoclonal antibodies (mAbs) that bind to specific targets with high affinity to achieve the desired biological effects. Nevertheless, not all mAbs possess the required drug-like properties to be developed and manufactured into viable drug products. The developability can be defined as the likelihood that a candidate will successfully advance to late-stage development and marketing license applications. Therefore, the candidate must remain stable during storage and through manufacturing unit operations. To this end, several analytical profiling methods should be employed in order to evaluate the suitability of molecules for further development, such as physicochemical properties, and minimum propensity to aggregate or to elicit unwanted immunogenicity. This includes several advanced analytical techniques coupled with miniaturized high-throughput experimental setups focused on characterizing a wide range of biochemical, biophysical, and in vivo properties [5].

The expected outcome of employing these workflows is to identify candidates that possess the best properties, such as high solubility, minimal physicochemical liabilities, and the flexibility to withstand variable process conditions that are

typically encountered during manufacturing and shipment. Furthermore, these evaluations are usually conducted in minimal buffer solutions in order to select the most stable candidates based on their inherent properties.

The candidate evaluation and selection process occur at the interface of discovery and development prior to the initiation of formal CMC development for a given new biological entity (NBE) using a tiered approach as depicted in (Fig. 1). Antibody libraries based on yeast display or other selection concepts are capable of identifying mAbs with high specificity and selectivity for therapeutic targets. Furthermore, automation enables rapid screening to identify multiple mAb candidates [28]. After library screening selection, hundreds of candidates with appropriate potency and selectivity are moved forward to the first stage of pre-formulation development. At Stage 1, the primary sequence of the candidates is screened in silico to determine several key properties including the following:

(a) pI and molar extinction coefficient
(b) Sequence liabilities (hot spots), which are residues and sites that are susceptible to aggregation and/or chemical degradation such as oxidation, deamidation, and isomerization
(c) Solubility and viscosity

Based on these analyses, potential protein engineering opportunities are identified and may be implemented to address any potential sequence-related issues and to improve drug-like properties. The outcome of this stage is a few tens of candidates that are advanced to Stage 2 [5, 7].

In Stage 2, the set of assays summarized in Table 1, or an appropriate variant thereof, is performed on a relatively large number of molecules in order to select up to five candidates to move forward to Stage 3. In order to accommodate the high number of molecules while only a few milligrams of material are available, the assays should be designed to enable high-throughput analysis and to be material and time efficient.

In Stage 3, a more in-depth characterization and drug-like property profiling are performed on the selected candidates from Stage 2 (Table 2) in order to identify the lead and backup molecules. At this stage, a thorough review of the drug-like properties provides the risk assessment and adherence with target molecule profile (TMP) and suitability for advancement into CMC development. Alternatively, the candidates may be reengineered to remove liabilities from the primary sequence associated with instability, and the testing is repeated [5–7].

Several risk assessment tools such as failure mode and effects analysis (FMEA) can be employed to estimate the risk of developing a particular candidate. A weighted score is assigned to each of the pre-formulation assays based on prior knowledge, experience with similar molecules, experience of others in the literature, and the anticipated contribution of the results to the overall molecular profile of the candidate. The predictability of the assays can then be categorized into having a strong impact (weight scale of 10) to having little or no impact (weight scale of 1) on predicting the development risk (Tables 1 and 2, third column). Next, the development risk can be estimated by comparing the results with established benchmark

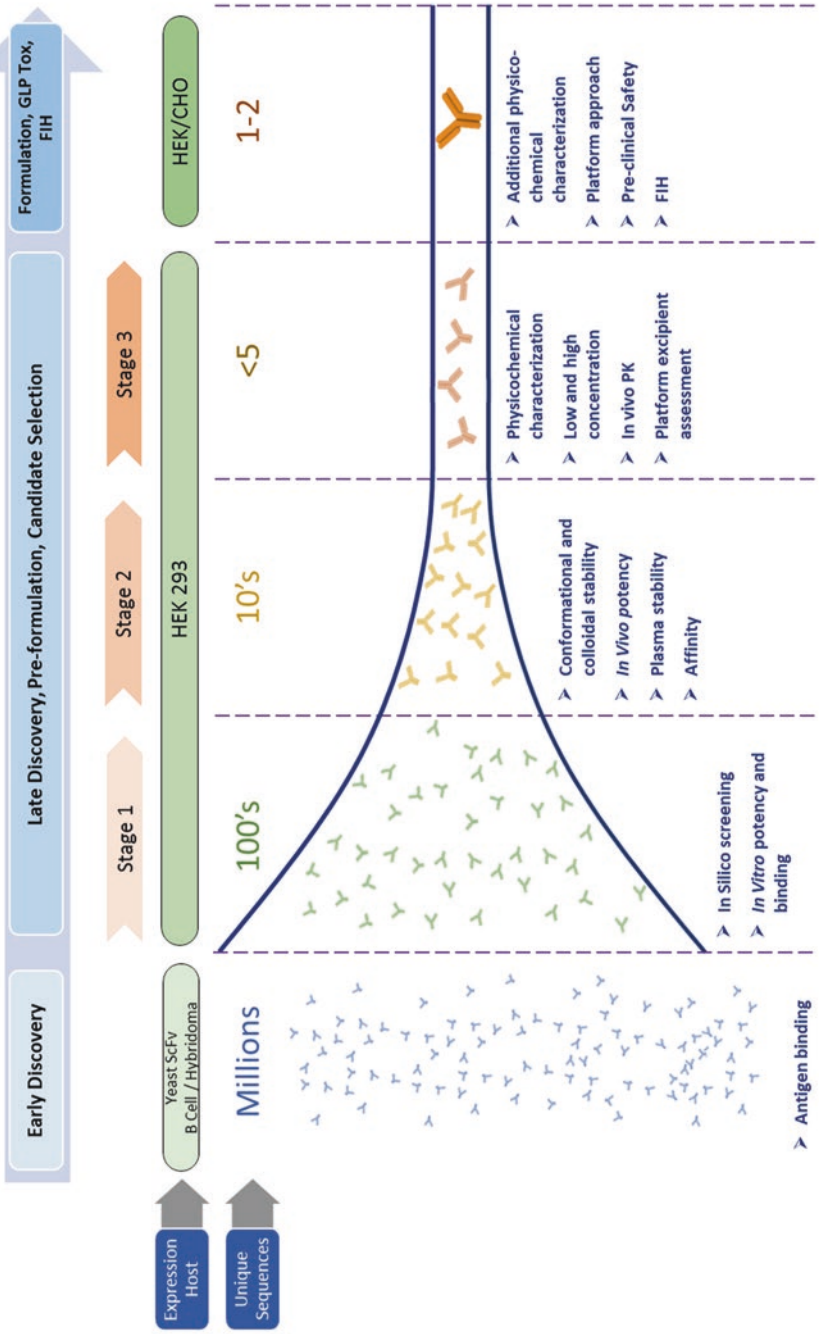

Fig. 1 General outline of the stages of pre-formulation screening and candidate selection process

Table 1 Overview of Stage 2 pre-formulation assays performed to enable a risk-based assessment and ranking of the drug-like properties for early candidate selection

Parameters	Assays	Weight scale	Proposed acceptance/ ranking criteria	Approximate drug substance needs	Conditions
Colloidal stability	Absorbance at 280 nm	10	≥100% of target	X mg	Target pH in platform buffer Concentrate to target mg/mL
			50–100% of target		
			<50% of target		
	Appearance by visual inspection	10	Clear		
			Opalescent		
			Phase separation/gelling/ precipitation		
Aggregation	Size exclusion chromatography (SEC)	10	Low % monomer loss after specified time	X mg (concentrated material from solubility assay)	Elevated temperature: Concentration: target mg/mL Initial and later time points Target pH in platform buffer
			Interim % monomer loss after specified time		
			High % monomer loss after specified time		
			Total SEC peak area after incubation: ± specified % of T0		
Conformational stability	Dynamic scanning fluorimetry (DSF)	7	T_{onset} ≥ high set point °C	X mg (concentrated material from solubility assay)	Target pH in platform buffer.
			T_{onset} = interim °C		
			T_{onset} < low set point °C		
Biological stability	In vitro plasma stability by SEC	7	≤Established % HMW per day	X mg	Rodent plasma

Table 2 Summary of Stage 3 analytical characterization and the drug-like property profiling for candidate selection

Parameters	Assays	Weight scale	Proposed acceptance/ ranking criteria	Drug substance needs	Conditions
Colloidal stability	Absorbance at 280 nm	10	≥100% of target	X mg	Target pH in platform buffer
			50–100% of target		
			<50% of target		
	Appearance by visual inspection	10	Clear		
			Opalescent		
			Phase separation/ gelling/precipitation		
Colloidal and chemical stability	Visual inspection, SEC, icIEF	10	Low % monomer loss after specified time	X mg	Target mg/mL at elevated, ambient and refrigerated temperatures Initial and later time points
			Interim % monomer loss after specified time		
			High % monomer loss after specified time		
			icIEF: report pI, % acidic, % main, % basic species		
Freeze-thaw stability	Visual inspection, SEC, icIEF	10	Low % monomer loss after specified F/T cycles	X mg	Freeze-thaw stability at target mg/mL and cycles icIEF: final cycle
			Interim % monomer loss after specified F/T cycles		
			High % monomer loss after specified F/T cycles		
			icIEF: report pI, % acidic, % main, % basic		
Solution stability: reversible self-association	SEC	10	Low % monomer loss after specified time	X mg (use solubility sample)	Internal or referenced procedure for investigating reversibility
			Interim % monomer loss after specified time		
			High % monomer loss after specified time		
Conformation stability	Differential scanning calorimetry (DSC)	7	T_{onset} ≥ high set point °C	X mg	Target pH
			T_{onset} = interim °C		
			T_{onset} < low set point °C		

(continued)

Table 2 (continued)

Parameters	Assays	Weight scale	Proposed acceptance/ ranking criteria	Drug substance needs	Conditions
Activity (binding)	Surface plasmon resonance (SPR)	7	Report results	X mg	Initial and later time points. Thermally stressed sample
Chemical stability	Mass spectrometry	10	Report results	X mg	Initial and later time points. Thermally stressed sample Reduced and nonreduced intact mass Disulfide and peptide mapping

criteria. These standardized pre-defined ranges allow assigning molecules to low, medium, or high development risk classes based on the results of the assays as indicated in Tables 1 and 2 [8, 29].

2.3 Novel Antibody-Based Formats

Recently, several novel antibody-based biologic formats have been engineered in order to improve potency, increase circulation half-life, expand functions, enable specific delivery of drugs and effector proteins to the site of action, and enhance tissue penetration. Examples for these new modalities include Fc fusion proteins, antibody-drug conjugates (ADC), bispecific antibodies, antibody fragments, etc. Compared to antibodies, several of these modalities display poor solubility and/or stability depending on how they are constructed. For instance, the solubility and stability of ADCs may be influenced by the chemistry of conjugation, the location and degree of conjugation, and the chemistry of the linker and payload.

The pre-formulation screening of most of these modalities is very similar to that described for antibodies in the preceding subsection. One special case that requires additional testing is ADCs. In general, the screening and candidate selection of ADCs is similar to antibodies but also requires coordinated workflows wherein the screening and selection of the antibody against a target antigen and the screening of linkers and payloads to enhance efficacy need to occur simultaneously.

In the experience of the authors, as well as others as reported in the literature, ADCs are more easily destabilized by thermal stress, although the ADC and the parent antibody may have similar secondary and tertiary structures. For example, for species with a drug-to-antibody ratio (DAR) of 6–8, conjugation renders the CH2 domain less stable to thermal stress such that ADCs rapidly form aggregates at

40 °C [12]. Also, the conformation and dynamics of a model ADC compared to the parent antibody have been shown to be different in the CH2 domain near the hinge region and at the CH2-CH3 domain interface [13]. As such, special attention should be paid to the conformational stability of ADCs during screening. Additionally, hydrophobic interaction chromatography (HIC) and reversed phase chromatography should be routinely used during screening to monitor the distribution profile of the DAR and to quantitate the amount of free toxin, respectively.

3 Formulation Development Strategies for Early and Late Stages

Once a candidate has been identified to move forward to first-in-human (FIH) studies, drug product formulation development activities start. The development of a formulation for antibodies and related modalities is typically done in a phase-appropriate manner depending on the required speed to enter clinical trials and to be ready for commercial distribution. Accordingly, the strategies followed in developing a formulation for early- and late-stage programs can be quite different in the scope and extent of the studies that are conducted. In this section, the phase-appropriate approach to formulation development of biologics is discussed [6, 30].

3.1 Strategy for Early-Stage Formulation Development

Typically, only a small fraction of the new molecular entities (NME) in clinical trials move forward to successful marketing license applications. As such, biopharmaceutical companies are constantly developing and refining "fit-for-purpose" strategies to minimize the efforts dedicated toward early-stage drug product development while enabling speedy entry to FIH studies. These strategies are designed to provide flexible and stable dosage forms for phase 1 and 2 clinical trials that meet certain quality requirements pertaining to safety, stability, purity, and flexibility in dosing via different routes of administration.

In general, mAbs are robust molecules and are mostly formulated as liquid and filled in vial or prefilled syringe (PFS) presentations. In contrast, many of the antibody-based novel formats (ADC, bispecifics, diabodies, etc.) are vulnerable to several physical and chemical instabilities, have limited stability in liquid state, and usually end up in lyophilized presentations for commercial, especially if they are intended to be administered intravenously. For ADCs in particular, the properties of the payload and linker may alter the intrinsic properties of the parent mAb, which will affect the physical and chemical stability of the molecule.

As a result, for molecules intended for liquid presentation for commercial distribution, the development of a frozen liquid drug product for early-stage clinical trials is recommended. This is mainly because long-term stability in the frozen state is almost guaranteed for most molecules and also because the development time can be

too short that the development of a liquid formulation for FIH is not feasible. On the other hand, for modalities that will be eventually presented as lyophilized powder for commercial, development of a lyophilized product for FIH is typical. The benefit of using this two-way approach is that the relevant experience and stability data collected over 2–3 years of clinical studies will be relevant and useful to help guide further commercial formulation and process development and optimization. Additionally, it aligns with the implementation of a platform approach for analytical methods, as well as process development, for both the drug substance and the drug product. Moreover, it can lessen the burden of performing comparability studies. The overall approach of formulation development for FIH studies is outlined in Fig. 2.

Frozen Liquid Drug Product

A frozen liquid formulation shares several components with that of a liquid formulation in order to achieve sufficient stability during processing and storage of the drug substance, as well as during fill and finish operations. The solubility and stability of the proteins are greatly influenced by the pH of the drug product solution. Accordingly, pH should be the first parameter selected in the design of the formulation. For antibodies that have a pI in the range of 8–9, a pH in the range of 5.5–6.5 generally provides suitable solubility of the protein. For certain modalities, especially at high concentrations, the addition of salts or other solubilizers may also be required to improve solubility. In accordance, a review of the information in the public domain shows that many of the commercialized mAbs are formulated at pH $\leq$ 6.5 (Table 3, data acquired from the PharmaCircle database). Therefore, a pH in the range of 6.0–6.5 can be adapted for early development. In this pH range, L-histidine has been commonly used as a buffering agent as it has a pKa of 6.0 and, thereby, will have maximum buffering capacity at the desired pH range. Moreover, L-histidine does not pose pH shift issues during freezing and thawing as is the case with some buffers including sodium phosphate.

To protect the protein against liabilities arising from exposure to different solution and process conditions, and to be able to achieve frozen storage stability as well as enough stability at room temperature ($\geq$7 days) and 2–8 °C ($\geq$2 weeks) for processing during manufacturing, the addition of disaccharides and/or polyols is typical. Sucrose and sorbitol have been widely used in both liquid and lyophilized formulations of several protein drug products. Both are also effective cryoprotectants that protect proteins against freezing-induced denaturation. They are also preferentially excluded from the surface of the protein, which helps protect it against conformational instability in the solution state. Sucrose and sorbitol at concentrations of approximately 8–9% (w/v) and 5% (w/v), respectively, usually provide sufficient protection and appropriate isotonicity.

Similar to mAbs, a formulation strategy for ADCs is to preserve the hydration state, charge distributions, and higher-order structure. Formulations that optimize pH, low salt concentrations, and carbohydrates may provide adequate stability [31]. But, other excipients may also be considered due to the hydrophobic nature of the linker-

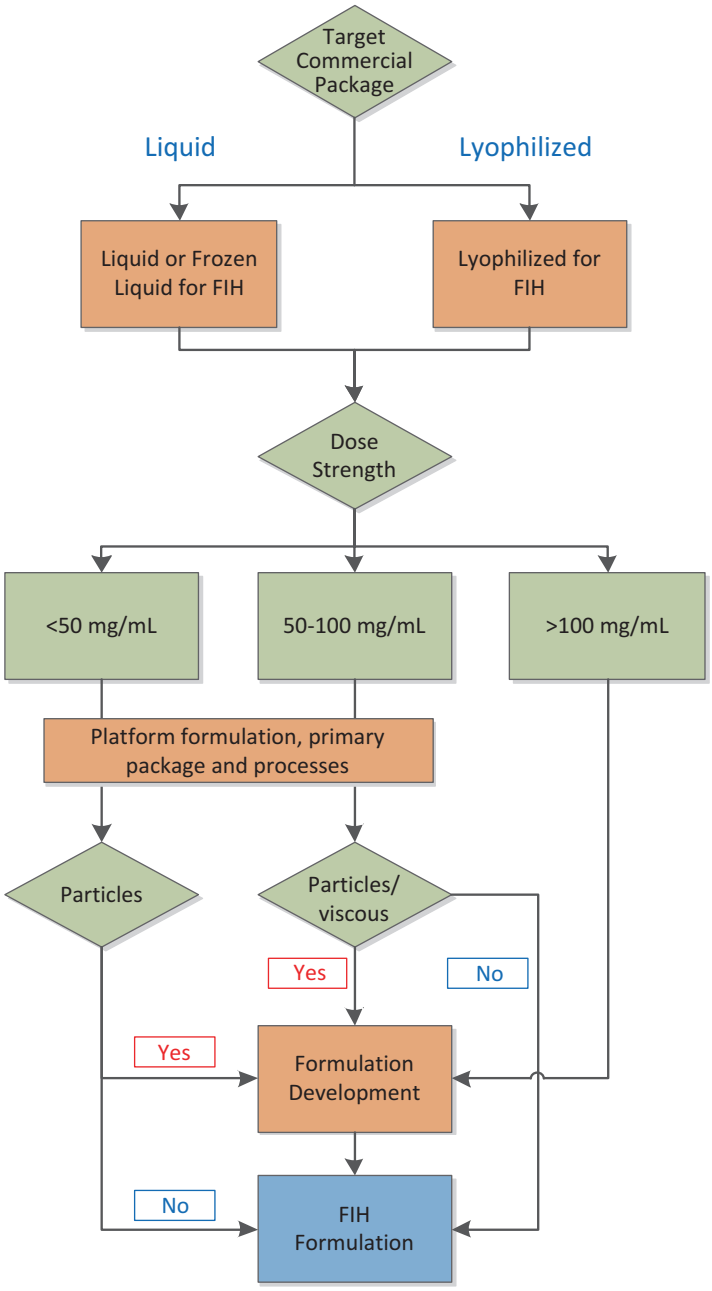

Fig. 2 General outline of the platform approach for early-stage development

Table 3 Formulation compositions and general properties of a partial list of the commercialized monoclonal antibodies or related modalities

Product name	Dosage form	Concentration mg/mL	pH	Formulation
KEVZARA (sarilumab)	Liquid	200	6.0	Each prefilled syringe or prefilled pen delivers 1.14 mL (200 mg or 150 mg) of KEVZARA in a solution containing arginine (45 mM), histidine (21 mM), polysorbate 20 (0.2% w/v), sucrose (5% w/v), and water for injection
ILARIS (canakinumab)	Lyophilized	150	~6.2	92.4 mg/mL sucrose, L-histidine and L-histidine HCl, 0.6 mg/mL polysorbate 80
XOLAIR (omalizumab)	Lyophilized	125	6.0	Clear to opalescent, 125 mg/mL omalizumab, 90 mg/mL sucrose, 1.7 mg/mL L-histidine hydrochloride monohydrate, 1.1 mg/mL L-histidine, 0.3 mg/mL polysorbate 20
REPATHA (evolocumab)	Liquid	140	5.0	Clear to opalescent, colorless to pale yellow solution, 140 mg evolocumab, acetate (1.2 mg), polysorbate 80 (0.1 mg), proline (25 mg) in water for injection, USP. Sodium hydroxide may be used to adjust to a pH of 5.0
RAPTIVA (efalizumab)	Lyophilized	100	~6.2	Approximately 82 mg/mL sucrose, 4.5 mg/mL L-histidine hydrochloride monohydrate, 2.9 mg/mL L-histidine, 2 mg/mL polysorbate 20
SYNAGIS (palivizumab)	Lyophilized	100		47 mM histidine, 3 mM glycine, 5.6% mannitol
SIMPONI (golimumab)	Liquid	100	~5.5	0.88 mg/mL histidine and histidine HCl monohydrate, 41 mg/mL sorbitol, 0.16 mg/mL polysorbate 80
STELARA (ustekinumab)	Liquid	90	5.7–6.3	1 mg/mL L-histidine and L-histidine HCl, 76 mg/mL sucrose, 0.04 mg/mL polysorbate 80
HUMIRA (adalimumab)	Liquid	50	~5.2	6.2 mg/mL sodium chloride, 0.86 mg/mL sodium citrate, 1.3 mg/mL citric acid monohydrate,12 mg/mL mannitol, 1 mg/mL polysorbate 80
CAMPATH (alemtuzumab)	Liquid	30	6.8–7.4	8 mg/mL sodium chloride, 1.44 mg/mL dibasic sodium phosphate, 0.2 mg potassium chloride, 0.2 mg/mL monobasic potassium phosphate, 0.1 mg/mL polysorbate 80, 0.0187 mg/mL disodium edetate dihydrate
AVASTIN (bevacizumab)	Liquid	25	6.2	60 mg/mL trehalose, 5.8 mg/mL sodium phosphate monobasic monohydrate, 1.2 mg/mL sodium phosphate dibasic anhydrous, 0.4 mg/mL polysorbate 20

(continued)

Table 3 (continued)

Product name	Dosage form	Concentration mg/mL	pH	Formulation
HERCEPTIN (trastuzumab)	Lyophilized	21	~6	Approximately 20 mg/mL a,a-trehalose dihydrate, 0.5 mg/mL L-histidine HCl, 0.32 mg/mL L-histidine, 0.09 mg/mL polysorbate 20, 1.1% bacteriostatic water
VECTIBIX (panitumumab)	Liquid	20	5.6–6.0	5.8 mg/mL sodium chloride, 6.8 mg/mL sodium acetate
ROACTEMRA (tocilizumab)	Liquid	20	6.5	15 mM phosphate, 50 mg/mL sucrose, 0.5 mg/mL polysorbate-80
ARZERRA (ofatumumab)	Liquid	20	6.5	8.55 mg/mL sodium citrate, 0.195 citric acid monohydrate, 5.85 mg/mL sodium chloride
SOLIRIS (eculizumab)	Liquid	10	7.0	Each 30 mL vial contains 300 mg of eculizumab, polysorbate 80 (6.6 mg) (vegetable origin), sodium chloride (263.1 mg), sodium phosphate dibasic (53.4 mg), sodium phosphate monobasic (13.8 mg), and water for injection, USP
LUCENTIS (ranibizumab)	Liquid	10	5.5	10 mM histidine HCl, 10% trehalose, 0.01% polysorbate 80
REMICADE (infliximab)	Lyophilized	10	~7.2	50 mg/mL sucrose, 0.05 mg/mL polysorbate 80, 0.22 mg/mL monobasic sodium phosphate monohydrate, 0.61 mg/mL dibasic sodium phosphate dihydrate
RITUXAN (rituximab)	Liquid	10	~6.5	9 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80
ZENAPAX (daclizumab)	Liquid	5	~6.9	3.6 mg/mL sodium phosphate monobasic monohydrate, 11 mg/mL sodium phosphate dibasic heptahydrate, 4.6 mg/mL sodium chloride, 0.2 mg/mL polysorbate 80
SIMULECT (basiliximab)	Lyophilized	4	6.5	1.4 monobasic potassium phosphate, 0.20 mg/mL disodium hydrogen phosphate (anhydrous), 0.32 mg/mL sodium chloride, 4 mg/mL sucrose, 16 mg/mL mannitol, 8 mg/mL glycine
ERBITUX (cetuximab)	Liquid	2	7.0–7.4	8.48 mg/mL sodium chloride, 1.88 mg/mL sodium phosphate dibasic heptahydrate, 0.42 mg/mL sodium phosphate
REOPRO (abciximab)	Liquid	2	7.2	10 mM sodium phosphate, 150 mM sodium chloride, 0.001% polysorbate 80

drug combination. Therefore, the goal of formulation development is to disrupt the hydrophobic effect of the combination. The introduction of a highly hydrophobic linker-drug combination may include the addition of excipients such as cyclodextrins and/or surfactants which may improve the colloidal stability. The properties of cyclodextrin make it suitable for forming an inclusion complex with the payload.

The third key component of the frozen formulation is polysorbate (or other similarly functioning surfactants), which serves as a surface-active agent and/or chaperone to minimize surface denaturation at the different interfaces (air-water, ice-water, or solid-water) that a protein typically encounters (i.e., at the surface of the container and the liquid or the surface of ice during freezing and thawing). For example, polysorbate 20 and 80 have been used in protein formulations and have been shown to play a critical role in frozen drug products to protect against denaturation at the ice interface in the drug substance, as well as in the frozen drug product.

In general, the frozen drug product presentation provides good flexibility to deliver a wide range of dosage strengths for FIH studies by using different fill volumes in vials with different sizes taking into consideration the risk of vial breakage. In this vein, a process/primary package design space obtained from historical data and prior knowledge is important for the successful development of a frozen drug product. For example, knowledge of the safe range of fill volume and freezing temperatures to be used for each vial size is critical to the development of the freezing process of the drug product vials.

For early-stage clinical trials, a dose strength of 100 mg per vial is preferred for dosing convenience. For that, a 3–5 mL fill in a 10 or 20 mL glass vial, respectively, is suggested to avoid vial breakage. In addition, the impact of the freezing temperature and storage time on the container closure integrity (CCI) of the vial is also important to define. Once a platform design space for the freezing process and the primary container configuration is established, a platform formulation of the components described above can then be determined for most antibodies and related modalities.

Lyophilized Drug Product

In general, the platform formulation used for the frozen liquid drug product can also be used for lyophilization with or without some modifications depending on the dose strength. For target protein concentrations more than approximately 25 mg/mL, the frozen drug product formulation can be used as is. In addition to their role as cryoprotectants, sucrose and trehalose may also be used as lyoprotectants to stabilize proteins during and after drying. For more potent modalities having low concentrations (e.g., <20 mg/mL), the addition of a crystalizing excipient like mannitol or glycine can be used to make the lyophilization process efficient and robust for scale-up and technology transfer while providing mechanical strength to the cake against shipping related stresses. As such, two platform formulations can initially be considered for FIH studies. For more details on the development and optimization of a lyophilization formulation and process, see chapters "Development of Robust Lyophilization Process for Therapeutic Proteins: A Case Study" and "Peptide Drug/Device Combinations"of the book.

Shipping Simulation Study

Physical stresses due to freezing and thawing, adsorption at interfaces, agitation-induced instability, and shipping could potentially lead to physical instabilities such as aggregation and subvisible and visible particle formation. In the experience of the authors as well as other published work, the effects of these stresses may not become apparent until the drug product has been placed on storage stability for some period of time [32]. Therefore, in order to further understand the protective effects of surfactants (mainly polysorbate) on protein stability and product quality, simulated shipping stresses are recommended to be exerted on the drug product primary package prior to shelf-life stability studies.

In order to save time on the development and optimization of a commercial formulation at later stages, it is critical to perform small-scale agitation or shaking studies and/or subjecting the product to transport simulation using the FIH/early-stage formulation both in vials and syringes (if applicable) before placing them on long-term stability. This approach enables an early determination of the propensity of the molecule to form particles and/or aggregates upon exposure to and/or interaction with the siliconized glass surfaces of vials and in PFS during transportation. The transport simulation study should include temperature, pressure, and vibration profiles that mimic standard ground and air transport conditions. As such, this data set can help guide the future course of the commercial formulation development.

3.2 Strategy for Late-Stage (Commercial) Formulation Development

Late-stage development typically starts around the time the molecule will be advanced to Phase 2 trials. Timelines may vary depending on the therapeutic indication and the length of the trials and also depending on whether or not the molecule is on accelerated path. As in early stage, the primary goal of late-stage development is to determine the optimal drug formulation composition and primary container configuration that can maintain the efficacy and safety of the drug as it is processed during the different drug substance and drug product operations through administration to the patients. For a late-stage program, however, this goal requires extensive knowledge of the drug product including its physicochemical properties, potential interactions with the excipients, raw material characteristics and variability, properties of the different materials used for administration in the different regions, and how these variables might impact the target product profile (TPP). Therefore, it is critical that late-stage formulation development starts with the definition of the commercial TPP and builds upon the knowledge gained in earlier stages [30].

Typically, a commercially viable drug product formulation should have at least 2 years of shelf-life stability at 2–8 °C and should be robust against the different stresses encountered during manufacturing due to freezing and thawing, sterile filtration, filling, inspection, packaging, and transportation. Additionally, if the product is designed for administration by the patient or caregiver at home, it may

encounter additional stresses including temperature excursions, room temperature storage, light exposure, and other unknown patient compliance issues. Together, these stresses may lead to changes in the safety and efficacy profiles. Degradation pathways are not expected to be the same for each molecule and are dependent on several physicochemical characteristics such as hydrophobicity, amino acid sequence, pI, and the target concentration. For example, in formulations of high concentration antibodies, self-association, opalescence, and viscosity are usually the major challenges, whereas in low concentration formulations, the typical issues include dose accuracy, air-water interface effects, and adsorptive surface loss. Such problems can be exacerbated by the properties of the target container closure system being a vial or PFS. In light of the complexity of these interacting factors, the importance of identifying these challenges and devising strategies to mitigate them early on can't be overstated. In this section, a general approach for development of commercial formulations is outlined. The focus is on the development of a liquid formulation in a vial or PFS presentation. The development of a lyophilized formulation is discussed in chapter "Development of Robust Lyophilization Process for Therapeutic Proteins: A Case Study" of the book.

Step 1: Analytical Characterization and Degradation Pathways Elucidation

Key analytical assays must be evaluated and developed prior to the initiation of the formulation development studies. Since mAbs are homogenous in nature, FIH and/or platform assays can be employed for early formulation stability studies. Concurrently, optimized molecule-specific assays are developed using forced degradation samples. In early stages, forced degradation studies are performed to elucidate the main degradation pathways of the molecule, to identify and develop appropriate analytical assays to characterize the degradants, and to conduct preclinical toxicology studies. Similar studies can be performed in later stages with the scope of developing and validating the stability-indicating assays for the commercial presentation.

In this vein, the guidelines of the international council for harmonization (ICH) (ICH Q1A (R2), ICH Q1B, ICH Q5C) and the FDA guidance for industry "INDs for Phase 2 and Phase 3 Studies, Chemistry, Manufacturing and Controls Information" indicate that stress conditions should be used to identify the stability-indicating assays. Such studies can be performed on a pure (minimal buffer solution) representative drug substance. Notably, an excessive level of degradation may not be representative of degradation under normal manufacturing and storage conditions and may complicate the findings of the studies.

Examples of the quality attributes to monitor in these studies include some or all of the following: primary structure, size variants, charge variants, oligosaccharide profile, glycation, PEGylation, and potency. These attributes can be monitored following the exposure of the formulation to several stress conditions or agents such as

elevated temperature, relevant freezing and thawing conditions, extreme pH conditions, oxidizing (chemical agents such as hydrogen peroxide or relevant metals), light and UV exposure, and relevant agitation conditions.

The aforementioned conditions should be defined case by case for the commercial product. In these studies, the use of reference material and several time points to understand the kinetics degradation is highly recommended. Examples of the recommended stress conditions are listed in Table 4; Fig. 3.

Step 2: FIH Formulation Assessment

Prior to the initiation of commercial formulation development activities, storage stability data collected throughout the duration of the clinical studies should be reviewed. Based on the clinical data, the protein concentration and the dose strength per container for commercial oftentimes change from those used for FIH studies. If the FIH stability data shows that there are no liabilities, one can proceed to evaluate the feasibility of using the FIH formulation for the new target protein concentration, as long as the concentration remains within a reasonable range, and the formulation is suitable for the commercial primary container configuration and route of administration. If no liabilities are observed in the feasibility studies, then upfront manufacturability and robustness studies can be performed, and a commercial formulation is recommended.

If issues with any of the pCQAs arise when using the FIH formulation, extended development work should be performed. A general outline of the systematic approach followed for the extended development phase to identify a liquid commercial formulation for antibodies is discussed in the following sections. These studies are designed to establish the robustness of the selected formulation following a sequence of univariate experiments. In this chapter, design-of-experiment (DOE)-based robustness studies will also be discussed. The reader is also referred to Chap. 13 for an in-depth discussion of the use of high-throughput methods in formulation development using multivariate studies.

Table 4 Example of the typical conditions used for forced degradation studies

Temperature conditions	Typical oxidation agents	Agitation conditions	Light conditions	pH extremes
Temperature: 25 °C Time points: 0, 1,2, 6, 12 weeks Temperature: 40 °C Time points: 0, 1, 2, 4, 8 weeks	Agent: 0.1–1% H_2O_2 Temperature: 25 °C Time points: 0–3 days	Speed: 200–400 rpm Temperature: 2–8 °C Time points: 24 hours	ICH Q1B Temperature: 25 °C Light: NLT 1.2 million LUX hours	pH: 3 and 11 Temperature: 40 °C Time points: 0, 1, 2, 3, 7 days

Adapted from Ref. [33]

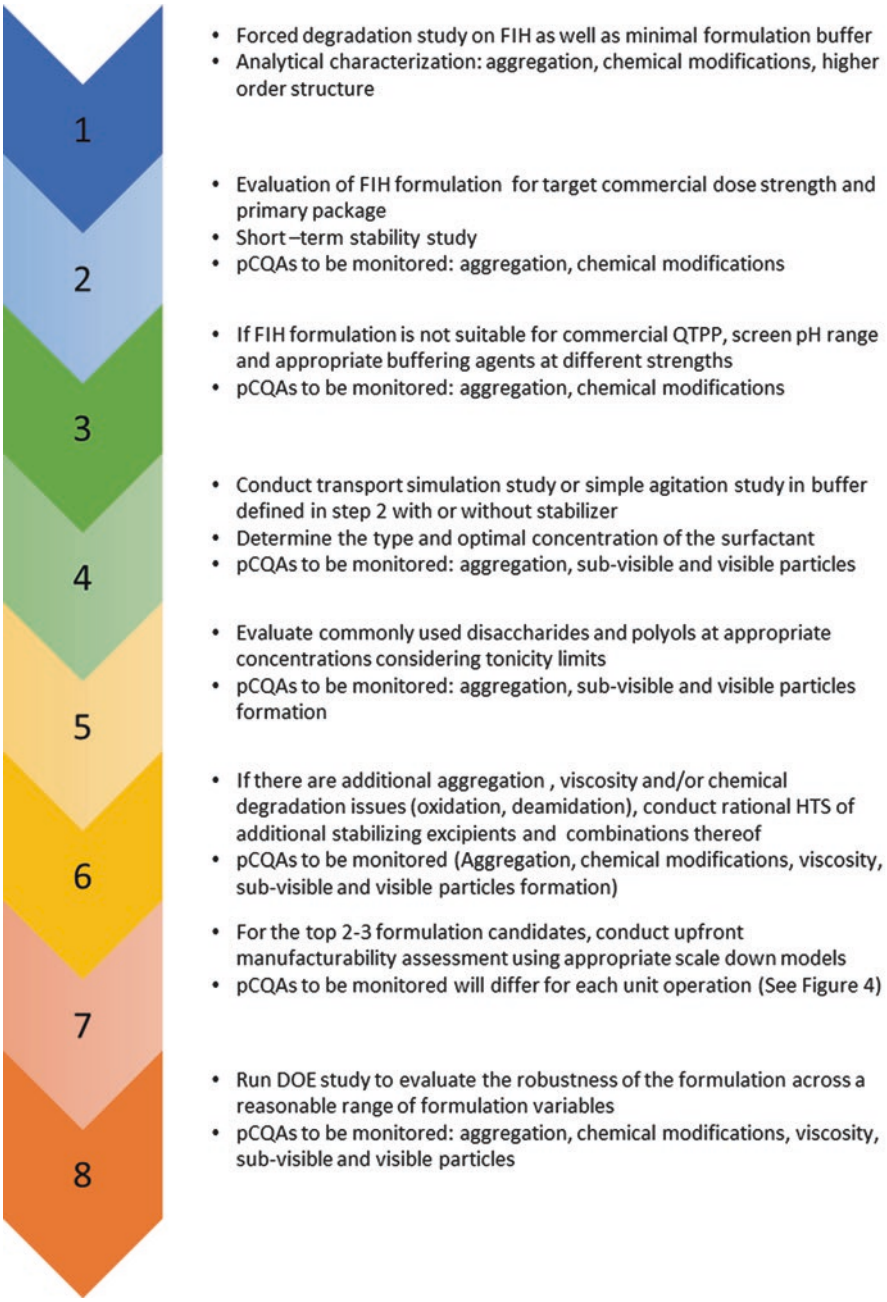

Fig. 3 General outline of the workflow for late-stage formulation development

Step 3: pH and Buffer Screening Study

The solubility, stability, and viscosity of a protein solution are significantly affected by the pH of the solution. At different pH values, the ionization state of the charged amino acid groups changes and may interfere with the electrostatic interactions required for maintaining the native protein folding [34, 35]. Also, various pathways of chemical degradation such as deamidation are very sensitive to changes in the solution pH. Therefore, it is important to identify the optimal pH range, buffer species, and ionic strength at an early stage.

To this end, a pH study in the range of 4–7.5 with increments of 0.2–0.5 units should be performed while keeping other formulation components (such as protein, stabilizer, and surfactant concentration) constant. The study can also be conducted in a simple buffer without other excipients depending on prior knowledge of the molecule. Selection of a buffer species with pKa value close to the target pH in the concentration range of 15–25 mM is usually sufficient. At high concentrations, proteins also act as buffering agents, which in some cases may eliminate the need for using a buffer.

Selection of the buffer species may also depend on the chosen delivery route. For example, citrate buffer may cause a stinging effect during subcutaneous injection. Buffering agents like acetate, succinate, glutamate, histidine, and phosphate have pKa values that cover the pH range typically used in formulations and are commonly used in drug products. Since changes may not always be initially observed, a short-accelerated stability study at different temperatures should be set up to evaluate such effects. The outcome of this study would be the identification of the optimal pH range and buffering agent concentration to be used for the following studies.

Step 4: Agitation Study

Protein molecules have the propensity to denature and/or form aggregates and particles upon shaking in vials or PFS due to exposure to the air-water interface and protein interactions with glass or siliconized surfaces. The denatured protein can act as a nucleus for the formation of larger aggregates and particles.

If the results of the transport simulation study performed during early development (if any) suggest that the molecule has a propensity to form aggregates and/or particles, a more extensive systematic agitation study should be performed to define the concentration of polysorbate that is needed to protect the protein against such stress. Other surfactants such as poloxamers can also be evaluated as an alternative if polysorbate causes oxidation issues. A simple setup that involves an orbital shaker and a speed of approximately 200–400 rpm with and without siliconized glass beads can be used to screen a range of polysorbate concentrations as has been described previously [32].

Step 5: Selection of Stabilizer

As indicated earlier, the main objective of the forced degradation study is to thoroughly understand degradation pathways of the protein, which enables the formulation scientist to devise appropriate stabilization approaches. Disaccharides, amino acids, and polyols are examples of commonly used stabilizers that are used to protect proteins against various stresses. Among these stabilizers, sucrose, trehalose, and sorbitol serve as cryoprotectants, tonicifying agents, as well as preferentially excluded solutes that enhance conformational stability [23, 24]. Although higher concentrations of these excipients may be beneficial, the solution tonicity, especially for subcutaneous administration, should typically be limited to 270–330 mOsm/Kg. Although sucrose has been widely used in marketed products, trehalose can also be used at low pH values where sucrose undergoes hydrolysis.

In this study, the pH and buffer species and the polysorbate concentration determined in the preceding studies are used as formulation constants. Also, the FIH formulation can be included as a reference for direct comparison with the performance of the new formulations. Correlations between the accelerated and long-term stability data of the FIH formulation can be helpful in evaluating the commercial formulations. The degradation profiles of the antibody in the different formulations for up to 3 months of shelf-life can then be used to define the top candidates to be moved forward.

Step 6: Extended Formulation Studies

In certain cases where one or more instabilities are observed in the preceding studies and could not be mitigated by optimizing the pH and buffering species, sugar concentration, and/or surfactant type and concentration, extensive systematic screening of formulations is then triggered. In this subsection, common challenges and appropriate mitigation strategies are described.

Viscosity Study

Development of a commercial formulation of high protein concentration is generally hindered by high viscosity and the propensity for reversible self-association. Such colloidal instability may impact fill and finish operations and syringeability. High viscosity also negatively impacts the manufacturing of the drug substance during UF/DF operations, where the protein may be concentrated by approximately 1.3–1.5-fold above the target concentration and then diluted back for the final compounding step. Whenever possible, a recommended upper limit for UF/DF operations is approximately 10 cP at 20–25 °C, and for delivery through an auto-injector, the upper limit should be approximately 6 cP.

Over the past three decades, substantial work has been done to understand the mechanisms underlying the high protein concentration-induced colloidal instabilities and the possible approaches to mitigate them. The interaction parameter (K_D) and the second virial coefficient (B_{22}) are commonly used to quantitate the overall net pro-

tein-protein interactions leading to self-association and high viscosity. Typically, K_D is most negative (i.e., attractive interactions) near the pI and more positive away from pI (i.e., repulsive interactions) [25]. Yet, at pH values far from the pI, the addition of salts renders intermolecular interactions less repulsive due to charge screening.

Several studies investigated the effects of several small-molecule excipients on viscosity, aggregation, and stability of model IgG1 antibodies [36–39]. These excipients include several amino acids and their salt forms (alanine, proline, valine, glycine, serine, histidine HCl, lysine HCl, arginine HCl, and sodium glutamate) and several salts (sodium chloride, sodium acetate, sodium sulfate, and ammonium chloride). The results of these studies showed that charge-charge interactions between the antibody molecules are the key determinants of the high viscosity. The counterion of the amino acid may also play a significant role in modulating viscosity.

Because a large variety of salts and excipients can be used to reduce viscosity and improve both solubility and stability, a simple design of the viscosity study is needed to overcome throughput issues, especially in the absence of an automated high-throughput system. The simplest approach is to use a concentrated solution of the antibody in minimum buffer at the target pH value or range and dilute back into concentrated stock excipient buffers to achieve the target protein and excipient concentrations. In this study, the stabilizer and surfactant concentrations will be fixed based on the preceding studies. Lyophilization may also be used as a concentration method wherein the formulation including polysorbate, buffer, and sugar is lyophilized in small vials and then reconstituted to the target protein and excipient concentrations using stock excipient solutions. This approach may eliminate the stress of concentrating the antibody to levels significantly higher than the target concentration. Standard stability-indicating assays should then be used to evaluate stability, solubility, and viscosity after incubation for short period of time at different accelerated and stress conditions. Moreover, evaluation of the syringeability and break loose and gliding forces (BLGF) should be conducted to verify the suitability of the target formulation viscosity at relevant temperature conditions. Furthermore, scale-down filling studies should also be conducted on the top formulation candidates to confirm the suitability of moving them forward.

Oxidation

Several amino acids in the protein primary sequence such as cysteine, histidine, tryptophan, and tyrosine are susceptible to oxidation with the fastest rates typically observed for methionine. The sulfur atom in methionine residues can be oxidized in the presence of reactive oxygen species into methionine sulfoxide in a reversible manner. The oxidation of methionine can potentially impact the bioactivity of the antibody, as well as elicit unwanted immunogenic responses [40]. In IgG1 antibodies, oxidation of two conserved methionine residues located at the interface of the CH2 and CH3 domains can decrease thermal stability [41, 42], protein A binding [43, 44], FcRn binding [43–45], and circulation half-life of IgG1 antibodies [46]. In addition, oxidation of methionine residues in the complementarity determining regions (CDRs) could impact antigen binding and hence the efficacy of the molecule. Accordingly, it is essential to characterize the effect of methionine oxidation

(if any) on the structure, stability, and biological activity of the antibody in the final drug product configuration.

In this regard, it is important to first investigate the root cause of oxidation and whether it occurs during storage, upon exposure to metal surfaces, is induced by oxidative degradants of polysorbate, or occurs due to exposure to light. Inclusion of antioxidants, such as methionine, sodium thiosulfate, ascorbic acid, BHT, BHA, sodium bisulfite, glutathione, and propyl gallate, is common in parenteral products of small molecules to protect the drug against oxidation by scavenging free radicals and/or dissolved oxygen. In therapeutic protein products, however, methionine has been the most commonly used antioxidant. The minimum effective levels (molar ratios of protein to antioxidant) that inhibit temperature-induced oxidation are generally observed to be 1:5 and 1:25 for methionine and thiosulfate, respectively.

In addition to using antioxidants, filling the vial headspace with nitrogen and implementing tight control strategies on the impurities in raw materials, especially polysorbate, are also commonly used strategies. The oxidation study is, therefore, recommended to be conducted in the commercial primary package using the formulation components identified in the preceding studies, and testing should be conducted after exposure to relevant oxidation stress conditions such as hydrogen peroxide, UV light, relevant LUX hours, and relevant amounts of appropriate transition metals.

Deamidation

Deamidation is another common chemical modification of antibodies that may impact the efficacy of the molecule. The deamidation reaction converts asparagine or glutamine to aspartate or glutamate, respectively, via a succinimide intermediate. Isomerization then follows and forms iso-aspartate or iso-glutamate, respectively. Deamidation and isomerization are pH dependent, as well as sequence dependent. For example, the deamidation rate is fastest at high pH values and at asparagine residues followed by glycine or serine residues. In order to mitigate these reactions, pH optimization and the use of divalent cations are typically tested, with pH being the more effective approach. Deamidation reactions are base-catalyzed and increase between pH 5 and 8, whereas isomerization reactions are acid-catalyzed and occur usually at pH 4–6. Since pH also has a significant impact on solubility and aggregation, it is recommended that deamidation is monitored and that a sweet spot for the pH value is determined early on during development.

Aggregation

Excessive aggregation (>5% HMW species) in the form of RSA or irreversible aggregation can be addressed by optimization of the pH, increasing concentrations of the stabilizer and/or polysorbate within reasonable ranges, and/or the use of other types of surfactants and excipients (e.g., nonpolar amino acids, cyclodextrin, polyanions, etc.). If aggregation is determined to be a critical issue at the target commercial dose strength and primary package configuration, HTS of different

stabilizers and the combinations thereof is recommended as the first step. Data from the preceding studies for pH and buffer screening, surfactants, and stabilizer levels will be helpful to guide the HTS study design. The best 2–3 formulations identified from the HTS study should then be evaluated for their suitability for the intended primary package and route of administration.

Preservatives

In the case of multi-dose formulations, preservatives may have to be added to the formulation to prevent bacterial growth as the primary container is reused several times. Benzyl alcohol is the most common preservative used, but it has been shown to induce protein aggregation in β-sheet proteins similar to antibodies. The optimal concentration of a preservative that effectively inhibits bacterial growth and does not cause protein instability should be evaluated using the final formulation and primary package and by implementing administration procedures and environmental conditions that mimic those used by the patient and/or the caregiver. In addition to benzyl alcohol, m-cresol has also been used in several sterile parenteral products including biotherapeutics.

Step 7: Upfront Manufacturability Assessment

Once formulation screening has been concluded, the top 2–3 formulations are moved forward to the next step to assess their manufacturability. Indeed, formulation screening studies should be designed so that all formulation parameters are kept within ranges suitable for manufacturing operations based on prior knowledge as well as the intended primary package. Nevertheless, upfront assessment of the manufacturability of the formulation using appropriate scale-down models is highly recommended before moving the formulation forward to the engineering runs and primary stability batches.

During the manufacturability assessment studies, each formulation is subjected to a series of unit operations mimicking those encountered by the drug substance and the drug product during manufacturing such as a freezing and thawing, mixing, filtration, holding in stainless steel vessels or disposable bags, filling, inspection, labeling, and secondary packaging (Fig. 4). In doing so, temperature and light exposure of the product should be monitored. Finally, the drug product vials will then be subjected to shipping conditions using a transportation simulation testing system (TSTS) before being placed on accelerated and real-time storage stability or used for clinical in-use studies.

For each unit operation, appropriate scale-down models should be used considering the manufacturing operations at scale. Throughout the process, samples should be pulled before, after, and, in some cases, during the unit operation and analyzed to delineate the impact of each of them on pCQAs. Both PFS and vials should be evaluated as primary containers to collect manufacturability and stability data for the formulations. Depending on the target delivery mode, on-body delivery systems (OBDS) may also be evaluated.

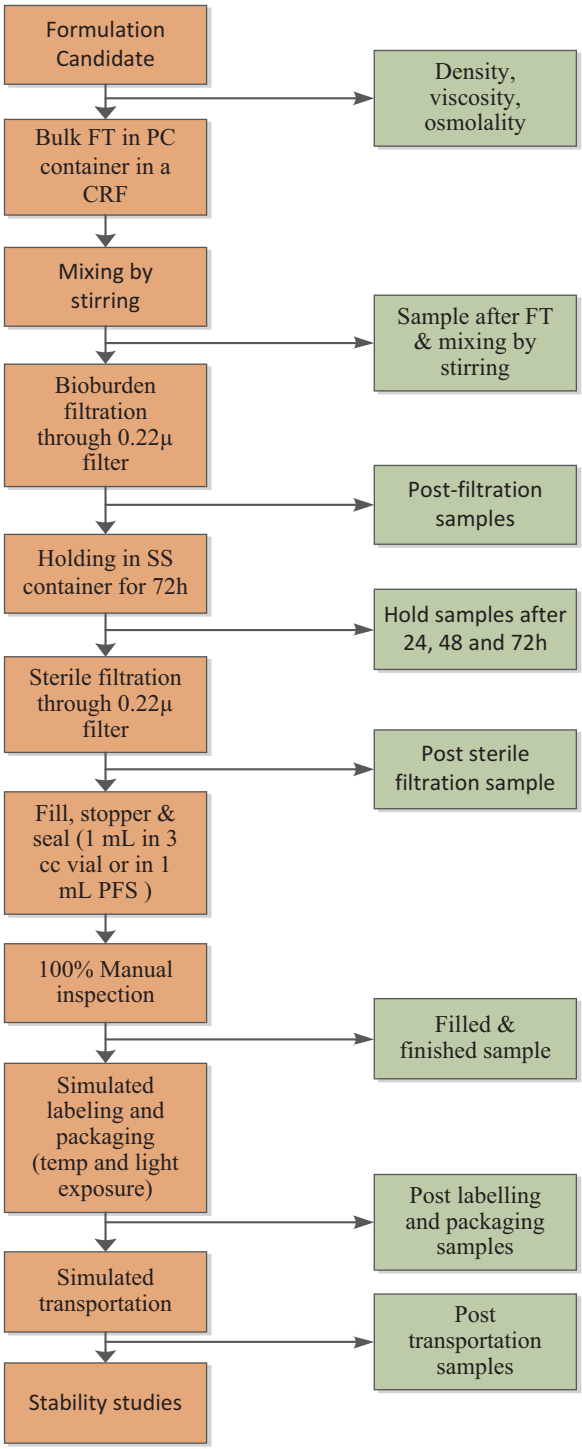

Fig. 4 Flowchart of the upfront manufacturability assessment workflow

In-process and drug product samples from the scale-down studies will be analyzed for relevant pCQAs. The overarching aim of the study is to define the compatibility of the formulation and the manufacturing process to achieve the TPP and to identify and mitigate any liability by fine-tuning formulation and/or process parameters as needed. As such, the commercial formulation recommendation will be based on the manufacturability and stability data (typically 6 months) from this study.

Step 8: Formulation Robustness

The goal of formulation robustness study is to determine the risk of having slight variations in the final drug product composition and configuration on pCQAs as it is processed during unit operations and administration to patients. A preliminary list of the formulation parameters that can impact pCQAs is created and ranked using a risk ranking tool wherein each formulation parameter is evaluated for its main effects, as well as the interaction effects with the other parameters. The main and interaction effect rankings are then multiplied to generate an overall severity score that is then used to determine the type and the number of the studies that need to be performed (for instance, univariate vs. multivariate) [8]. At this stage, it is critical that experts from development as well as from commercial manufacturing work together in order to obtain a meaningful risk ranking.

To illustrate the formulation robustness approach, the following case study is presented [47]. All the formulation parameters that can potentially impact the drug product quality attributes along with the severity numbers are listed in Table 5. Formulation parameters that scored severity ≥8 were categorized as critical parameters. Definitions for the relative impact and rank scoring are outlined in Table 6, the results from the initial risk assessment are summarized in the Table 7, and the corresponding DOE is shown in Table 8. This assessment identified buffer and stabilizer concentrations as parameters to be evaluated in univariate studies. The solution pH and surfactant and protein concentrations were identified as the parameters to be evaluated in a multivariate study.

Target formulation and multivariate study ranges for the antibody are given in Table 9. The stability of the antibody in the formulations was evaluated at 2–8 °C,

Table 5 Scoring criteria for risk ranking

Severity score	Experimental strategy
≥32	Multivariate study
8–16	Multivariate, or univariate with justification
4	Univariate acceptable
≤2	No additional study required

Table 6 Definition of main effect impact and scoring

Impact description	Score
No impact	2
Minor impact	4
Major impact	8

Table 7 Example of a risk ranking matrix of the formulation parameters

Formulation parameter	Proposed design space range		Control space range		Main effect score (M)	Rationale for (M) Main effect	Interaction score (I)	Rationale for (I) Interaction effect	Severity score ($M \times I$)	Potential interaction parameters	Recommended characterization studies
	Low	High	Low	High							
Solution pH (5.3)	4.7	5.6	5	5.6	8	Physical and chemical degradation	4	Physicochemical degradation of excipient and/or mAb, additive effect	32	mAb concentration and polysorbate 20	Multivariate study with temperature
Surfactant concentration (0.01%)	0.005%	0.02%	0.005%	0.015%	8	Physical and chemical degradation	4	Physicochemical degradation of mAb, additive effect	32	mAb concentration and pH	Multivariate study with temperature
Protein concentration (25 mg/mL)	20.0 mg/mL	30.0 mg/mL	22.5 mg/mL	27.5 mg/mL	4	Potential for inadequate efficacy and/or overdose	4	May have additive effect, dripping and drying effects at the nozzle and inside the pump	16	pH, excipients (buffer, surfactant)	Multivariate or univariate with temperature
Buffer species acetic acid/acetate (20 mM)	10 mM	30 mM	15 mM	25 mM	2	Upstream/downstream process ensures tight control	1	N/A	2	None expected	No study
Stabilizer/tonicifier (9%)	5%	13%	7%	11%	2	Potential for HMW <4% or >15% Upstream process ensures tight control	1	N/A	2	None expected	No study

Adapted from Ref. [47]

Table 8 Example of a formulation robustness study DOE

Pattern	pH	Polysorbate 20 (% w/vol)	mAb (mg/mL)	Acetic acid/acetate (mM)	Sucrose (% w/vol)
0000	5.3	0.01	25	0	0
− + + + −	5.0	0.015	27.5	25	7
− + + − +	5.0	0.015	27.5	20	9
− + − − −	5.0	0.015	22.5	20	7
+ − − − −	5.6	0.005	22.5	20	7
− − + − −	5.0	0.005	27.5	20	7
+ + − − +	5.6	0.015	22.5	20	9
+ + + − −	5.6	0.015	27.5	20	7
+ − − + +	5.6	0.005	22.5	25	9
+ + + + +	5.6	0.015	27.5	25	9
− − − + −	5.0	0.005	22.5	25	7
+ − + − +	5.6	0.005	27.5	20	9
− − + + +	5.0	0.005	27.5	25	9
− + − + +	5.0	0.015	22.5	25	9
+ − + + −	5.6	0.005	27.5	25	7
+ + − + −	5.6	0.015	22.5	25	7
− − − − +	5.0	0.005	22.5	20	9
00000	5.3	0.01	25	0	0

Table 9 Example of a formulation design space constructed based on formulation robustness DOE data

		Design space lower limit	Control space lower limit	Target	Control space upper limit	Design space upper limit
Drug substance	pH	4.7	5.0	5.3	5.6	5.9
	Acetic acid/ acetate (mM)	10	15	20	25	30
	Sucrose (% w/vol)	5	7	9	11	13
	Polysorbate 20 (% w/vol)	0.005	0.005	0.01	0.015	0.02
	Protein concentration (mg/ml)	65	65	75	85	85
Drug product	pH	4.7	5.0	5.3	5.6	5.6
	Acetic acid/ acetate (mM)	10	15	20	25	30
	Sucrose (% w/vol)	5	7	9	11	13
	Polysorbate 20 (% w/vol)	0.005	0.005	0.01	0.015	0.02
	Protein concentration (mg/ml)	20	22.5	25	27.5	30

25 °C, and 40 °C for the drug product in the primary package and at −20 °C and 2–8 °C storage conditions for drug substance.

The analysis of the DOE data using the predictive profiler function (Fig. 5) indicates an increase in aggregation upon storage at 40 °C for 3 months and 2 years at 2–8 °C. Aggregation also increased at pH 5.3 and as the protein concentration increased. A formulation design space was constructed based on these results (Table 9) where the design space limits for acetic acid/acetate, sucrose, and protein concentration are within the characterized ranges. As aggregation increased with pH, the upper limit of the design space is set to 5.6.

In this example, the results of the robustness study provided good understanding of the multivariate relationships between the formulation parameters and pCQAs. The results generated using these studies can also simplify the implementation of post-approval changes if needed.

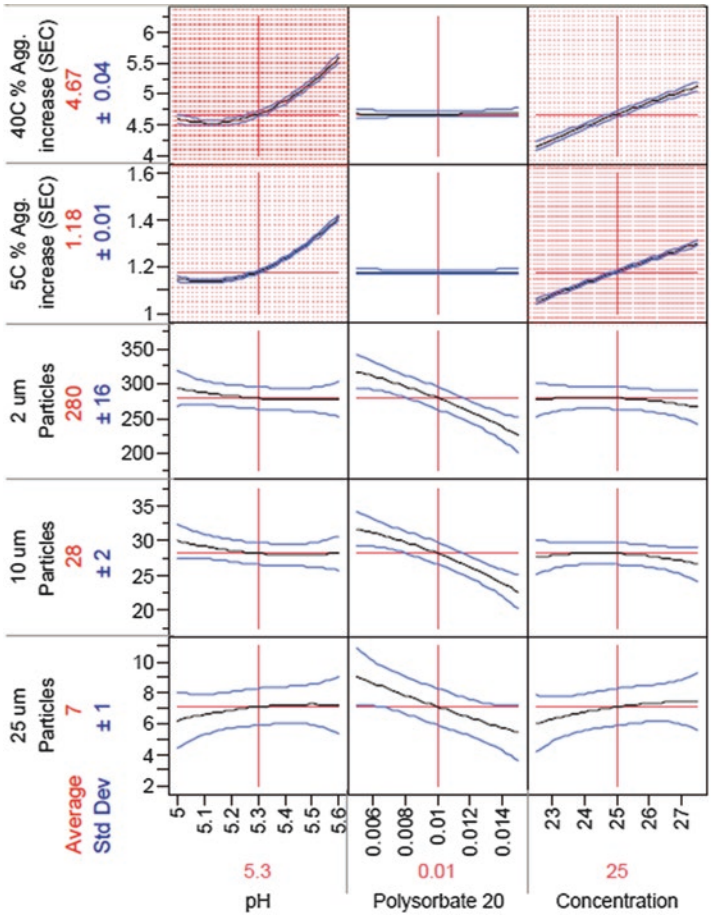

Fig. 5 Prediction profiler depicting aggregation and particulate matter as a function of pH, polysorbate 20 concentration, and protein concentration at 2–8 °C for up to 2 years and 40 °C up to 3 months. (Adapted from Ref. [29])

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Chapter 13
Formulation Development for Biologics Utilizing Lab Automation and In Vivo Performance Models

Michael Siedler, Sabine Eichling, Martin Huelsmeyer, and Jonas Angstenberger

1 Introduction

Formulation development for biologics in the past was predominantly focused on assuring the stability of the drug product during processing and upon shelf life. Nowadays, formulation development is becoming more and more challenging due to the increase in:

- Molecule format diversity, e.g., such as bispecific antibodies and antibody-drug conjugates [1–5].
- Formulation functionality, such as increased dosages (>200 mg of protein), large volume for subcutaneous (s.c.) delivery (>2 mL), [6–7] controlling/increasing bioavailability, and reducing pain sensation of s.c. administration.
- Expectations by authorities to provide substantially more data that would allow to identify and understand the Critical Quality Attributes (CQAs) of a given molecule, which determine the quality, i.e., safety and efficacy of a new drug product.
- Adequate data to calculate the Formulation Design Space and demonstrate non-significant impact of formulation composition on the CQAs within the Design Space.

Figure 1 summarizes how these new challenges in conjunction with the increasing expectations for an improved product and process understanding by following a Quality by Design development paradigm inevitably demand new analytical tools and models capable of generating the required data.

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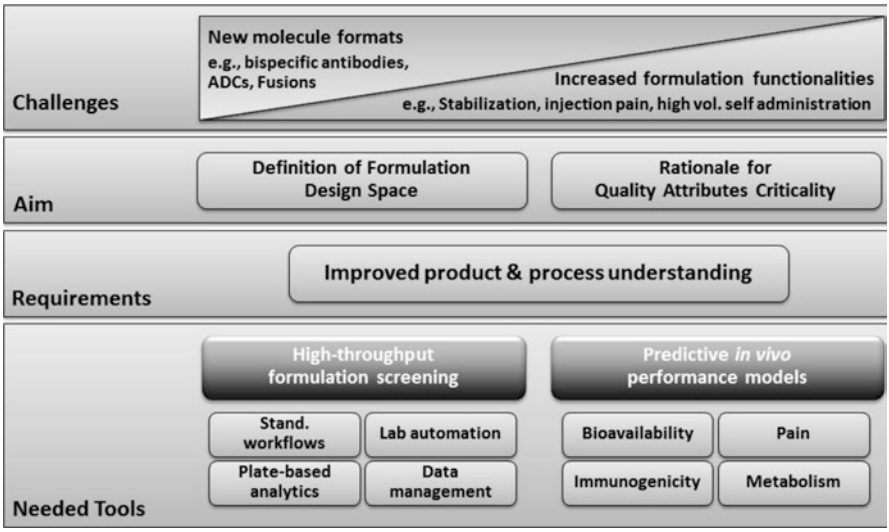


Fig. 1 Implications of enabling Quality by Design in current formulation development

For state-of-the-art biotherapeutic development, two main areas can be identified as a strategic imperative:

- A high-throughput formulation screening platform that would allow for the efficient evaluation of "multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters" to calculate the Formulation Design Space and demonstrate nonsignificant impact of formulation composition on drug product quality within the Design Space [8].
- In vivo performance models that would enable characterizing the formulation based on biological criteria such as pain on injection, in vivo stability/metabolism, bioavailability or immunogenicity of a given molecule construct, or formulation after administration. Furthermore, these models would allow to study and determine the biorelevance of the potential Critical Quality Attributes for a given drug product. Ideally, this would allow for a data-based definition of the CQA criticality.

Therefore, this chapter aims to provide a detailed description of how these tools can be implemented in these two areas and utilized to further improve and complement drug product development.

1.1 QbD in Formulation Development for Biologics

Modern biopharmaceutical development, as outlined by the Quality by Design (QbD) initiative [8–10], is striving for a systematic scientific understanding of the molecule, its mechanism of action, and how possible changes to the molecule dur-

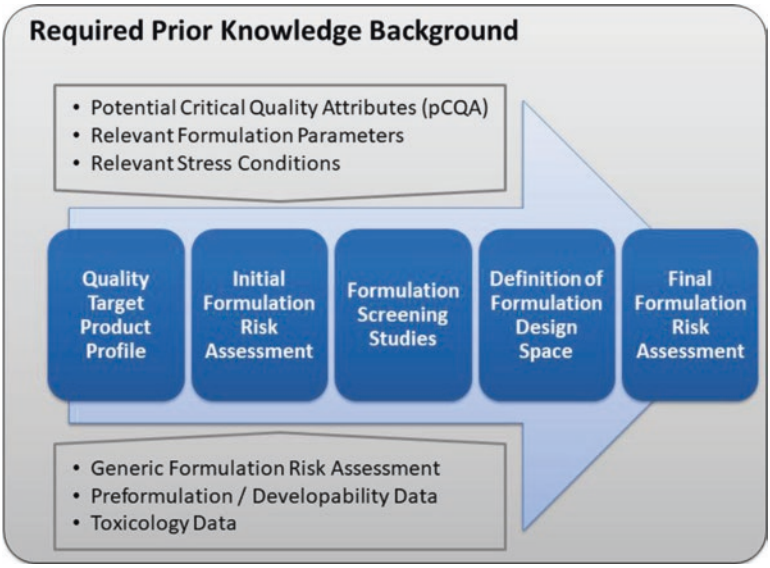


Fig. 2 Overview of a risk-based formulation development process according to Quality by Design

ing drug substance manufacturing, fill-and-finish, product distribution, and administration could alter the safety and efficacy of the final product. Interestingly, implementing QbD in development seems to be mainly focused on the manufacturing process. Quite a few articles focus only on how formulation robustness studies using Design of Experiments can justify a Formulation Design Space [10–12]. There are only a few examples in the literature that describe a holistic approach on how QbD elements can facilitate formulation development [13, 14].

As depicted in Fig. 2, the starting point is always the definition of the Quality Target Product Profile (QTPP) of the drug product, as it relates to quality, safety, and efficacy, considering, e.g., the routes of administration, dosage form, bioavailability, strength, and stability.

The QTPP will summarize all potential Critical Quality Attributes (pCQA) of the drug product, based on Prior Knowledge so that those product characteristics having an impact on product quality can be systematically identified and subsequently studied and controlled.

CQAs are defined according to ICH Q8 (R2) and Q9 as "a physical, chemical, biological or microbiological properties or characteristics that should be within an appropriate limit, range, or distribution to ensure the desired product quality." [8, 9].

In combination with the revised notification MAPP 5016.1 by the FDA Office of Pharmaceutical Quality (OPQ) for Applying ICH Q8 (R2), Q9, and Q10 Principles to the CMC review, it became an expectation to determine the CQA for a given drug product [15].

Due to the complex nature of biologics, the assigned criticality will fall into a "continuum of criticality" that reflects the complexity of structure-function relationships

in large molecules and the reality that there is uncertainty around attribute classification due to their inherent heterogeneity and variety of possible posttranslational modifications [13]. This will become even more complex for novel molecule formats such as bispecific antibodies and antibody-drug conjugates.

Therefore, until there is sufficient toxicological and clinical experience established for a novel molecule, it will be more accurate to utilize the term "potential Critical Quality Attributes" (pCQA) [16].

Nevertheless, based on the available Prior Knowledge such as compendial requirements or by expectations from authorities, certain "standard" or "obligatory" CQAs can be identified for a respective molecule format and dosage form as shown in Fig. 3.

Ideally, they may already being used during molecule engineering to design quality into the molecule [17].

It should be noted that the assigned criticality of a respective CQA might change with the increasing level of scientific understanding during the development. A CQA that has been initially assigned a high severity and likelihood (due to the absence of specific data) can be re-assigned, if the respective data would justify it and vice versa.

Once all potential Critical Quality Attributes for a given molecule have been identified, they can be used for conducting an initial formulation risk assessments to identify all those formulation parameters, manufacturing/primary packaging, and storage conditions that potentially could have an impact on the final product quality. The level of impact on the CQAs will be determined in order to identify gaps of the existing Prior Knowledge. Table 1 provides an example of such an initial formulation risk assessment.

The outcome of this assessment will be utilized to guide the formulation screening studies that are needed to acquire the necessary understanding on the relationship between the respective formulation parameters and the drug product CQAs, in other words to explore the desired Knowledge Space. The results of the formulation screening studies will allow for calculating the Formulation Design Space that will ensure final product quality and will be vital information for defining a suitable Control Strategy during the process development.

As a last step, a final formulation risk assessment needs to be conducted to demonstrate that all risks previously identified are mitigated using the proposed Formulation Design Space and Control Strategy.

2 Enabling QbD in Formulation Development by High-Throughput Screening

The formulation of any given biotherapeutic is of utmost importance to assure the stability of the therapeutic protein during processing, shipment, over shelf life, and upon administration.

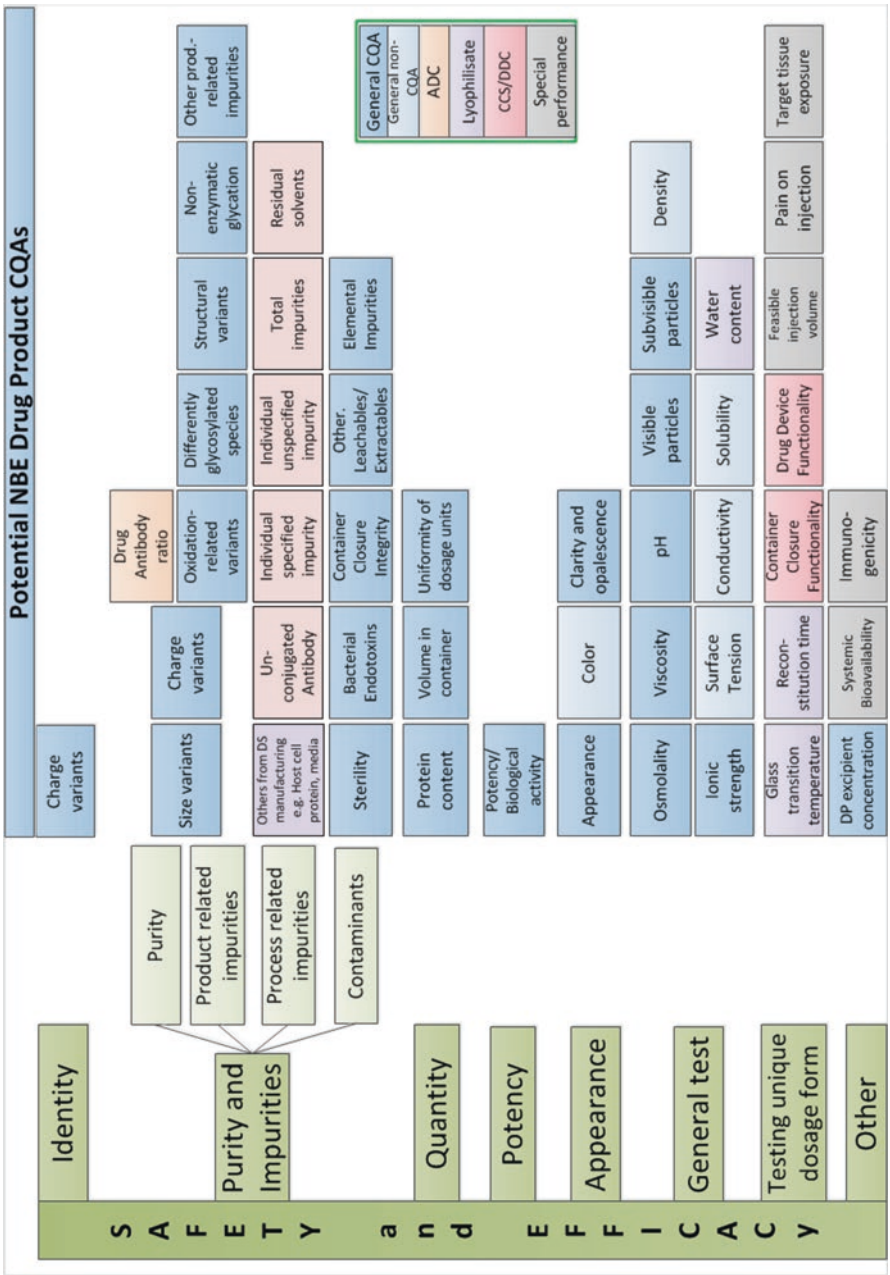


Fig. 3 Overview of potential Critical Quality Attributes (pCQAs) for biotherapeutics. General pCQAs which are applicable to all molecules are highlighted in blue, additional pCQAs specific for ADCs, lyophilisates, or the primary packaging components and devices are highlighted in orange, magenta, or red, respectively. Finally, pCQAs that are affecting the safety and efficacy via a specific biological response are highlighted in gray

Table 1 Example of an initial risk assessment for describing the correlation between the formulation parameter and the respective Critical Quality Attribute by using the risk levels high, medium, low, and none

Criticality Score	7	10	7	7	5	5		10	7	5	10	10	5	5
Critical Quality Attributes / Formulation Parameters	Protein Content	Potency	Mono.	HMW	Frag.	Acidic	Main	Vis.	Subvis.	Clarity	pH-Value	Syringe App. Forces	Osmol.	Visco.
mAb conc.	high	none	med	med	none	low	low	med	med	low	low	med	low	high
pH	none	low	high	high	high	high	high	high	high	high	n.a.	med	low	high
Isotonizer selection	none	low	med	med	low	med	med	med	med	med	none	none	low	med
Isotonizer conc.	none	low	high	med	med	low	low	med	med	med	none	none	high	low
Surfactant selection	none	low	med	med	med	med	med	high	high	med	none	none	low	none
Surfactant conc.	none	low	high	high	high	high	high	high	high	high	none	none	low	none
...														

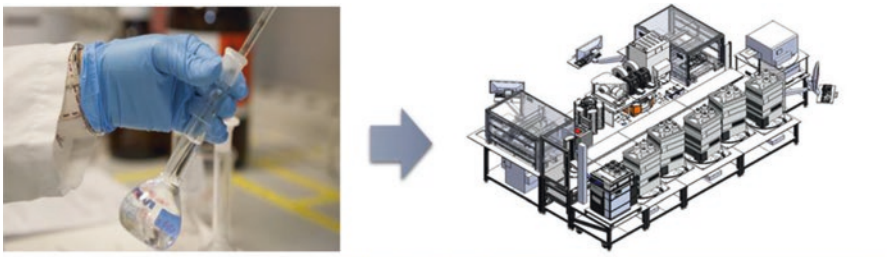


Fig. 4 Comparison of a traditional formulation development and a high-throughput formulation development approach following quality by design

One can even consider modern developability screenings usually implemented as part of the molecule design and candidate selection as a first step in formulation development, since the aim is to identify candidates that minimize stability issues during formulation development [17].

Therefore, such a developability assessment can be considered the first element of a systematic drug product development approach as required by QbD.

When comparing the traditional formulation development with the QbD approach as depicted in Fig. 4, it becomes clear that the fundamental difference is in the amount data required to gain a broad product and process understanding. These data are required not only for identifying an optimal formulation composition but also

for characterizing a much broader area around a potential optimum, i.e., the Knowledge Space that is the prerequisite to define the Design Space which is defined according to ICH Q8(R2) as:

> The multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality. [8]

Consequently, a Formulation Design Space is the outcome of multivariate experiments with relevant formulation parameters such as:

- Protein concentration.
- pH,
- Ionic strength.
- Stabilizer type.
- Stabilizer concentration.
- Surfactant type.
- Surfactant concentration, etc.

Even when utilizing a DoE approach, the required number of formulation compositions that needs to be tested can easily reach up to 100 and above, depending on the number of parameters and levels to be explored.

Since it is simply not possible to generate such amount of data by classical stability studies in the intended primary packaging system, suitable scale-down models are required.

Scale-down models represent an important and well-established element within the QbD paradigm. They are being used throughout drug substance and drug product development to sufficiently characterize the various unit operations, as a prerequisite to define the Design Space. However, as with every model, adequate justification is required to assure that the outcome of a scale-down model is predictive to the intended full scale.

With regard to a formulation screening, this means that there should be no significant and relevant difference in stability whether the formulation will be studied in the selected down-scale container, such as a 96-well plate or in the actual primary packaging materials.

Ideally, rigorous statistical testing will be used for all relevant pCQAs to demonstrate nonstatistical difference between scale down and representative full scale.

There are an astonishing number of examples in the literature that describe how certain biophysical analytical methods can be converted and utilized to enable a high-throughput characterization [18, 19, 20].

Often, these methods are not directly measuring a given pCQA but provide an indirect measure that can be utilized to draw conclusions about the stability of the molecule. Since these methods typically require only minute amounts of material, they are of particular interest during early developability screenings when only very limited amounts of material are being available.

Furthermore, tremendous progress has been made by instrumentation companies not only to improve the sensitivity of analytical methods but also to increase the

throughput of the equipment to get more data with less material in shorter times by using automation, such as for sample exchange.

Interestingly, there are only very few examples that describe a holistic approach on how a transition from a traditional toward a high-throughput formulation development can be achieved by taking advantage of every improving lab automation. A first example was described by scientists from Novartis [21]. It was basically a robotic liquid handling system that included basic analytical methods as in-process controls such as pH measurements. It was utilized to compound the various formulation compositions and also was used to prepare different dilutions required for subsequent analytical characterization. It was also one of the first times that some key elements of advanced lab automation such as automated data handling and data visualization were discussed.

It is remarkable that up to now an integrated fully automated formulation screening that covers the whole process from compounding, applying of stress conditions (e.g., elevated temperature, freeze/thaw, and mechanical stress), analytical characterization, data management, and data evaluation has not been described. It is due to the complexity and flexibility of the required workflows as well as the difficulties in combining the different analytical instruments and their software that did prevent the use of lab automation in formulation screening to the same level as it is standard in other areas such as discovery or bioanalytics.

Therefore, the following section aims to discuss the six key elements that are shown in Fig. 5 required to leverage the full potential of the lab automation for formulation development.

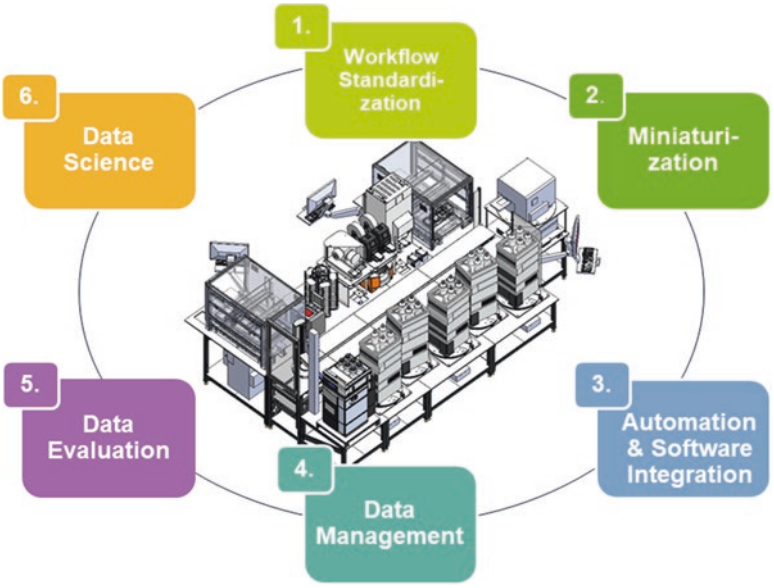


Fig. 5 Overview of the key elements required for an efficient and fully integrated high-throughput formulation screening using lab automation

2.1 Overview of Elements Required for an Automated High-Throughput Formulation Screening

Workflow Standardization

Formulation development for proteins is an expensive, labor-intensive, and time-consuming process. As a result, the biopharma industry has largely adapted a strategy of custom-designed research programs for each new molecule. Given the unique nature of each molecule, a high-throughput platform research strategy is capable of identifying an optimal formulation with less effort and stronger scientific validity and confidence based on the information-rich data set that are generated [19, 21, 22].

This may be accomplished through intelligent study design, but it also depends heavily on high-throughput automation systems for sample preparation and analysis, as well as plate-based assays for analytical testing.

Workflows in an automated or robotized setting require thorough planning as they cannot be changed fast and easy. Such workflows are composed of harmonized subroutines and usually involve multiple devices (such as liquid hander, chromatography system, plate reader, and others) that all need some kind of programming to execute a number of predefined tasks.

To assure a seamless interaction of all components involved, we have introduced standardization into our workflows. In this context, modern high-throughput technology relies on standardized sample containers. We use multiwell plates (MWP) that are available in numerous flavors, but they all have the same footprint to enable robotic handling. Standardization is also required in the succession of stresses and analytics during formulation development screening to limit the effort of programming for highly variable processes.

Screening Design

When it comes to the design of workflows for formulation development of liquid biotherapeutics, it is, of course, important to adhere to established principles. The ultimate goal of pharmaceutical development, including formulation and manufacturing process development, is to consistently deliver a stable and safe product. The formulation parameter of strongest impact on protein stability is undoubtedly the pH value. Therefore, this value needs to be optimized early within the entire process. In a second stage, a range of excipients can be tested for their stabilizing abilities. To limit the number of theoretical formulation compositions, we have defined groups of excipients with similar stabilizing principle (e.g., sugars, sugar alcohols, surfactants, antioxidants, salt, etc.), and only one representative of each group is used for the screening. Furthermore, we limit its concentration to one typical value. This approach leads to a lead formulation that we call “prototype formulation.” In a last stage, the concentrations of all formulation components are optimized in a comprehensive DoE type of screening (together with pH variations) to calculate the Formulation Design Space and demonstrate the formulation robustness.

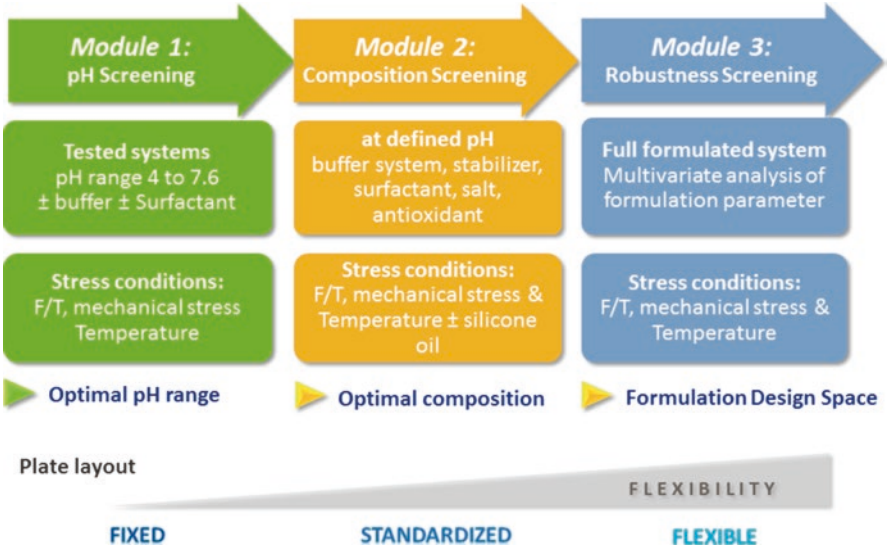


Fig. 6 Modular high-throughput formulation screening concept allows for standardization and flexibility

The outlined three stages in formulation development as depicted in Fig. 6 are the foundation for our modular standardized screening approach. All three modules are performed at least once during product development – modules 1 and 2 usually prior clinical Ph 2 and module 3 after POC.

Module 1 is a *pH screening* in the range of pH 4.0–7.6 in a universal citrate-phosphate buffer that is capable to buffer the entire pH range. Additionally, the protein is formulated in buffer-free medium to simultaneously evaluate the stability behavior under low ionic conditions. The samples are run through a set of standardized stress conditions including freeze/thaw, mechanical, and various temperature stresses to gain a basic understanding about the protein's stability. As the pH varies, the preferred pH range for optimal stability can be deduced from analytical readout. Both buffered and nonbuffered solutions are tested with and without detergent, revealing whether or not detergent is beneficial for stabilization. This screening module is fully standardized, has no flexibility, and is always carried out in the same manner.

Module 2 is termed *composition screening* and investigates a variety of formulation compositions to determine which excipients are most favorable for stability. While holding the pH at the preferred value (module 1), different buffers, stabilizers, detergents, antioxidant, salt, etc. are tested and analyzed. Usually, one representative per excipient group is enrolled. The same stress methods as in module 1 are employed, and additionally, tolerance to silicon oil can be tested. The composition screening results in a lead and several back-up formulations regarding the assessment which composition should be used.

Module 2 has some degree of flexibility as the setup varies depending on module 1 outcomes and specific project requirements that are reflected in the choice of excipients. It is possible to test a lot of different excipient compositions at only one typical concentration to identify those that are particularly suitable, just as well concentration series of only a few excipients can be employed which have shown to be useful before. The stability study, however, i.e., the succession of stress and analytical experiments, is identical for all samples.

Regulatory guidelines (ICH-Q8(R2)) state the requirement that "Critical formulation attributes and process parameters are generally identified through an assessment of the extent to which their variation can impact the quality of the drug product," in other words, the need to assess the robustness of a formulation. The guidance further advocates "building quality into products" by science- and risk-based approaches and recognizes statistical experimental design as one of the tools that enable a scientific risk-based approach.

In accordance with this regulatory framework, the robustness of a lead formulation is assessed in a *module 3* screening campaign using a DoE approach. This last stage of HTS is used for evaluating the optimal composition and proving the formulation robustness. As the stability studies are dependent on the outcome of module 1 and module 2, this screening is variable and complex in execution. But still, due to the modular design of stress tests and analytics, major parts of the standardized workflow can be utilized.

The level of standardization at the stage of sample compounding and at stress assay/analytical evaluation is summarized in Fig. 7.

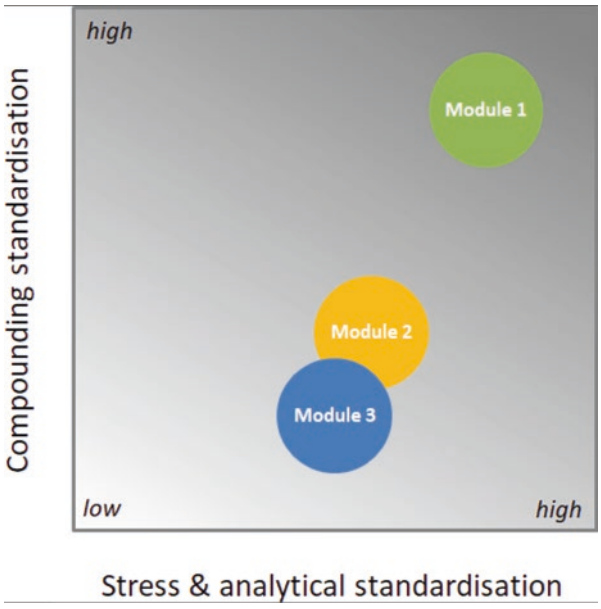


Fig. 7 Different levels of standardization during screening campaigns. The necessary higher level of flexibility in modules 2 and 3 is reflected in lower standardization of compounding what is in turn related to the individual properties of the development candidate

Standardization of Methods and Assays

Another area where standardization plays a critical role is analytical methods and stress assays. In order to make assays and methods broadly applicable, we have developed standardized platform approaches. This avoids time-consuming evaluations prior to screening campaigns to prove suitability for the intended use. During method development, we pay attention to employ a range of molecules with differing properties to ensure the assay or method can accommodate them all and ultimately make the method reusable for any new molecule. This is in contrast to analytical QC where each and any method is an individualized development.

The standardization of methods has a further immense advantage. All data generated within the screening campaigns are fully comparable, even though methods are not validated. All data come with the same quality and error, which is a huge advantage for subsequent data evaluation. As an example, even if the same method is performed in two labs, for instance, chromatography, small variations in buffer composition or pH, column dimensions, pressure or oven temperature, etc. will end up in small result changes. Whether a 2% difference in the results can be regarded as insignificant or not is impossible to judge. This problem is omitted when all data are generated within the same system with known error ranges.

Standardization is also applied in the succession of stress assays. In early versions of HT screening, multiple stress assays were applied subsequently, for example, freeze/thaw (f/t) followed by temperature stress or mechanical followed by temperature stress. Not only made the combination of stress models data handling difficult, we found out that degradation effects from multiple stress models are basically independent. This opened the way to a parallelized and much easier workflow.

Let us make that a bit more tangible: The old workflow started with plate preparation, i.e., dispensing and compounding the samples into MWP. This plate then ran through f/t stress. Subsequently, references were added onto the plate (after the first stress to avoid influence from the stress model) and analyzed. Additionally, a subset of samples from the plate was mirrored onto another MWP and put on 40 °C (to be able to distinguish between temperature-based and nontemperature-based degradation). A part of the analyzed plate was then supplemented with silicone oil and subjected to mechanical stress and subsequently further analytics. Finally, this plate was subaliquoted to three daughter plates and put on temperature stability at 5, 25, and 40 °C and analyzed after predefined pull points up to 3 months. To summarize, the various screenings had samples with a huge diversity of stress and stress combinations as summarized in Table 2.

After a thorough evaluation of data, it became clear that a combination of stress models is not necessary and does not provide additional stability insight. Therefore, we established three parallel routes each of which addressing one single stress type: f/t, mechanical, and temperature as shown in Fig. 8**.** This workflow is much less complex and less prone to errors yet provides all necessary information to discriminate good from poor formulation compositions.

Table 2 Overview of stress conditions used in the initial workflows. Some samples were subjected to f/t alone (row 1) or a combination of f/t and mechanical stress (row 3). The most complicated combination joins four stress models, such as f/t, mechanical, silicon oil, and 25 °C incubation (row 6)

Screening no.	f/t	mechanical	Silicon oil	5 °C	25 °C	40 °C
1	X					
2						X
3	X	X				
4	X	X	X			
5	X	X	X	X		
6	X	X	X		X	
7	X	X	X			X
8	X	X		X		
9	X	X			X	
10	X	X				X

This modular setup of the standard workflow has also the advantage that branches can be plugged in and out depending on project needs without impacting the rest of the screening. This build-in flexibility was missing in the initial workflow version.

Miniaturization

Robust analytical methods are key for formulation development of biopharmaceutics. Formulation development is seeking for those environmental conditions that confer highest storage stability for the protein under investigation. This means as well that the analytical methods employed should be capable to detect any change in stability with high security as stability is closely connected with product safety. In the context of formulation development, this is a particular challenge as analytical methods have to work under a much wider range of conditions (i.e., variations of pH, excipients type and concentration interference, DS concentration, etc.) than, for example, QC methods which are usually validated for one specific formulation condition. Under any formulation composition condition, a robust assay performance must be assured to discover molecular liabilities.

At the beginning of formulation development, a set of analytical methods needs to be defined based on the predefined CQAs. Table 1 provides an example of an initial risk assessment for describing the correlation between the formulation parameter and the respective Critical Quality Attribute. Ideally, a high-throughput screening should be capable of providing analytical results for all required CQAs.

Going from traditional analytics to a high-throughput setting, all methods and technologies need to be miniaturized to cope with small sample volumes in multiwell plates. Even more, one sample of a few tenths of microliters needs to be shared by a dozen methods. What can be achieved so far is summarized in Table 3 comparing

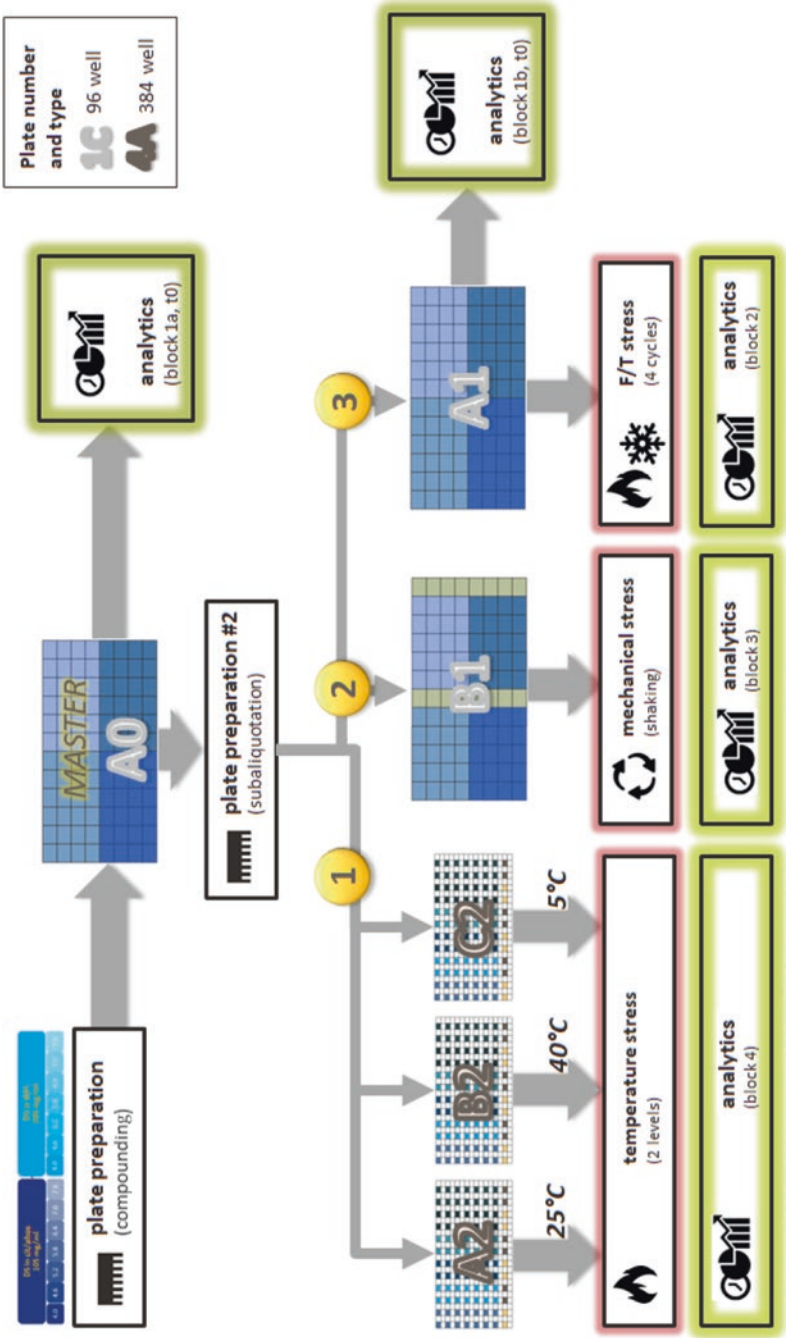


Fig. 8 Sketch of the most recent workflow utilized for HTS formulation development

Table 3 Comparison of typical sample volumes required by standard analytical methods and optimized high-throughput screening methods

Purpose	Method	Consumption with stand. devices [µl]	Consumption* with HTS methods [µl]
In-process controls	pH	5000	2
	Fill-height	n/a	0
	Concentration (SE-UHPLC, AUC)	500	(0)
	Conductivity	2000	0
Physico-chemical characterization	Turbidity	2000	0
	SE-UHPLC	50	0.4
	CEX-UHPLC	50	0.4
	HI-UHPLC	20	4
	DSF	50	18
	Sub-visible particles	5000	15
	Viscosity	2000	10

5 - 2000x reduction

per measurement

the sample consumption for one analytical measurement under HTS conditions with that under classical QC conditions:

Downsizing of methods can be more or less demanding, and it needs to be considered that the miniaturized method should not move too far from the traditional QC method to be able to compare results from formulation development with later QC testing. Additionally, the new method must be automatable from both a workflow perspective and equipment capabilities.

For example, the translation from traditional *HPLC technology* to modern UHPLC equipment is relatively straightforward. With appropriate choice of column materials and method development, sample requirements can be reduced more than 100x to below 1 µl (UHPLC-CEX, UHPLC-SEC). Additionally, analysis times decrease dramatically, without substantial loss in resolution (e.g., a standard runtime for SEC would be 30 min by HPLC vs. 8 min by UHPLC). As a large number of samples need to be processed within a HT screening, fast analytics and time economy are important factors also with regard to limit evaporation during processing.

On the other hand, apparently simple methods could pose huge challenges in a HT setting. One of those is *pH measurement*. As outlined in the workflow standardization, pH is the most important formulation parameter to assure stability and therefore needs to be monitored. While the value is easily accessible using a glass electrode in a standard lab, such electrodes do not match the size of a 96 MWP or even 384 MWP. There are special solid-state electrodes available that fit into a 96-well plate; however, dipping an electrode into 96 sample wells with intermitted rinsing and drying and the risk of cross-contamination of samples suggests to refrain from this approach. Instead, we developed a plate-based mix and read assay that uses imaging as summarized in Fig. 9. First, 2 µl of sample is transferred to a 384

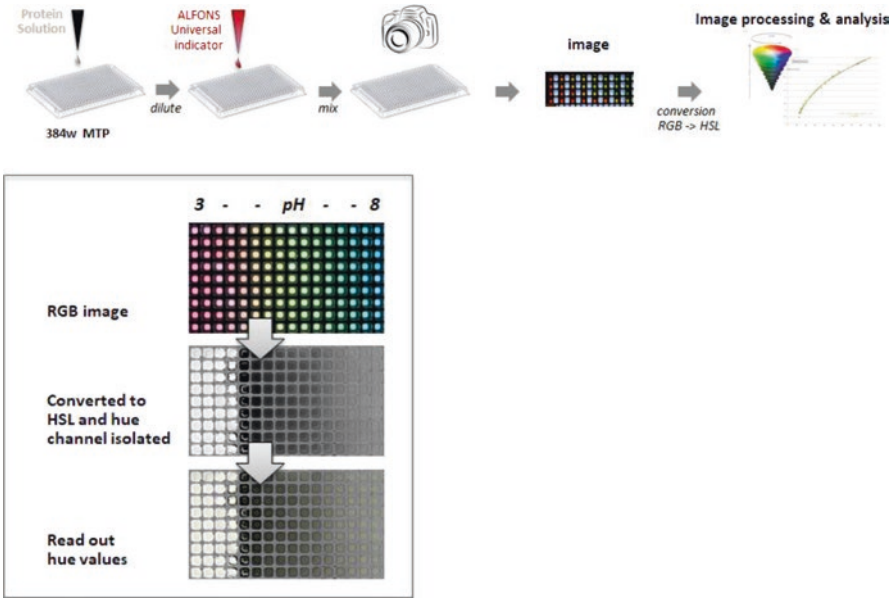


Fig. 9 Process chart of a miniaturized pH determination method

MWP, and a proprietary mix of pH indicators is added. The indicator mix is usable in the relevant pH range of 3–8. A photo is taken after mixing which is processed using ImageJ [23]. The key step in processing is converting the RGB color information to the HSL color space. This step makes the assay insensitive to imaging errors (e.g., exposure, sharpness), and the transformation to HSL color decouples color information from brightness and saturation. The entire color information (what is the measure for pH) is now stored in a single channel that can easily be translated to a pH by using a standard curve. The assay is robust, is easy to perform, is amenable to automation, and does not need any specialized equipment.

Finally, HT screening needs *some* methods and methodology that are only necessary in this particular setting. When dealing with very low volumes, evaporation of solutes becomes a big problem. As the formulation composition is meant not to change over time, the effect needs to be controlled as much as possible. Evaporation can take place during analytical testing or incubation. In the first case, it is inevitable (e.g., during transmission measurements, wells must be open), and hence the analysis times should be optimized to a minimum. The latter, incubation, especially at elevated temperatures for longer time periods [see workflow chapter], requires a well-chosen pair of multiwell plate and sealing foil together with an optimized sealing protocol to assure tight sealing of the wells and avoid loss of solvent or exchange of solutions. In any case, the ability to detect loss of solvent is essential and an important in-process control. We have established a protocol, where *fill height* measurements are being done together with transmission experiments to determine

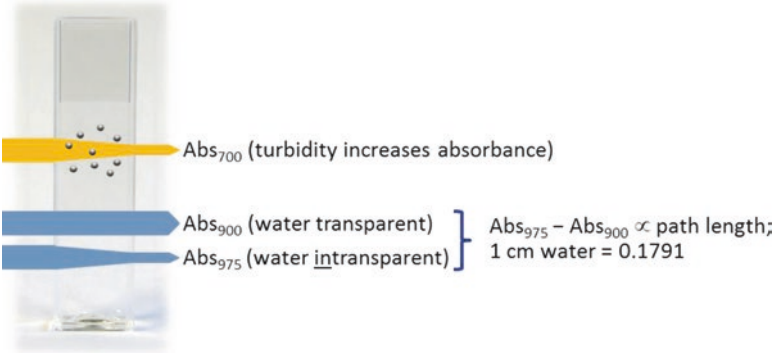


Fig. 10 Illustration of the measurement principle of turbidity determination. The absorption of water in the IR region is exploited to determine path length

sample turbidity in a plate reader according to Fig. 10. The beauty of the approach is that neither additional sample preparation nor extra analysis steps or equipment are necessary. The method uses the property of water to be transparent for light at wavelength 900 nm, but to absorb light at wavelength 975 nm. The difference of absorptions ($A_{975} - A_{900}$) is proportional to the path length which can be calculated knowing that 1 cm of water has absorption of 0.1791. Excipients do not disturb the method as none of them absorbs above 700 nm.

It should be mentioned that the path length is not only required as fill height IPC but is also necessary for transmission measurements. Keep in mind that we are using multiwell plates that lack the well-defined path length of cuvettes.

Up to now, analytics in small volumes were portrayed as challenging. But small volumes also come with advantages, even more, low consumption of drug substance is *the* unique feature of a HT screening. DS is a precious material and usually in short supply in particular at the beginning of drug product development. Any means to generate high content data from minute amounts of DS are therefore highly appreciated. Already in the current development state, Abbvie's HTS platform is able to deliver unprecedented data sets in highest quality from only a few gram of DS. To produce similar records using traditional approaches would have consumed at least 100x more DS what is in turn a huge cost saving for the company.

Unfortunately, not all CQAs are amenable to a high-throughput setting, which is mainly due to technical limitations (Table 1). As such, potency assays using living cells should therefore be performed in a specialized bioassay lab. Another example would be force measurements, related to packaging material performance that would also require a specialized material science lab. Establishment of miniaturized methods was a stepwise process in our case. Facing limited resources, we started off with the most important techniques such as chromatography and then successively added and improved assays according to gaps and potential CQAs. This process is still ongoing, and further methods addressing posttranslational modifications such

as oxidation or deamidation could be helpful for the creation of a standardized interface for mass spectrometry measurements.

It is well known that packaging materials can influence the stability of biopharmaceutics; therefore, the question is often asked whether or not formulation data produced in (plastic) MTPs are representative for typical packaging materials. We have verified the analytical results generated in the HTS setting (96w MTP) with those generated in established primary packaging materials (glass vials and syringes): we could not detect meaningful differences between the packaging materials; SEC and CEX chromatographic data from HTS are fully comparable to data generated in vials or syringes as depicted in Fig. 11. The same is true for PETG bottles that are used as containers for storage and shipping of DS.

Automation and Software Integration Strategy

Liquid Handling (as a Starting Point for Partial Automation)

Prior to automation initiatives in the formulation development of biologics, several lab groups supported pipeline biologics manually. Each group engaged in an individual/customized molecule development dependent on molecule and project needs. The advantage was a tailored approach taking specific molecule liabilities into account. The disadvantage was a lack of standardization of workflows and development activities through different ways of reporting.

As a first step toward standardization, a small high-throughput team was founded. They used a liquid handling system in combination with a minor set of plate-based analytics, e.g., liquid chromatography. With the use of a liquid handling system, the experiment planning was standardized using Excel templates, translating the pipetting schema into automation. All further steps, transferring plates, stressing plates, and analyzing plates, as well as data handling and documentation within the electronic lab notebook (eLN), were manual steps.

Beside this automation initiative, the implementation of Quality by Design (QbD) into the formulation development process took place, which led to a clear definition of critical stresses, meaningful analytics, and the overall formulation workflow.

Workflow Requirements and QbD Defining Automation Strategy

The implementation of QbD principles defined the key elements of our scientific workflow. It was clear that we needed to reduce the amount of drug substance resulting in the usage of multiwell plates (96-well plates and 384-well plates). Depending on the module (*see* sections "Screening Design" and "Standardization of Methods and Assays"), different plate layouts (formulation compositions) are tested, leading to flexible pipetting of the mother plate. The workflow including several daughter plates was standardized as each daughter plate should be exposed to a defined stress

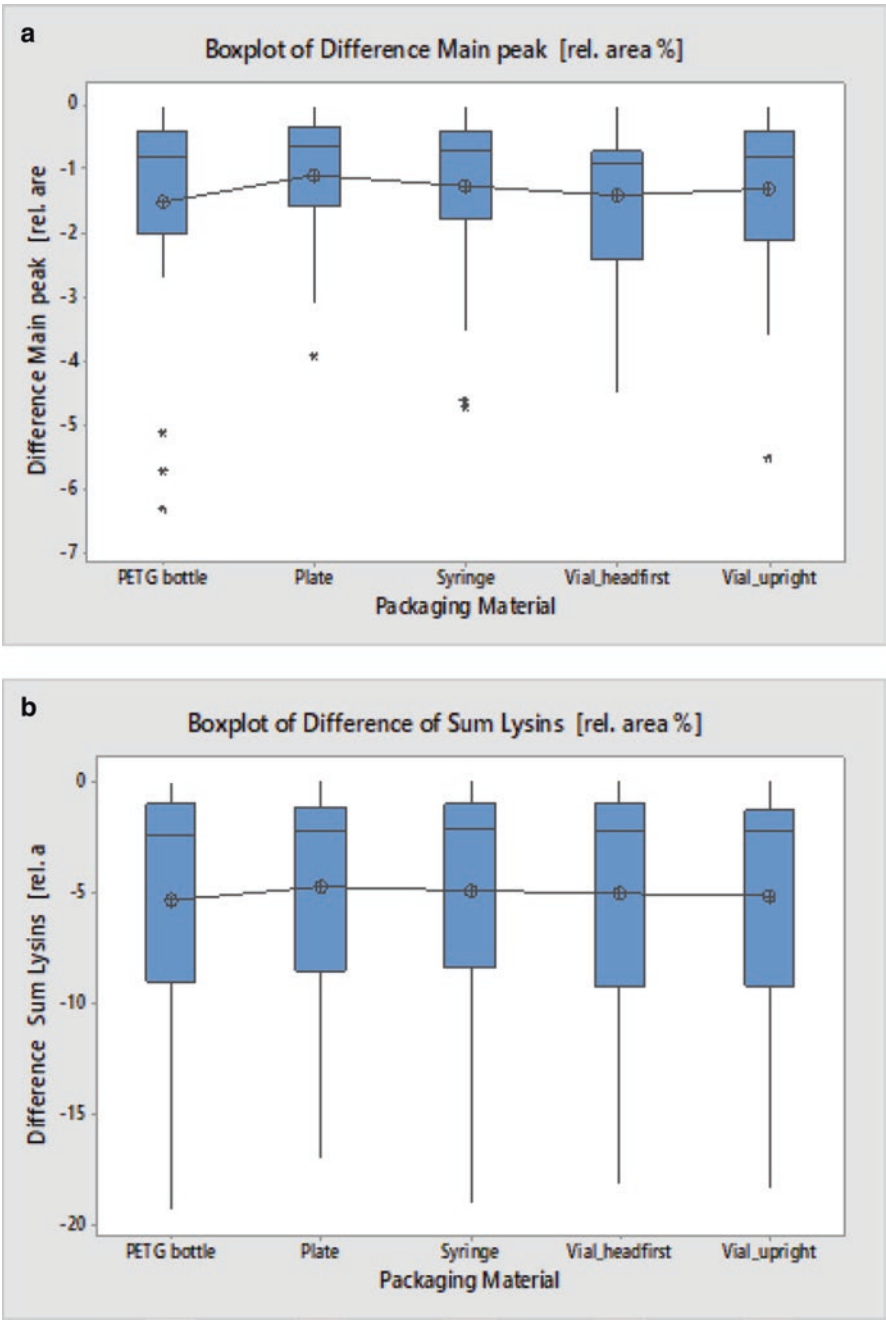


Fig. 11 Comparison of 96-well plate down-scale model results with established primary packaging materials does not show statistically significant differences. (**a**) Size exclusion chromatographic (SEC) data boxplot of different monomer of various packaging materials after 6 months at 25 °C storage. (**b**) Cation exchange chromatographic (CEX) data boxplot of different main peak of various packaging materials after 6 months at 25 °C storage

model following analytical tests. These analytical tests were defined by the respective potential Critical Quality Attributes.

With this standardized workflow, we were able to define an automation strategy including equipment specifications. As a starting point, the existing hardware (e.g., liquid handling system) should be combined and expanded into one fully automated process.

High Throughput of Formulation Conditions and Design Space

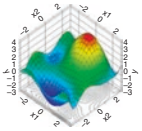
From an automation perspective, our standardized workflow is different compared to "classical" high-throughput approaches to be used in early developability screenings during molecule design and lead candidate selection. Usually a screening funnel approach is used to select promising molecules with, ideally, fast and meaningful analytical assays. In such scenarios, one key performance indicator is the amount of processed molecules.

In contrast, our workflow processes just one molecule in various conditions. A more precise description of our high throughput might be a screening of formulation conditions instead of biologics pipeline candidates (see a summary in Table 4).

As a consequence of our screening concept, each module defines key parameters of the following module (e.g., output module 1 is the pH and input of module 2 is the pH range), and the overall result is a Design Space with more than 90.000 data points for each candidate. In contrast to classical high-throughput screenings, we analyze one candidate deeply, instead of many candidates more superficial – which are done upstream in the preformulation group.

Another very critical workflow characteristic is the length of a screening. We already mentioned that usually fast high-throughput cycling times (e.g., one run per night) are desired. In our case, we have stress methods ranging from several hours (e.g., freeze/thaw stress) to several days (e.g., mechanical stress) and finally several weeks (e.g., temperature stress). This results in temperature incubation

Table 4 Comparison of our formulation conditions screening with a classical screening funnel

	Screening funnel	Formulation conditions screening
Screening type		
Screening characteristics	Many molecules per screening Molecule selection Fast decision Short cycling times Plate loss can likely be repeated	One molecule per screening Molecule understanding Knowledge increase Long cycling times Plate loss results in a potential complete failure

periods of several weeks in which the automation line is more or less in an idle state. There are several solutions for such a scenario. One potential solution might be using some kind of stacking and intelligent scheduling. Another more robust approach might be sequential screenings. Having this said, the cycling time per module/run is about 2–3 months. Additionally, the analytical methods range from several minutes (e.g., turbidity measurement in the plate reader) to several days (e.g., liquid chromatography).

Another very important topic is the availability of material and costs. As we test just one candidate, a loss of a mother or daughter plate results in a complete failure instead of a classical screening with 100x identical iterations of different compounds. Overall, this could result in losing several million dollars and even worse a significant delay in development times.

Automation Strategy: Fully Integrated Versus Decentralized Islands

With the described characteristics of our workflow (see above), the general decision of fully integrated or decentralized islands automation was made. We decided to go for a fully integrated automation line due to workflow length/characteristics, equipment usage, and space limitations.

Decentralized island is usually split from each other by workflow characteristics. A common scenario is one island for sample preparation and a second or several islands for analytical methods. If we transfer this to our workflow, we only do have a liquid handling system for sample preparation, which is not very practical. Another potential split might be splitting stress models from analytical blocks (sum of all analytical techniques after a stress model). Each stress model is just one single instrument (e.g., freeze/thaw unit) with very diverse stress times a split does not make much sense either. Additionally, temperature stress is combined with analytical blocks. If we use the incubation at 25 °C as an example, the corresponding daughter plate is incubated for 7 days, analyzed, and incubated for additional 14 days and again analytically analyzed. If mechanical stress models and analytical blocks were split from each other, several manual steps in-between these islands were needed.

As the stress and analytical parts are interlinked, another consequence of separate islands would be to duplicate several instruments like sealer, de-sealer, or centrifuge as we have several of these steps between stress and analytics. One example might be the freeze-thaw stress, and following centrifugation, shaking and de-sealing before the turbidity can be measured. Additionally, a plate that was analyzed by liquid chromatography has a punctured well sealing, which needs to be centrifuged, de-sealed, and sealed for further temperature incubation. This would result in much higher costs and a greater footprint of the automation line. In Fig. 12, a short part of the fully integrated workflow is shown, which resulted in a decision for a fully integrated automation line as described above.

An additional aspect might also be error recovery. In separated islands, not all instruments would be available, especially out of working hours with no possible

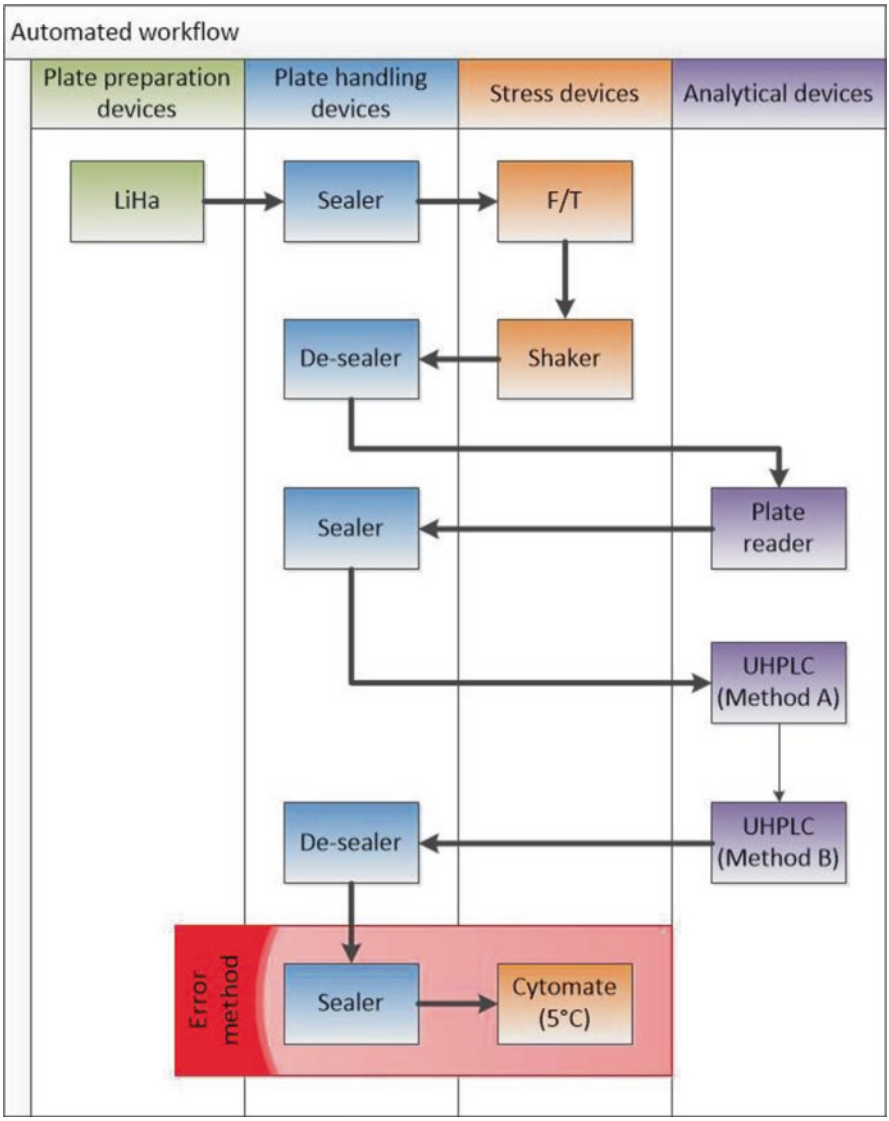


Fig. 12 Overview of a small part of the fully integrated formulation screening workflow that results in a fully integrated automation approach

manual intervention – and each run is out of working hours with duration of 2–3 months. In an ideal scenario, predefined error handling always secures the corresponding plate tightly sealed in the 5 °C incubation chamber. This is only possible, if the sealer and incubator is always available, which is only the case in a fully integrated automation line (see the section marked in red at the bottom of Fig. 12).

Implementation Challenges

We integrated sample preparation, mechanical stress models, and analytics in one fully integrated automation line. One very obvious consequence is that if one part of the automation is not functional, the whole automation line is not functional anymore. One solution, which was not possible for all instruments, is redundancy. Due to that and because of long runtimes per plate, we included several liquid chromatography systems in the automation line.

From an automation perspective, the liquid chromatography is special, as the automation line needs to control not only the hardware (scientific instrument) but also the chromatography data system (CDS). This led to tremendous changes in the IT infrastructure and a lot of efforts in driver development and as a consequence to massive delays.

In the "classical" setup, the automation line is a separated network construct that harbors and connects all instruments of the automation line. The advantage of this isolation is that no harmful network traffic (e.g., antivirus updates) is interfering with the automation run. The disadvantage is that data handling becomes more and more complex. And in our case, a CDS system located in the IT data center (outside of the laboratory and automation network) is not possible.

We had several experts from internal IT and the vendor involved to come up with an IT infrastructure that on the one hand separates the automation network but being able to connect to a CDS system within the AbbVie infrastructure. This took approximately 1 year and led to massive delays during implementation.

In parallel, automation drivers usually talk directly to the hardware located in the laboratory. In the liquid chromatography/CDS construct, it has to talk to the hardware (e.g., automation interface for plate loading not supported by the CDS) and the CDS or rather the control unit connected to the CDS servers. The development and testing of this driver were rather complicated and time consuming and lead to additional delays during the implementation phase.

Intelligent Automation as a Paradigm Shift

With our workflow for formulation development, we challenge several common settings of automation. The first and one of the major differences is the "just one" plate setting limited by drug substance availability instead of a screening funnel with numerous plates of different compounds. The already mentioned consequence is that the loss of this plate, or one of the unique daughter plates, results in a loss of the whole run. A loss of material worth several million dollars and the delay in development is very critical and should be prevented with intelligent automation and corresponding error handling.

Intelligent automation is, in contrast to the classical and static workflow, an adaptive workflow based on the workflow itself, similar to an autonomous driving car that needs to react and adapt to the environment. Similar things are possible, if all data (log files, analytical data, and additional sensors) are used to adapt the workflow while it is running.

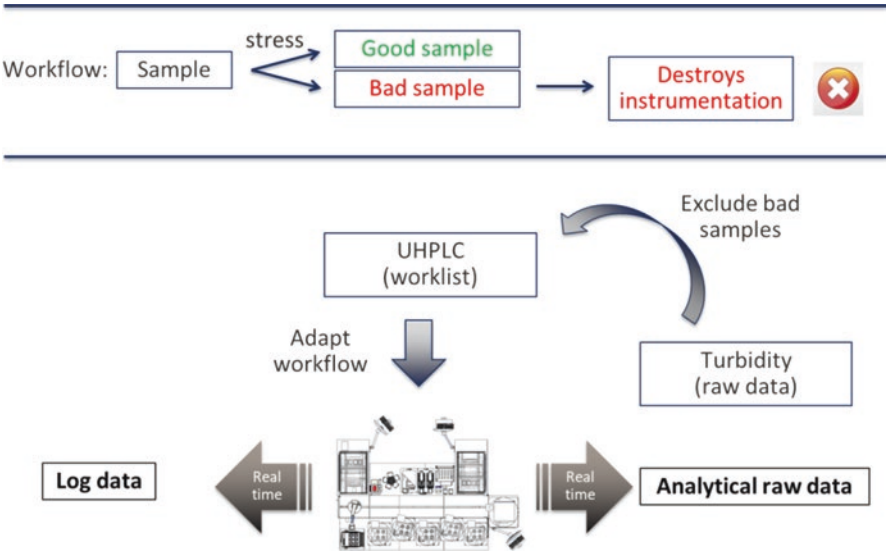


Fig. 13 Basic example of intelligent automation, adapting the workflow near real time to increase its robustness

A simple but descriptive example is again the liquid chromatography. From a scientific perspective, the liquid chromatography is a very common and widely used and well understood method; nevertheless, it is very sensitive. In our workflow, we stress samples in many ways by freeze/thaw, mechanic, or elevated temperature, and most probably, we will have bad conditions within our Design Space, to be more specific, to identify the border of the Design Space. Due to this, the likelihood to analyze bad conditions with liquid chromatography is close to 100%, which affects the robustness dramatically. On the other hand, good conditions on the plate should be tested. Therefore, we use the robust plate reader turbidity measurement to identify bad conditions and exclude them from the liquid chromatography worklist. With this simple feedback loop, the robustness of the whole workflow is significantly increased. Additional information from log files and further sensors will help to increase the robustness and improve the workflow itself. This example is shown and illustrated in Fig. 13.

A classical relational database approach with a fixed data model might not be the best solution for such data processing and the usage of huge log and sensor data in combination with analytical data. Because of that, we invested quite some effort improving the data handling, which is described in the following chapter.

Data Management

High-Throughput Data Handling Following QbD

Scientific workflows start with experimental planning and setup. Even at this stage, important information such as formulation composition and corresponding physical well position are defined. Additionally, the workflow itself (e.g., which stress models

are used) needs to be defined in this phase and has a major impact on how data and metadata will be processed. In a QbD approach, all information need to be tracked ranging from this planning phase to the final screening data table and its evaluation (e.g., visualizations and statistics) used for the development report. One important part of this data lineage is the experimental data generation in the laboratory and potential aberrations along the process. These aberrations might be errors in the workflow (e.g., shorter mechanical stress due to hardware errors) or errors during analytics (e.g., measured values out of range due to hardware malfunction) which all need to be documented. Based on the error description, a risk assessment needs to be done followed by a drop of affected data points.

Area of Conflict: Manual Data Handling for Automated Workflows

In the past, all of the documentation described above was done manually in lab notebooks. This has the consequence that most documentation was done chronologically and in very heterogeneous quality. Some people documented extensively, others rather cryptic, and only some might document shortly but precise. If one had to write development reports or even filing documents, several lab notebooks and/or summary reports need to be manually "mined" to combine and evaluate corresponding results. The documentation process was time and labor intensive including text writing, text reading, and evaluation at the end.

This process did not change much with introduction of the electronic lab notebook as the documentation procedure was kept more or less the same. Over time, standardized eLN templates were generated for standardized lab processes. This increased data quality, but it reduced flexibility significantly. From a time perspective, different templates were combined to document one process, which was very time intensive and led to various errors. These errors were either human errors or caused by processes that did not fit to the templates properly.

Nevertheless, as a first start, the ELN was used for documentation, and with additional standardized templates, a data import was performed. This upload was feeding a data mart connected to a visualization tool. With this construct, users were able to access individual data sets and evaluate data they generated.

Several pain points led to a change in data storage and documentation strategies.

- First of all, the process changed on a regular basis affecting the eLN templates and the connection with the data mart and visualizations negatively leading to down times.
- With increasing complexity of the workflow, the data handling template became bigger and more complex, reducing the degrees of freedom. At the end, it was not really maintainable or adaptable to our complex workflow.
- Additionally, with increasing sample numbers from automation, IT infrastructure and the eLN vendor discouraged that approach as all data had to pass the eLN before entering the data mart.

The documentation process itself was still time consuming and was dependent on lab personal attending the experiment in the laboratory. With increasing automation, however, more and more steps are not performed from scientists during working hours, but rather by robotics potentially and not surveyed by human beings. As a consequence, a process description has to be based on log files generated by the instruments themselves or afterward based on data/controls.

Evolution of Data Handling Strategies: From eLN to Hadoop

As the eLN had some major limitations, the need arouses in exploring several other potential options for data handling and for more automated documentation.

The first idea was to test several prebuild software solutions (e.g., from Genedata or ACD Labs) to store and process our data. With these software tools, both vendors supplied import filter and a small set of visualizations were available. Additionally, a tool-specific programming language could be used to program additional user-specific import filter and visualizations. This approach was on one hand relatively expensive, and, on the other hand, not very intuitive, as noncommon programming languages were used. Having that in mind, we wanted to build our own solution with a database to store the data in combination with visual dataflow software for data processing. Within our test, we evaluated several database types (relational SQL and non-SQL) and visual dataflow software vendors. The outcome was that we might need different databases for different tasks (analytical data vs. log files) and that most dataflow software tools lacked suitable connectors for databases we potentially wanted to use. Additionally, commonly used programming packages (e.g., SciPy) with very valuable functions were not available. The only work around was to re-program parts of those packages or build long sequences of graphical steps. Due to that, we were looking for other solutions that might fit a greater purpose and could potentially become a blueprint for data handling in general.

We found one solution that covers all of that: the Hadoop framework that contains various databases, additional connectors to external data sources and tools (e.g., visualization), as well as already included processing/data evaluation tools like the Data Science Workbench in which “classical data science” programming languages (Python or R) with all available packages can be used. Besides, the data processing in distinct Docker containers would be able to separate different processes and log them. In addition to that, our Cloudera Hadoop cluster contains the governance tool Navigator, which is able to track data lineage. For code versioning, a direct connection to the enterprise GitHub repository was established helping to very precisely organize the data handling. As a frontend, we use a self-designed tool called “HTS Studio” that remote-controls the Hadoop cluster and triggers uploads and predefined jobs within the Data Science Workbench. All user interactions with the HTS studio are logged as well.

Data Integrity in Automated Data Handling

We included the data integrity aspect already in the selection of tools we intended to use for data handling. In all test scenarios, data integrity was one key KPI to fulfill. The Cloudera Hadoop framework offers the governance tool Navigator, in which the whole data lineage is visible from data upload to the final data table, which is used for visualizations and statistical data evaluation. We achieved that by designing the processes and programming code in the Data Science Workbench in such a way that it is automatically tracked and logged in the Navigator tool. In addition to the Navigator logging, the manual upload via the "HTS Studio" is tracked (who uploaded what content and why) and version-controlled as well. Potentially, some data (e.g., liquid chromatography data from Chromeleon CDS) need to be re-evaluated/re-integrated as part of the data cleaning process which would result in a second upload. This is possible; both versions are logged and the latest version is used for the following data handling.

For data integrity, errors and process deviations are key challenges. To cover that, we included the "drop planner" into the "HTS Studio." This small application can change the status of a data point or several data points from valid to invalid. With that status change, the user has to define a drop reason via a predefined pull-down menu and sign for that action. It is important to state that data points are not deleted, they are flagged. As a consequence, mining of drop reasons and nonrobust process steps that potentially need improvement or workflow adaptations is possible, as well as investigations why data points are dropped and who dropped them.

In general, errors are documented in the eLN either with manual explanation or referenced to a log file followed by a short risk assessment if that resulted in a data drop or if the status was kept as valid. A potential next evolutionary step would be to utilize automated decision-making algorithms by defining defined criteria (e.g., out of method range) that leads to an automated drop of data points. This automated drop is flagged as well including a status, if the drop was made by an algorithm (which can be traced back to algorithm and code version used) or manually (described above). All documentation about process aberrations and data drops is referenced in the development report document summarizing the whole screening run.

A Data Framework for Standardization and Flexibility

A very important KPI for our data handling and during the tool selection process was the availability of two general data paths. One path was briefly described in the chapters above and focuses on the development report generation and the corresponding data handling, a rather standardized path.

Using analytical data and log files in a structured way to improve process robustness is most probably rather standardized as well.

The second path is rather exploratory and uses the available data in a non-predefined manner for data mining and data science. In general, this exploratory

path does not need the same amount of data integrity and logging as the standardized parts explained before. Nevertheless, it needs to be completely separated from the data pipeline leading to the development report. If the exploratory path generates knowledge and processes that can be transferred in a standardized path, it is included in the process described above.

The standardized data handling pipeline for the development report consists of several stages:

- Planning (e.g., definition of plate layout by well plate planner function in "HTS Studio").
- Upload of analytical data generated in the laboratory (manual or automated).
- Description of workflow in the eLN (manual or automated).
- Automated data merge and preprocessing (e.g., done by predefined algorithms in Docker jobs run in the Data Science Workbench documented in eLN/GitHub and in a corresponding data management document/eLN documentation).
- Manual data cleaning process documented in the eLN.
- Visualizations and statistical evaluation described in the eLN.

For the exploratory data evaluation, a screening data table containing all performed screenings is generated and can be used within a secure and separated Docker environment in the Data Science Workbench. Besides, all analytical instrument files, metadata files, and log files are available and can be used for user-specific data analyses. These data can be stored in Hadoop databases and visualized with the directly linked visualization tool Spotfire, evaluated within the Data Science Workbench or downloaded for external tools like Minitab. See Fig. 14 as

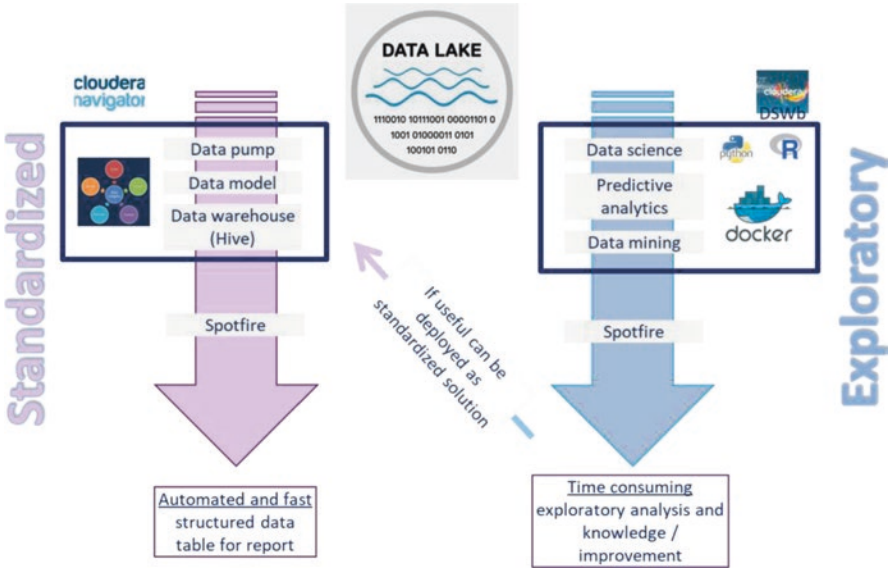


Fig. 14 Hadoop, a data framework for standardized and data integrity driven workflows, as well as exploratory advanced analytics improving Knowledge Space

an illustration of the Hadoop framework usage for standardized and data integrity driven workflows and exploratory knowledge improvement by the use of advanced analytics.

Data Evaluation

The final step for the data handling in the HTS formulation screening is the data evaluation including a visualization of data by using Spotfire (TIBCO, Inc.) and a statistical analysis using Minitab (Minitab, Inc).

It starts with visualizing the processed data obtained by the Python script using Spotfire. This tool provides the possibility to link or embed data that is used for visualization. For high-throughput screenings, the embedded function is being used to include a snapshot of the data in the analysis file, so that the analysis file is independent of the data source and can be used offline/stand-alone. As a consequence, each data change requires a new data upload to Spotfire and a new version of visualization.

In a subsequent step, the statistical data analysis is being conducted. It is the first time that the outcome of the screening results is reviewed by an analyst as a whole and analyzed for trends and correlations. Dependent of the scope of the screening, not all parts can be statistically analyzed due to limited data sets. The data sets with sufficient data points are spitted in subsets based on stress condition. Based on the individual subset, a linear regression of each response variable with interactions through second order (A∗B) and terms through second order (A∗A) of the linear and categorical factors is performed using backward elimination (alpha ≤ 0.05). The statistical model is accepted when R-square > 70%.

The significant factors affecting the response variable are visualized best by using main effects plots. The optimal formulation condition (e.g., pH value) or composition is calculated by minimizing changes in the pCQAs (e.g., decrease in monomer and increase in aggregates and fragments) in combination with a respective predefined pCQA impact score listed in Table 5. The pCQA impact score is being used to determine the impact of formulation conditions on the overall stability.

Table 5 Example of predefined pCQA impact scores (1: low criticality, 10: high criticality)

Quality attribute	Criticality score
Size variants (monomer)	10
Size variants (aggregates)	7
Size variants (fragments)	5
Size variants (reversible self-association)	5
Charge variants (main isoforms, acidic and basic species)	7
Clarity and opalescence	5
pH value	10
Unfolding T_{on} and T_m	1

Table 6 Examples of pCQA ranges for SEC and CEX results to be used for data evaluation

Threshold	Decrease in monomer (%)	Increase in HMW (%)	Increase in LMW (%)	Decrease in main isoform (%)
Normal	5	2.5	2.5	5
Wide	10	5	5	10

pCQAs are evaluated using a response optimizer function and overlaid contour plots. Response optimization helps to identify the combination of variable settings that jointly optimize a single or a set of pCQAs. It is used to evaluate the impact of multiple variables on a response. For instance, Minitab calculates an individual desirability for each pCQA based on its respective impact score and by the requirement to stay within a certain range (i.e., normal or wide), based on the linear regression model. Examples for pCQA ranges to be used for the optimization of a factor are listed in Table 6.

Observed statistically significant differences within the variation of a method are not relevant. Hence, no statistical evaluation is performed for responses within the method variation. Furthermore, no data modeling is performed if data sets are too small.

Compiling of a Summary Report

The HTS formulation screening is summarized and its outcome is documented in a written report. The report is archived in a suitable document management system as soon as the documentation and the report review are finalized. The report is used as a source document to support submission for filing documents and intellectual property.

Data Science

Usage of Process Data, Log Files, and Analytical Data for Advanced Analytics/ Data Science and Intelligent Automation

The Hadoop framework has the advantage that all files are stored in the data lake and are available in “native” form. A potential new question one might have and wants to answer with the available data in the cluster needs a specific data processing and most probably results in a different data set and/or different table and data structure. This is very time consuming, but it can be done if the needed programming skills are available. Nevertheless, quite some questions might be answered with a standard data table generated from all screenings, which we call the “all screening data table” in a Hadoop database. This data table has the same structure compared to the already mentioned screening data table (metadata, analytical data,

processed data, and drop status). Nevertheless, the all screening data table evolves, whereas the individual screening data table used and saved in the eLN is locked in the status of the corresponding summary report. This does not only include additional screenings but might also include different processing of instrument data resulting in slightly different processed data compared to the locked screening table. This all screening data table is linked to Spotfire and can be downloaded and used in external tools or alternatively be analyzed within the Hadoop cluster. With the Data Science Workbench tool, individual Dockers for Python or R can be generated and used for advanced analytics as it harbors all needed data science, machine learning, predictive analytics, or even deep learning algorithms. All those algorithms do their job within the Docker and do not harm any other process. Another advantage is that the distributed Hadoop cluster improves the performance (based on the algorithm) compared to a stand-alone PC the scientist or data scientist might use.

From an automation perspective, not only the analytical data but also log files and sensor data from the automation line might be very interesting and could be used for further analysis. The value of those log data can be increased by linking them with the corresponding workflow metadata and the analytical data. As an example, we already covered the analysis of valid and invalid data points including the corresponding error description and improving the process by the nearly real-time usage of information like the turbidity measurement to exclude harmful samples for the UHPLC by removing them from the CDS worklist. Increasing understanding of the equipment and their log files helps to improve the workflow in many ways and can be used to optimize workflows, experiments, methods, and the automation implementation itself. As a start, we are using Spotfire to analyze and visualize liquid handling and automation runs as all data are available within the Hadoop cluster according to the process outlined in Fig. 15.

Fig. 15 Using automation data (analytical data, log files, and sensor data) to improve the process/workflow by using the CRISP-DM cycle [26]

Data FAIRization and Sharing Data in an Enterprise Data Platform

The description of data handling using Hadoop is a common use case for this kind of environment. Nevertheless, Hadoop is prominently known as a Big Data tool, and Big Data is defined by five V's:

- Volume (data size).
- Velocity (speed of change).
- Variety (different forms of data sources).
- Veracity (uncertainty of data).
- Value (business value).

A benchmark with the use case reveals that we have the need for velocity (sensor and log data), variety (different data source within the automation line and sensors), and veracity (different data qualities), and all data have a clear business value for compiling a summary report or continuous improvement of the scientific workflow and implementation with automation equipment. If we look at the volume capability of Hadoop, we do not have Big Data as the amount and size of our data files are tiny compared to other industries (e.g., Google, Amazon, or Ebay). Because of that, an evolution into an enterprise data platform usable within the whole company does make sense and is the next logical step.

In most industries, use cases are shown implementing such data lake structures for companies or divisions to tear-down data silos [25]. The approaches used in those use cases are very similar to the tools and data inputs we are using within Hadoop. Additionally, we confirmed our strategy with several consultancies as we wanted to implement as much state-of-the-art as possible.

Over time and in discussion with other groups interested in such a data handling construct in Hadoop, we realized that our data input with instrument files is only usable with the knowledge we have as a group who generated the data. Second, even if we fed the same data format from the same instrument into the data lake, the question of comparability between groups and methods is not possible to answer with the data; additional expertise from stakeholder groups is needed.

To summarize the findings we had:

- Data are not findable as we only have rudimental instrument files lacking metadata.
- Data are not really accessible as we do not know what we have as no common ontology is used.
- Data are not interoperable, as we do not have similar standards (based on data structure and naming conventions).
- Data are not reusable, as the only starting point is our data model, but not the raw data (which is not self-explaining due to missing metadata and common ontology).

These phenomena are not unique to our use case, and a general strategy to solve these challenges is the implementation of FAIR principles into the data handling [24].

The concept of findable, accessible, interoperable, and reusable data is very generic, and practical implementations are not published yet. The Allotrope consortium aims to develop and define industry-wide standards to implement FAIRization concepts. Once available, they will be tested in a proof-of-concept (PoC) within our Hadoop cluster and depending on the outcome will be used as a blue print for an enterprise data platform. This PoC includes the ontology defined from the pharmaceutical industry for the pharmaceutical industry as well as an ADF container format capable of storing all kinds of analytical and log data including essential metadata definitions defined in the consortium as well. As pharmaceutical industry vendors participate within the Allotrope consortium as well, even some instruments offer a data export into ADF containers automatically populating essential metadata fields for the instrument/technique already today, and as Allotrope is an industry standard, an increasing amount of instruments will have this export functionality of the shelf in the near future. Besides, we are working on a strategy to compare and use data from similar methods by alignment between those scientific functions and groups using the Allotrope ontology and comparable control samples.

Vision for In Silico Supported Formulation Screenings

With our described data handling strategy, we are able to support the biologics pipeline with development data for liquid formulation in a secure process following data integrity and QbD principles. Besides, the growing data set can be used for data mining and process optimization. As soon as other data, from groups earlier in the development pipeline, are available in the cluster data evaluation, data mining and training of predictive analytics algorithms are possible to predict molecule parameters. Implementation of FAIR principles potentially with Allotrope concepts will fasten and enable such advanced data analytics combining data from different groups and sources.

As we are defining liquid formulation options for pipeline biologics, it would make sense to train predictive analytics algorithms with the intention to reduce experimental effort and reduce cycling times.

Currently, we are exploring an approach using a molecule sequences database, modeled three-dimensional structures, and extract molecule features that can be used to train predictive analytics algorithms to predict analytical outcomes (e.g., hydrophobicity). These molecule parameters are measured analytically within the high-throughput screening and could be used to train the algorithms. For these trainings, analytical results from early developability studies could increase accuracy as well.

The basic concept shown in Fig. 16 would be to use combined data sets in an enterprise data platform, ideally in a FAIRized format to train algorithms that predict formulation characteristics for new and unknown pipeline molecules reducing the experimental effort.

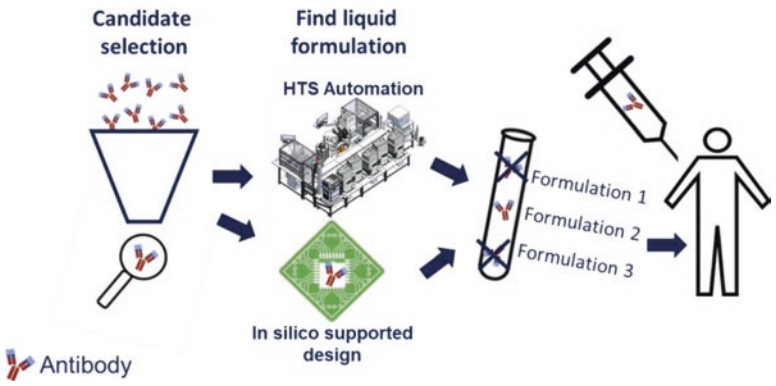


Fig. 16 Using predictive analytics trained by real-world stability data from our automation line to in silico support formulation development

3 Enabling QbD in Formulation Development by Predictive In Vivo Performance Models

3.1 Introduction: Predictive In Vivo Performance Models to Understand the (Bio)-Relevance of Critical Quality Attributes (CQAs)

The aim of formulation development for proteins is to achieve sufficient physical and chemical stability for adequate shelf life of the drug product. Today, a whole armament of analytical characterization methods with an ever-improving sensitivity is available to determine and understand the potential degradation mechanisms that may occur between drug substance, drug product manufacturing, and over shelf life up to administration.

However, very little is known about the relevance of formulation properties after injection.

Therefore, it is important to extend formulation development beyond physicochemical stability in the primary packaging, toward the interaction of the formulation and the biotherapeutic with the biology upon injection.

A good example of the mismatch between analytical capabilities and the lack in understanding the respective medical risk – in other words the biorelevance – was the discussion about a potential analytical gap for characterizing subvisible particles and the recommendation to develop analytical methods capable to characterize the smaller subvisible particle range between 0.1 and 10 μm [27]. Although there was no clear evidence, the assumption was that these small particles may act as a virus-like particle and be involved in breaking the immune tolerance for a given biotherapeutic [28].

The perspective by the pharmaceutical industry was agreement that there is certainly value to have better analytical methods for characterization and quantification for guiding development. However, it was also clearly stated that without under-

standing the immunogenic potential of the various particle species, little would be gained by just generating more data [29].

Interestingly, in aftermath, the research did focus primarily on improving the analytical instruments and methods. [30–32]

This leads to the situation that we have nowadays very powerful analytical methods and determine a whole variety of particle data as expected [33], but we have only a very limited understanding how these data are linked with a medical risk. [34, 35]

Therefore, there is an eminent need for developing predictive in vivo models that allow correlating analytical results with a biological readout [36]. For subvisible particles, this would be immunogenicity but could be also bioavailability, protein metabolism, or pain on injection depending on the CQAs.

This goes concurrent with the rising expectations by authorities, to provide substantially more data not only on physicochemical data of a given biotherapeutic but also on its respective biological performance. This would allow to identify and understand the Critical Quality Attributes of a given molecule, that determine the quality, i.e., safety and efficacy of a new drug product (Table 7).

Table 7 Overview of the expectation by authorities toward understanding biological performance

Regulatory expectation	Reference
Unless otherwise indicated by a specific guidance, this guidance recommends that the traditional *BE limit of 80–125%* for nonnarrow therapeutic range drugs remains unchanged for the bioavailability measures (AUC and Cmax) of narrow therapeutic range drugs	Bioavailability and bioequivalence studies for orally administered drug products – General considerations Guidance for Industry, July 2002
The ICH Q8(R2) guideline defined and formalized the required key elements of QbD. The starting point for all development activities is identifying the potential critical quality attributes (CQA): "a **physical, chemical, biological or microbiological properties or characteristics** that should be within an appropriate limit, range, or distribution to ensure the desired product quality"	ICH Q8 (R2), November 2009
The expected consequence of metabolism of biotechnology-derived pharmaceuticals is the degradation to small peptides and individual amino acids. Therefore, the metabolic pathways are generally understood. Classical biotransformation studies as performed for pharmaceuticals are not needed Understanding the behavior of the biopharmaceutical in the biologic matrix (e.g., plasma, serum, cerebral spinal fluid) and the possible influence of binding proteins is important for understanding the pharmacodynamic effect	Preclinical safety evaluation of biotechnology-derived pharmaceuticals ICH S6 (R1), June 2011
Evaluation of therapeutic protein products in the in vivo *milieu in which they function* (e.g., in inflammatory environments or at physiologic pH) may reveal susceptibilities to modifications (e.g., aggregation and deamidation) *that result in loss of efficacy or induction of immune responses* Such information may facilitate product engineering to enhance the stability of the product under such stress conditions. *Sponsors should consider obtaining this information early in product design and development*	Immunogenicity assessment for therapeutic protein products Guidance for Industry, August 2014

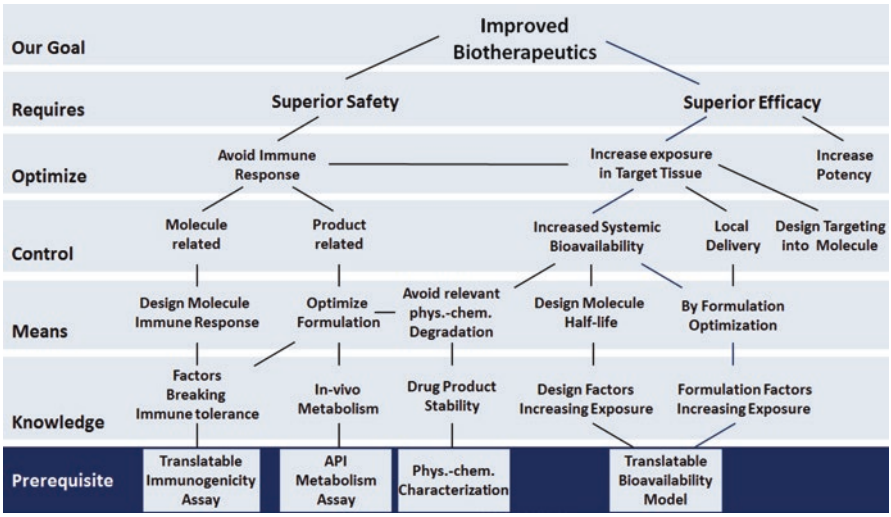


Fig. 17 Top-down overview of the key elements and their interrelationships that would allow for a rational design of improved biotherapeutics

Consequently, biotherapeutic development requires additional analytical capabilities that would also enable characterizing the biological performance (e.g., stability, functionality, immunogenicity, bioavailability) of a given molecule construct or formulation after administration.

Therefore, this section aims to provide an outline what needs to be done and how it can be achieved to develop improved biotherapeutics.

An overview of how to deliver biotherapeutics with improved performance (i.e., safety and efficacy) is depicted in Fig. 17. It visualizes how this goal is connected with the existing aspects in protein engineering and drug product development that needs to be understood.

As described above given the capabilities of modern analytical instruments and methods, it is probably fair to say that the main challenge we face as an industry in development is a direct result of lacking the required tools (i.e., assays and models) to study and understand the factors leading to:

- Breaking of immune tolerance.
- Understanding of protein metabolism.
- Bioavailability/increasing exposure of the API in target tissue.

The reason why the pharmaceutical industry as a whole has not yet been able to overcome these issues is mainly due to the complexity of the tasks, the required level of interdisciplinary collaboration across different functions, and the lack of adequate technological tools.

However, the realization that overcoming these limitations and establishing the enhanced scientific understanding how to design and develop the next generation of empowered molecules is vital to assure a sustainable development pipeline.

Therefore, in the following section, an overview about the aim and challenges in developing assays and models to determine metabolization and bioavailability of biotherapeutics will be provided.

Characterizing Protein Metabolism as a Tool for De-Risking CQAs

Biotherapeutics, such as antibodies, bispecific antibodies, and antibody-drug conjugates, contain a variety of chemical and physical modifications, which are being considered by the authorities as CQAs (see also section "Data Evaluation"). Tremendous effort is expended during process and formulation development in controlling and minimizing this heterogeneity, which may not affect safety or efficacy and, hence, may not need to be controlled.

Many of the chemical conversions also occur in vivo, and the knowledge about such in vivo metabolism can be applied to determine the potential impact on the biological activity and safety of a given therapeutic protein. Other attributes may affect the bioavailability and/or clearance and thereby alter drug efficacy. A high-level overview about the current development approach is summarized in the flow chart in Fig. 18. A more detailed list of the respective CQAs and their criticality score is provided in Table 5.

Therefore, the aim is to develop the tools and methods to gain the scientific understanding that is necessary to identify and assess the criticality of the various observed quality attributes and to focus the tremendous effort in drug product development only on relevant CQAs, which have a significant impact on the performance of a given biotherapeutic.

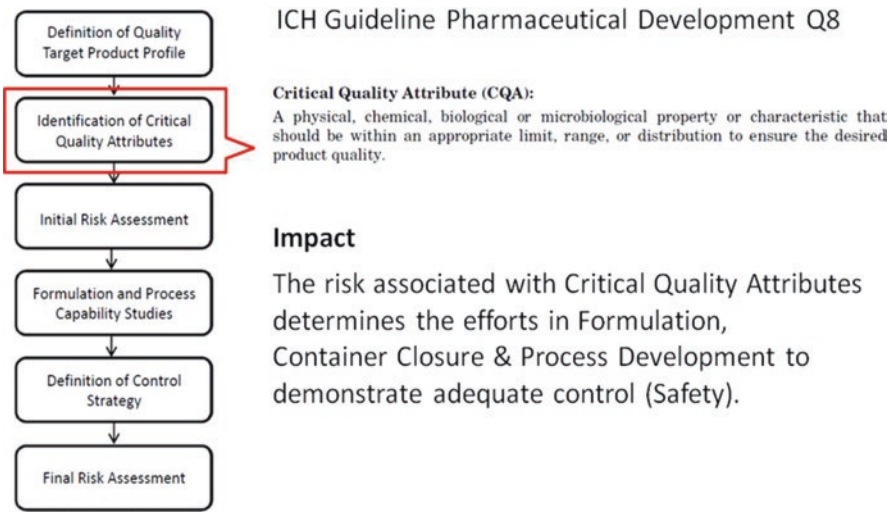


Fig. 18 Flow chart that outlines the expectation by the FDA and EMA for pharmaceutical companies to provide a systematic approach within development

The key principle to be used in these evaluations is how fast a respective attribute changes in vivo compared to the rate of mAb clearance. Together with the existing information about the formation rate of the attributes of interest in the drug product during processing and shelf life will allow an assessment of their criticality. An attribute that is changing only in the drug product and not in vivo may have greater potential to affect safety or efficacy and, thereby, reach the status of a Critical Quality Attribute that should be controlled during production and storage and vice versa.

Since in early development little is known about molecule-specific biorelevance of CQAs, therefore besides compendial requirements, industry-wide best practice is being followed to define product specifications. Whereas, in late phase development, clinical experience of the respective CQAs is increasing only within or below specifications that where initially set in early develoment. The consequence is uncertainty and ambiguity of the biorelevance of CQAs, leading to diverse opinions what is necessary to maintain and improve quality of biotherapeutics. Therefore, the need in developing predictive in vitro/in vivo models for a science-based evaluation of CQAs and their biorelevance is eminent.

Bioavailability

One preferred route of administration for monoclonal antibody formulations is by subcutaneous (s.c.) injection. This route is favored compared to an intravenous (i.v.) infusion because a more convenient self-administration by the patient is possible, and it is more cost-efficient for the health-care system as well.

A drawback of subcutaneously injected antibodies is their variable bioavailability, ranging from about 20% to about 100% of the dose compared to an i.v. injected antibody that by definition leads to 100% bioavailability [37].The reduced bioavailability is a consequence of the required transport of the protein from the injecting site, the subcutaneous fatty tissue to the lymphatic system, and the subsequent release into the central system, via the valve between the thoracic lymph duct and subclavian vein. Along this path, many factors could play a role in affecting the bioavailability such as:

Molecule related	Formulation related
Fc modifications (e.g., FcRn mutations, glycosylation, PEGylation) Size/molecular weight Hydrophobicity Charge (e.g., pI, distribution of the charge) Target mediated disposition	Conc./vol. pH Composition Active moieties (e.g., hyaluronidase)

Although some data are available about the general in vivo transport mechanisms and how certain modification in the primary sequence alter the pharmacokinetic of antibodies, little is known about what happens after s.c. injection, which formula-

tion factors may alter bioavailability, and what happens to the antibody, especially to the portion that do not reach the central system?

These uncertainties usually require for additional clinical bioavailability (BA)/bioequivalence (BE) studies when changes in the formulation/dosage form occur during development. In general, authorities would consider a bioavailability between 80% and 125% as bioequivalent given the inherent variance of clinical studies. [38]

What seems to be a drawback when changing the composition could become interesting, either as a life-cycle management option or as a formulation option for new proteins to achieve an improved bioavailability.

A prerequisite for any approach to enhance bioavailability, either by protein engineering or by formulation, would rely on an in-depth understanding of the interactions with the respective biologic environments and the various factors that govern the transport of the protein from the injection site (e.g., s.c. or i.v.) to the target tissue.

Therefore, analytical models are required to study the transport and to determine the effect of:

- Formulation parameters on the bioavailability.
- Stability of the protein (metabolites) in the respective tissues.
- In vivo posttranslational modifications on either potency or immunogenicity.

It is necessary to understand the relationship between formulation and molecule parameters and their biological consequences. There is little doubt that due to the ever-increasing need to differentiate the biological functionality and performance of a given molecule, the demand for testing such additional features will grow.

The lack in the availability of such predictive methods and models will result in either a delay of the development or the need to increase clinical testing which raises practical and potentially ethical concerns. Therefore, human studies can only be designed to proof a given mechanism. They would not allow performing series of studies necessary to understand the complex interaction with biology to develop an optimized prototype, e.g., formulation.

The example shown in Fig. 19 demonstrates the potential impact the formulation can have on the bioavailability of a monoclonal antibody. It shows the results of a clinical study, where the formulation showed an impact on bioavailability following s.c. administration. Formulation #3 showed a distinct increase in the area under the curve compared to all other formulations tested. This example indicates the necessity to understand the molecule and formulation parameter that are affecting bioavailability early in development.

Most preclinical in vivo models, however, are focused on molecule-specific pharmacodynamics, pharmacokinetic, or toxicology, and they are not intended of being used as a formulation screening tool.

Currently, the standard for PK studies is to use rats and monkeys species [39]. However, skin morphology of furred animals such as rats and monkeys is different from humans and pigs, due to the loose attachment of the subcutis to the muscle layer underneath. [40, 41] Additionally, these animals have a muscle called pan-

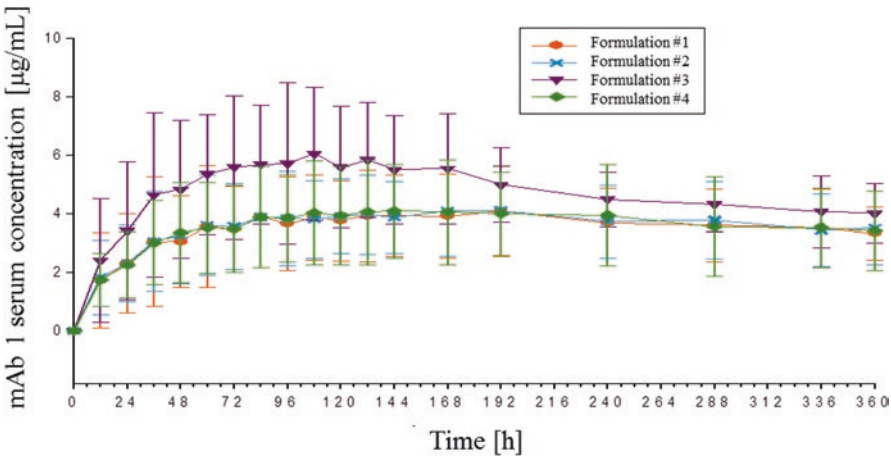


Fig. 19 Clinical study – formulation-dependent difference in PK profile of different antibody formulations after s.c. injection (dose = 40 mg; n = 23–24)

niculus carnosus, which is different from humans and pigs where the s.c. layer is tightly attached to the muscle underneath and has no or only a rudimentary amount of panniculus carnosus muscle.

The pig is an expensive but structurally more comparable model compared to the rat [42]. In general, the correlation of bioavailability between humans and animals is highly variable. [43, 44] In order to reliably predict effects on bioavailability, the scaling among species and model variability need to be better understood.

Nevertheless, s.c. in vivo studies can be used to study how biologics are transported and learn more about the complex interplay of the various processes involved in the transport of the protein from the injection site to the central circulation.

Furthermore, these s.c. models may be used for comparing the performance of different formulation compositions that would allow for a prescreening and allow the selection of the most promising formulation such as Formulation #3 in Fig. 8. Interestingly, there is almost no literature using PK model in such a way. Ideally, such studies are being complemented by measuring the metabolization of the protein over time to get information whether presystemic degradations might occur and potentially affect the bioavailability.

4 Concluding Remarks

Formulation development for antibodies has always undergone dramatic changes. From the beginning in 1890 the serum therapy was discovered by Von Behring that lead to the first therapeutic application of antibodies to the mind-boggling diversity of antibody-like molecule formats we a formulation scientist faces today. This chapter aims to provide the reader with a brief outlook of what can be expected for the

future. By looking back to where we came from, it was always the case that some disruptive technological innovation required from the formulation scientist to keep-up and develop new ideas to harness a new technology and convert it into viable drug product concepts. It will be certainly an interesting journey how the astonishing new capabilities that are almost within reach such as big data, artificial intelligence, lab automation, and in silico modeling will – once again – change the way we will be developing biotherapeutics in the not so far away future.

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Chapter 14
Practical Considerations in High Concentration Formulation Development for Monoclonal Antibody Drug Products

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1 Introduction

High-concentration protein formulation (HCPF) is a term that is used to describe protein formulations, mostly monoclonal antibody (mAb) drugs, at high protein concentration. The concentration is rarely defined, with typical protein concentration at or above 100 mg/mL for mAbs.

In the last two decades, monoclonal antibody (mAb) therapeutics have gained tremendous popularity due to their high target specificity, efficacy, and low toxicity. In parallel, high concentration formulations have assumed increasing importance in supporting drug product development. As shown in Fig. 1, the number of FDA-approved drug products with protein concentrations ≥100 mg/mL has significantly increased over the last decade.

A detailed list of the high concentration (≥100 mg/mL) mAb drug products approved by FDA in the past two decades is summarized in Table 1. The table includes approval year, formulation components, route of administration, and primary containers information obtained from the corresponding drug product labels. About 50% of the high concentration mAb products were approved by FDA in the last 3 years (2016–2018).

The need for high concentration drug products is usually driven by the desire to increase the drug dose without increasing the dose volume, leading to improved efficacy or reduced dosing frequency, as well as making possible additional delivery routes such as by subcutaneous (SC) injection. For example, the Trogarzo formulation (Table 1) contains 150 mg/mL of ibalizumab for IV infusion; the total dose volume required to deliver a 2000 mg dose is 13.3 mL. In comparison, a 100 mL dose volume would be required to deliver the same 2000 mg dose using a formulation

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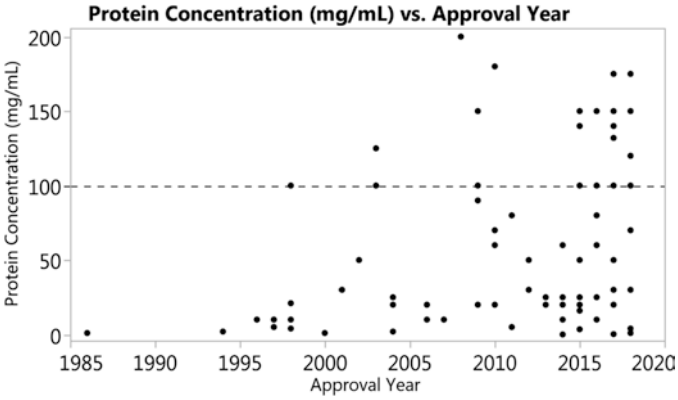

Fig. 1 Protein concentrations in FDA-approved mAb drug products

containing only 20 mg/mL of ibalizumab. As an added benefit, it is easier to transfer a smaller volume of drug product from the product vial into the IV bag for the infusion. In addition, higher concentration formulations are suitable for delivering the reduced dose volumes required for SC administration, which provides the option of relatively convenient, low-cost patient self-administration using readily available autoinjectors. Enabling patient self-administration reduces healthcare cost and relieves the burden on the healthcare system. In recent years, some products have switched from commercialized IV product to SC product by increasing protein concentration in formulations, such as Actemra SC (180 mg/mL), Herceptin SC (120 mg/mL), and MabThera SC (120 mg/mL). Finally, high concentration formulations lower the drug substance and drug product volumes, reducing processing and storage requirements during manufacturing.

While high concentration formulation drug product provides patients with convenience, important considerations must be built into the development of such formulations and products. Molecular interactions of mAbs, such as reversible and irreversible aggregation, and the solution viscosity may increase as protein concentration increases. In general, high concentration formulation development may encounter formulation, analytical, manufacturing, and delivery challenges.

Over the last decade, several excellent reviews have been published addressing high concentration formulation development: Shire and Shahrokh et al. have discussed solubility, stability, manufacturing, and analytical challenges in developing high concentration formulations with case studies [1, 2]; Warne proposed a platform approach for using high concentration formulations in early-stage clinical trials [3]; Hofmann and Gieseler reviewed different screening approaches and the use of predictive data to estimate protein solubility, viscosity, and stability at high concentrations [4]. Challenges in high concentration drug substance manufacturing and drug product fill/finish process have been recently reviewed by Piedmonte et al. [5] With the learnings from previously published work, this book chapter covers practical consideration and strategies in developing high concentration formulations and

Table 1 FDA-approved high concentration mAb drug products

Brand name	Generic name	IgG type	Approval year	Delivery	Dosage form	Protein concentration (mg/ml)	pH	Excipients	Primary container
Synagis	Palivizumab	IgG1	1998	IM	Lyophilized	100	6.0	Histidine, mannitol, glycine	Vial
Synagis		IgG1	2004	IM	Liquid	100	6.0	Histidine, glycine	Vial
Raptiva[a]	Efalizumab	IgG1	2003	SC	Lyophilized	100	6.2	Histidine, sucrose, PS 20	Vial
Xolair	Omalizumab	IgG1	2003	SC	Lyophilized	125	–	Histidine, sucrose, PS 20	Vial, PFS
Cimzia	Certolizumab pegol	Fab	2008	SC	Liquid	200	4.7	Acetate, sodium chloride	PFS
Cimzia		Fab	2008	SC	Lyophilized	200	5.2	Lactic acid, sucrose, polysorbate	Vial
Simponi	Golimumab	IgG1	2009	SC	Liquid	100	5.5	Histidine, sorbitol, PS 80	PFS
Ilaris	Canakinumab	IgG1	2009	SC	Lyophilized	150	–	Histidine, sucrose, PS 80	Vial
Actemra	Tocilizumab	IgG1	2010	SC	Liquid	180	6.0	Histidine, arginine hydrochloride, methionine, PS 80	PFS
Nucala	Mepolizumab	IgG1	2015	SC	Lyophilized	100	7.0	Phosphate, sucrose, PS 80	Vial
Praluent	Alirocumab	IgG1	2015	SC	Liquid	150	6	Histidine, sucrose, PS 20	PFS
Repatha	Evolocumab	IgG2	2015	SC	Liquid	140	5	Acetate, PS 80, proline	PFS
Repatha		IgG2	2017	SC	Liquid	120	5	Acetate, PS 80, proline	Cartridge
Cosentyx	Secukinumab	IgG1	2015	SC	Liquid	150	5.8	Histidine, trehalose dihydrate, PS 80, methionine	PFS
Cosentyx		IgG1	2015	SC	Lyophilized	150	5.8	Histidine, sucrose, PS 80	Vial
Anthim	Obiltoxaximab	IgG1	2016	IV	Liquid	100	5.5	Histidine, sorbitol, PS 80	Vial
Zinbryta	Daclizumab	IgG1	2016	SC	Liquid	150	6	Succinate, sodium chloride, PS 80	PFS
Tremfya	Guselkumab	IgG1	2017	SC	Liquid	100	5.8	Histidine, sucrose, PS 80	PFS
Kevzara	Sarilumab	IgG1	2017	SC	Liquid	132, 175	6.0	Histidine, sucrose, arginine, PS 20	PFS
Hemlibra	Emicizumab	IgG4	2017	SC	Liquid	150	6.0	Histidine, arginine, poloxamer 188, aspartic acid	Vial

(continued)

Table 1 (continued)

Brand name	Generic name	IgG type	Approval year	Delivery	Dosage form	Protein concentration (mg/ml)	pH	Excipients	Primary container
Siliq	Brodalumab	IgG2	2017	SC	Liquid	140	4.8	Glutamate, proline, PS 20,	PFS
Dupixent	Dupilumab	IgG4	2017	SC	Liquid	150	5.9	Acetate, histidine, sucrose, PS 80, arginine hydrochloride	PFS
Dupixent		IgG4	2018	SC	Liquid	175	5.9	Acetate, histidine, sucrose, PS 80, arginine hydrochloride	PFS
Ilumya	Tildrakizumab	IgG1	2018	SC	Liquid	100	5.7–6.3	Histidine, sucrose, PS 80	PFS
Emgality	Galcanezumab	IgG4	2018	SC	Liquid	120	5.3–6.3	Histidine, sodium chloride, PS 80	PFS
Trogarzo	Ibalizumab	IgG4	2018	IV	Liquid	150	6.0	Histidine, sucrose, sodium chloride, PS 80	Vial
Takhzyro	Lanadelumab	IgG1	2018	SC	Liquid	150	6.0	Citric acid, histidine, PS 80, sodium chloride, sodium phosphate	Vial
Ajovy	Fremanezumab	IgG2	2018	SC	Liquid	150	5.5	Histidine, sucrose, PS 80, EDTA	PFS

[a]Withdrew from market in 2009

EDTA ethylenediaminetetraacetic acid; Fab antibody binding fragment, *IgG* immunoglobulinG, *IM* intramuscular, *IV* intravenous, *PS* polysorbate, *SC* subcutaneous, *PFS* prefilled syringe

drug products for commercial use. Based on authors' hands-on experience as well as literature findings, the discussions in this chapter focus on formulation and analytical aspects, as well as primary container considerations for high concentration formulation development. Drug substance and product process development, device selection, and administration of high concentration formulation drug products are discussed in other chapters of this book in details.

2 Considerations on HCPF Properties

High concentration formulations typically contain ≥100 mg/mL of mAb, stabilized with excipients. With the increase in antibody concentration, the intermolecular interactions between the individual drug molecules, as well as the interactions between mAb and excipient, increase, leading to increased reversible and irreversible aggregation, phase separation, precipitation, solution coloring and opalescence, and increased formulation viscosity. In addition, potential shear thinning or shear thickening occurring at high drug concentration can lead to variability in fill volume during drug product manufacturing. Impurities such as host cell proteins, which are often controlled at very low levels in low concentration mAb formulations, may be co-concentrated in high concentration formulations, leading to increased interactions with drug and excipient and posing unexpected challenges in formulation stability.

2.1 Solubility

Solubility is defined as the mAb concentration at which the chemical potentials of the aqueous and solid phases are equal. When mAb concentrations are near or over the solubility limit (i.e., supersaturation), it may lead to opalescence, aggregation, turbidity, or even amorphous protein precipitation and crystallization, presenting challenges in formulation development, product manufacture, and stability. To achieve a stable formulation, the mAb concentration must be significantly below its solubility limit. Therefore, it is critical to understand the solubility of the mAb during the high concentration formulation development.

Solubility Limit

Two types of solubility are often reported in literature, kinetic and thermodynamic solubility. Kinetic solubility refers to the solubility measured while the solid phase is not stable. Several methods are available for characterizing mAb kinetic and thermodynamic solubility in aqueous solution. Kinetic solubility is usually determined from a pre-dissolved liquid stock solution by switching the solvent, concentrating

the antibody, or adding a precipitant. Thermodynamic solubility refers to the solubility measured while the solid phase of drug substance is present and stable. Thermodynamic solubility is measured by adding excess antibody solid into the solvent and measuring the antibody concentration in aqueous phase at equilibrium.

Methods for measuring mAb kinetic solubility include ammonium sulfate or PEG (polyethylene glycol) precipitation and dissolution of crystalline/amorphous antibody into aqueous medium [6–8]. When using the precipitation methods, addition of charged ammonium sulfate could result in pH shift of the buffer, which can significantly impact mAb solubility. PEG-induced precipitation could be effective in rank ordering the relative solubilities of studied mAbs but is ineffective in predicting mAb solubility [9]. Monoclonal antibody thermodynamic solubility can be evaluated by concentrating mAb solutions with ultracentrifugation and assessing for precipitation. Results obtained from this solubility evaluation should be interpreted with caution as shear stress generated by ultracentrifugation could lead to mechanical stress-induced antibody unfolding [10]. In addition, clogging of ultracentrifugation membrane by gel formation could lead to underestimation of the solubility. To measure the thermodynamic solubility of antibodies, mAb solid is required. When preparing mAb solid for measuring thermodynamic solubility, mAb might be partially denatured during the drying process due to the dehydration stress, interfacial stress, freezing stress, or thermal stress on antibodies [11].

Antibody solubility is dependent on antibody properties (such as amino acid sequence, posttranslational modifications, secondary and tertiary structure, solid/crystal form) as well as environmental factors (such as temperature, pH, excipients, and ionic strength). Since most of the high concentration mAb drug products are stored at 2–8 °C, the solubility at 2–8 °C is practically relevant. Although monoclonal antibodies are typically highly water-soluble due to the presence of numerous hydrophilic residues, their solubility at 2–8 °C can also be substantially impacted by the formulation compositions. For instance, formulation pH and ionic strength affect the solubility by modulating charge properties of the mAb and altering intermolecular hydrophobic and electrostatic interactions. Mariya et al. observed precipitation and gelation of mAb-G (pI ~9) at 91 mg/mL after increasing the solution ionic strength from 15 mM to 165 mM [12]. In this instance, the increase in ionic strength resulted in the reduced mAb-G solubility likely by shielding the net charge of antibody molecules, leading to decreased repulsive electrostatic interactions. This explanation was also supported by the diffusion interaction parameter, k_D, which decreased from −15 mL/g to −26 mL/g at pH 7 when the corresponding ionic strength was increased from 15 mM to 165 mM. In addition, across the tested pH range from 5 to 8, the k_D values are generally lower at the high ionic strength, indicating stronger attractive intermolecular interactions. The solubility of mAb-G also has a slight pH dependence, with solutions of mAb-G being clear at pH 5, compared to the milky white appearance observed at higher pH. Considering the pI of mAb-G is around 9, increasing pH leads to less net charge of the antibody and weaker repulsive electrostatic interaction as supported by experimental charge measurement and theoretical charge calculation.

Solubility Enhancement Strategy

Since the solubility of a protein is determined largely by its amino acid sequence, it is imperative to include solubility as a critical quality attribute in preclinical candidate developability assessments. This allows for selecting candidate molecules with high solubility under platform formulation conditions for further preclinical and clinical development [13]. During manufacturing of high concentration formulations, drug substance concentrations higher than 200 mg/mL are typically required for manufacturing 150 mg/mL drug product formulations due to the dilution resulting from excipient spiking and from the desire to achieve sufficient process yield. To support high concentration formulation manufacturing, it is crucial to understand if the candidate molecule is soluble at concentrations of >200 mg/mL. Candidates of lower solubility typically require extraordinary effort to achieve the desired product profile required for successful commercialization.

Monoclonal antibodies often exhibit pH-dependent solubility, with solubility increasing as the solution pH gets further away from the isoelectric point (pI). The further pH is away from isoelectric point (pI), the more net charge mAb carries, leading to stronger repulsive electrostatic interaction between molecules and higher solubility [12]. As mentioned above in the case of mAb-G, solubility was favored by lower pH values since it's away from pI. Luo et al. also observed that mAb-A (pI = 7.9), mAb-B (pI = 8.3), and mAb-C (pI = 6.5) all showed highest turbidity at pH values closest to their respective pI [14]. As the formulation pH moved away from pI, the solutions became clearer. Raut et al. observed lower light transmittance of antibody solution (mAb-A) around its pI, which is an indicator of lower solubility [15]. When examining antibody solubility at various pH, stability and viscosity of the antibody should be studied simultaneously and incorporated into the selection of the optimal pH.

Salts can also influence mAb solubility. According to electrostatic theory, at low salt concentrations, the protein solubility increases in proportion to the square root of the ionic strength (salt-in) due to a corresponding decrease in the electrostatic free energy of the protein resulting from counterion shielding [16]. Conversely, high salt concentrations usually disturb the electrostatic interaction between water and protein necessary for protein hydration, leading to reduction in protein solubility (salt-out) [16]. The definitions of "low" and "high" salt concentration are often antibody dependent. Priscilla et al. investigated the effects of sodium chloride addition to mAb5 formulations [17]. The inclusion of even 15 mM sodium chloride reduced the apparent solubility of mAb5 by more than 50%. The effects of salts on antibody solubility should be evaluated on a case by case basis. For protein formulation development, salt concentration is typically below 150 mM to keep the formulation isotonic.

Other excipients can enhance mAb solubilization through preferential interaction, preferential hydration, dispersive interaction, and hydrogen bonding [18, 19]. For example, arginine hydrochloride is a well-known solubility enhancer and has

been used in several high concentration commercial mAb formulations including Hemlibra, Dupixent, and Kevzara (Table 1). Luo et al. demonstrated that with an increase of arginine hydrochloride from 0 to 100 mM in the formulations, the solution became clear for all three antibodies (mAb-A, mAb-B, and mAb-C) regardless of their pI [14]. The solubility-enhancing effect of arginine hydrochloride was attributed to a reduction in attractive intermolecular forces, as supported by a decrease of negative k_D value. Chavez et al. observed higher solubility of a model IgG3 antibody in an arginine hydrochloride formulation comparing to acetate or histidine formulations, indicated by higher UV/Vis transmission at 410 nm [20]. The UV/Vis transmission at 410 nm is further enhanced with higher concentration of arginine hydrochloride at 200 mM.

Buffer type also plays a role in antibody solubility because many buffering agents used in protein formulations are also salts. As shown in Fig. 2, in addition to the pH effects on solubility, buffer type (citrate, acetate, histidine, or phosphate) also contributes to the solubility of an IgG1 antibody [21]. At pH 5, the solubility of this mAb behaved quite differently in four buffers, with an over threefold higher solubility in acetate buffer versus in citrate buffer.

All abovementioned factors, pH, ionic strength, salt, solubility enhancer, and buffer, may influence mAb solubility. Meanwhile it is also important to realize the theoretical limit of mAb solubility due to steric restrain. Garidel et al. mapped out the maximum solubility of mAb by packing molecules in a lattice [22]. Regardless of the packing models, it was concluded that mAb concentration above 500 mg/mL will be extremely difficult to achieve. In addition, the viscosity or osmolality limit may be reached before antibody concentration approaching 500 mg/mL.

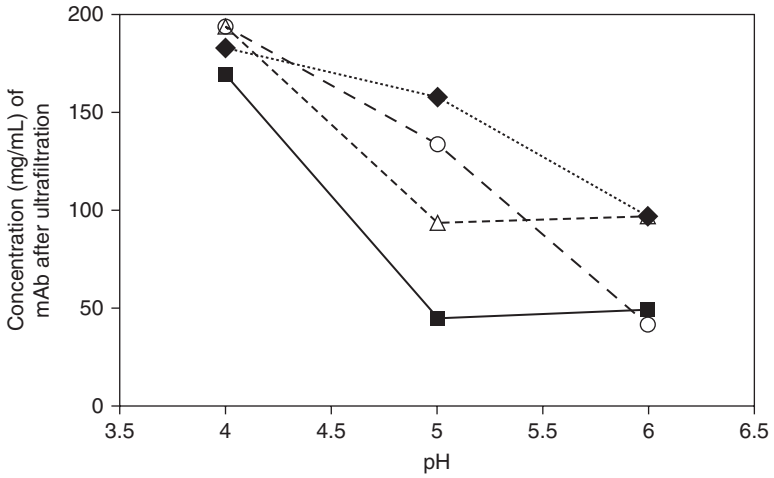

Fig. 2 Effect of buffer on the apparent solubility of an IgG1 mAb as a function of pH. 50 mM citrate (solid squares), 50 mM acetate (solid diamonds), 50 mM histidine (open triangles), or 50 mM phosphate (open circles)

2.2 Increased Aggregation

Aggregates formation in protein formulation is widely recognized as a critical quality attribute for drug product. As protein aggregates are potentially linked to immunogenicity [23], controlling the amount of protein aggregates in the final drug products is an important development goal. Minimizing the amount of protein aggregates in high concentration antibody formulations can be especially challenging.

Typical mAb aggregates can be classified based on their size: (a) aggregates ranging from dimer to oligomers, i.e., high molecular weight species (HMWS) characterized by size exclusion HPLC method, (b) submicron and subvisible proteinaceous particles with size range from ~50 nm to ~50–70 μm, and (c) visible proteinaceous particles larger than 70 μm. Although protein aggregation is a large and diverse topic [23–26], the discussion in this chapter is limited to soluble aggregates (HMWS), which comprise the most common form resulting from mAb aggregation and may lead to the formation of submicron, subvisible, and even visible particulate formation.

HMWS can form during many stages of a product manufacturing process and the product life cycle, including drug substance manufacturing (cell culture process, purification, and formulation), drug substance storage and handling, drug product manufacturing (freeze and thawing, mixing, hold in the bulk containers, filtration), drug product storage and handling, and finally, dose preparation and administration [27]. Formulation composition will impact HMWS formation at each step of manufacturing, handling, and storage. Practical considerations must be built into the development of stable formulations for both drug substances and drug products, and case studies are presented here demonstrating effective strategies for controlling HMWS formation.

Concentration-Dependent Aggregation

As HMWS formation typically involves two or more antibody molecules, HMWS formation rate is generally protein concentration dependent. The higher the antibody monomer concentration, the higher the rate of HMWS formation. This is generally true for the formation of dimer and other high molecular weight species. For liquid drug product, high concentration drug products generally have higher aggregation rates during the shelf life storage, or at accelerated or stress conditions comparing with low concentration formulations, despite efforts of formulation optimization.

As an example, Fig. 3 shows the %HMWS formation as functions of protein concentration at 5 °C, 25 °C, and 45 °C for an R-mAb1 formulation. With the same excipients and pH, the 150 mg/mL formulation showed significant higher aggregation rate than the 25 mg/mL formulation at all three temperature conditions.

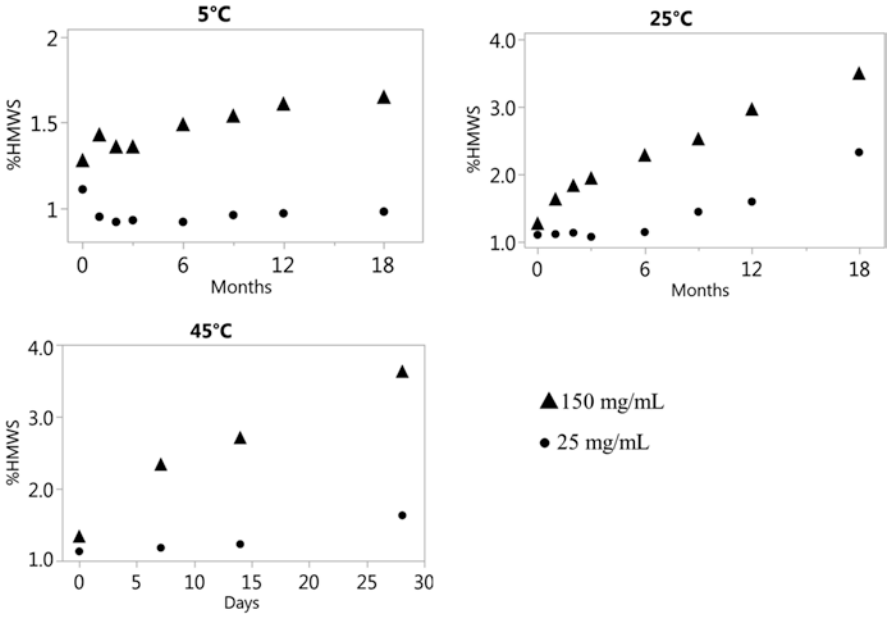

Fig. 3 %HMWS formation for R-mAb1 formulations at 25 mg/mL and 150 mg/mL

In this example (Fig. 5), both the 25 mg/mL and the 150 mg/ml formulations were prepared from the same lot of R-mAb1 drug substance. The initial %HWMS for 25 mg/mL formulation is 1.1%, 0.2% lower than that of the 150 mg/mL formulation. The HMWS in the 25 mg/mL R-mAb1 continues to dissociate during the first 2 months of storage at 5 °C. This indicated the dissociation of the HMWS when diluted to lower R-mAb1 concentration. The dissociable HMWS is a result of protein-protein self-interaction (or self-association) [28] and is in equilibrium with monomers. Such dissociable HMWS, predominantly dimers [29, 30], also form during the 2–8 °C storage in the high concentration mAb formulations. During the drug product development, HMWS formation is closely monitored and controlled at long-term storage even though these soluble reversible dimer species may retain their native structure and biological activity [29–31].

Minimizing Aggregation in Liquid Formulation

Optimizing formulation composition is one of the most effective ways in minimizing and controlling aggregation in high concentration formulations. A high concentration mAb formulation typically is composed of mAb, buffer, and excipients (stabilizers, viscosity reducers, and surfactants) at a defined pH. As shown in Table 1, commonly used buffers for high concentration formulations are histidine, acetate, citrate, and phosphate. Polyols, such as sucrose, mannitol, trehalose, and sorbitol, are mostly used as stabilizers. Amino acids including proline, glycine, and

arginine were used for several products either as stabilizers or viscosity reducers. The pH range for the high concentration mAb products is typically within 4.5–7.5. Salts including NaCl and $CaCl_2$ were used as viscosity reducers or tonicifiers. Polysorbate 20 and 80 are commonly used surfactants for their role in stabilizing protein against the interfacial stress. These excipients and pH must be carefully screened and optimized to minimize HMWS formation.

It is recommended that the excipient screening studies be conducted at the targeted high protein concentration when possible. This will ensure the HMWS studied are relevant and the degradation rate is representative to the final product formulation. The formulation pH often has the most impact on HMWS formation of mAb formulations and should be studied as early as possible. The pH range of the commercial high concentration mAbs, 4.5–7.5, is a good starting range. The buffers selected should have sufficient buffering capacity at selected pH, generally within one pK_a unit of the buffer agent. Although 10 mM of buffer agent is typical for many mAb formulations, higher buffer concentration, such as 20 mM or higher, has been used to ensure the adequate buffering capacity for high protein concentration formulation and in certain cases to improve the product stability. For example, 25 mM histidine buffer was used for 175 mg/mL Kevzara formulation (Table 1). High temperature stress conditions are commonly used for formulation screening. The typical stress conditions used for high concentration mAb formulation studies include 37 °C, 40 °C, and 45 °C. Thermal stresses accelerate the degradation rates to allow differentiation of formulation effects in a short development time, e.g., 2–8 weeks during formulation screening. Figure 4 shows an example of pH effect on %HMWS for a 150 mg/mL R-mAb2 formulation studied at 45 °C. The formation of HMWS is highest at pH 7.0 in phosphate buffer and pH 4.5 in acetate buffer. The pH range of 5.3–6.0 is optimal for further R-mAb2 formulation development.

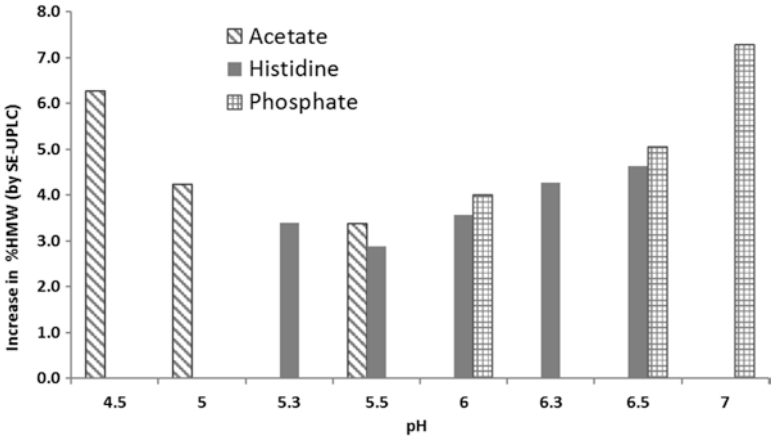

Fig. 4 HMWS increase after 28 days at 45 °C during the pH and buffer screening of 150 mg/mL of R-mAb2

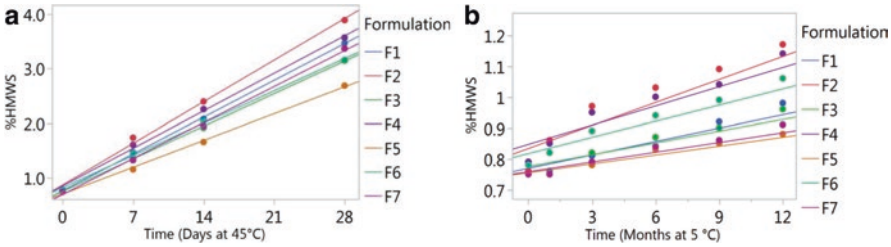

Fig. 5 %HMWS formation of 150 mg/mL R-mAb3 formulation under different excipient conditions at pH 6.2 (**a**) HMWS formation at 45 °C (**b**) HMWS formation at 5 °C

Similar to pH and buffer effect, excipients often have significant impacts on HMWS formation during product storage. Fig. 5a showed that changing the stabilizer type and amount impacts thermal stability of 150 mg/mL R-mAb3 at optimized pH of 6.2 under stress conditions (45 °C for 28 days). The stabilization effect of excipients observed under stress conditions also translated to the liquid drug product storage stability (Fig. 5b). In this R-mAb3 case study, F5 is the most stable formulation, and F2 is the least stable formulation under both stress and storage condition.

Stress Condition Selection for Formulation Screening

For formulation screening, the stability studies are usually performed at stress or accelerated temperature rather than at shelf life storage temperature to understand the degradation pathway of molecules and to shorten the development timeline. While such accelerated or stress stability testing allows prediction of the drug degradation rate for small molecules, which is typically described by the Arrhenius equation, it has been a challenge to apply this approach to determining the degradation rate of high concentration mAb formulations, particularly when it pertains to predicting the rate of HMWS formation. This is primarily due to different aggregation (HMWS formation) mechanism. For example, the underlying degradation mechanism at high temperature may primarily involve thermal unfolding, whereas aggregation at lower temperatures, such as 25 °C or 5 °C, is generally due to protein associations governed by colloidal stability [32–34]. Due to such difference in aggregation mechanisms, formulation selection solely based on stress stability sometimes could be biased. As an example, Fig. 6 illustrates the effect of temperature on the rate of HMWS formation for two high concentration R-mAb4 formulations (F1 and F2) that employed different stabilizers. From 45 °C stress stability, F1 appeared to be the more stable formulation regarding the HMWS formation. Interestingly, F2 was more stable regarding HMWS formation at 25 °C; and the same trend is also observed during the long-term storage (5 °C) that F2 is more stable than F1. The results indicated that 25 °C is a better accelerated condition predictive of long-term storage stability instead of 45 °C for the R-mAb4 development studies. Close examination of the SEC chromatogram (data not shown)

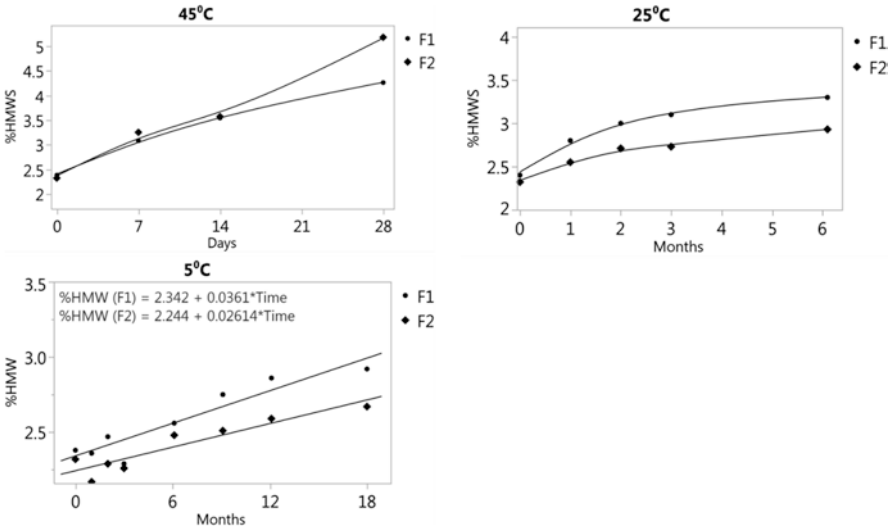

Fig. 6 Formation of HMWS of two R-mAb4 high concentration formulations at 45 °C, 25 °C, and 5 °C

revealed that under 45 °C stress, dimer formation followed by higher-order aggregates is the main aggregation pathway, while at 25 °C and 5 °C, the main aggregation pathway is the dimer formation. The results further confirmed the different aggregation mechanisms at different temperature stress conditions. In short, for high concentration formulation development studies, it is important to characterize mAb stability under different temperature conditions early in the development and select appropriate accelerated temperature conditions for formulation screening studies.

Aggregation During Drug Substance Frozen Storage

Most of the high concentration mAb drug products are kept at 2–8 °C for long-term shelf life storage. The bulk drug substance for manufacturing high concentration mAb drug products, however, is often stored under frozen conditions for varying periods of time prior to its use in manufacturing the final drug product. Ensuring the stability of the bulk high concentration drug substance during the frozen storage is an integral part of the high concentration drug product development.

As mentioned in Sect. 2.2.2, high temperature stress conditions such as 40 °C and 45 °C to accelerate protein aggregation are commonly used for formulation screening, and high concentration drug product formulation is often selected from such stress studies. However, high concentration formulation selection solely based on stress stability may put the frozen storage stability at risk. Figure 7 showed an example of frozen storage stability of 175 mg/mL R-mAb5 formulation. The 175 mg/mL R-mAb5 formulation was selected based on a stress stability screening study and has an acceptable long-term storage stability at 2–8 °C, with 0.5% increase

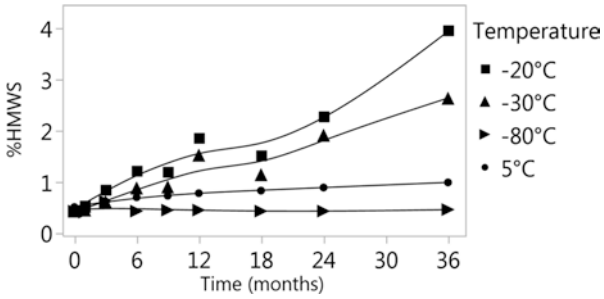

Fig. 7 Long-term storage stability of 175 mg/mL R-mAb5 formulation selected based on the stress stability

in HMWS after 36 months of storage. Interestingly, the same formulation was not stable when stored frozen at −20 °C or −30 °C, which are the preferred storage condition for drug substance.

Stress and/or accelerated stability of liquid formulation may be indicative of liquid drug product storage stability, but it may not translate into formulation frozen storage stability as protein-excipients interactions in solution state and in frozen state are very different. Protein stabilization by excipients at solution state has been well-reviewed [18, 19]. Some excipients stabilize proteins in solution by excluded volume effect and preferential interaction, other excipients may have different mechanism such as cohesive force, etc. All the excipient stabilizing effect results from fundamental interactions such as electrostatic and cation-pi interactions, dispersive forces, and hydrogen bonding between protein and excipient. Since the molecular solvation state, physical state, effective protein concentration, and molecular orientation and mobility are different between the liquid and frozen state, molecular interaction type and magnitude are different, resulting in different stabilization effect between liquid and frozen states. In general, sucrose is a superior stabilizer for the frozen storage comparing with other polyols, salts, and amino acids. Trehalose and sorbitol are also effective stabilizers for frozen storage of high concentration formulations. The ratio of stabilizers to mAb molecules should be optimized as part of the high concentration formulation development to ensure drug substance frozen storage stability.

Due to ice-crystal formation, cryo-concentration, and low temperature, freezing of a high concentration formulation may lead to crystallization of the formulation excipients, phase separation, and potentially protein cold denaturation [35, 36]. Conventionally, T_g' has been referenced as one of the factors to guide the choice of the frozen storage temperature for bulk drug substance in the absence of long-term storage data. It is based on the notion that molecular mobility is highly restricted below T_g' and storage of drug substance at a temperature below the T_g' would maintain long-term frozen storage stability. Selecting drug substance frozen storage temperature based on T_g', however, is often not reliable as frozen formulation stability cannot be explained solely by T_g' [37]. Currently there is no good accelerated model to simulate the drug substance frozen storage stability. An extended frozen storage time, such as minimum of 6 months, is typically required to observe frozen

state protein stability or instability [37]. For most of high concentration mAb formulations, −80 °C is a conservative storage condition for the bulk drug substance when stability at other frozen conditions is not known.

2.3 Increased Viscosity

Formulation viscosity is an important attribute due to its impact on not only the manufacturing process but on drug product handling and delivery. For example, when delivering a drug with an autoinjector, high viscosity requires high injection forces and prolongs injection time. Ideally, formulations intended for SC injection via autoinjector will have a viscosity below 10 cP at 20 °C, whereas below 20 cP is acceptable for manual injection [38].

Intermolecular interactions, such as electrostatic interactions, hydrogen bondings, van der Waals forces among solute molecules, as well as antibody networks and higher-order structures play important roles in determining solution viscosity [39]. At high mAb concentrations, due to molecular proximity, the interactions between antibody molecules, as well as between antibody and excipient molecules, increase, resulting in high solution viscosity [19]. The understandings on the viscosity of HCPF have been well-reviewed [40, 41]. Mechanistically, reversible cluster formation driven by the electrostatic interaction in high concentration formulations has been proposed and identified as a major cause of high viscosity [42–46]. Colloidal theories have been developed and applied to globular proteins to understand viscosity, but it is still difficult to model or calculate high concentration solution viscosity for monoclonal antibodies adopting anisotropic shapes.

Different mAb drug candidates, based on individual sequences and higher-order structures, have different viscosity properties. It is highly recommended to screen and select candidates with low viscosities during candidate screening to select the molecules with desired viscosity profiles to reduce risks associated with late stage and commercial development. Similar to solubility assessment, characterization of a candidate's viscosity in a defined formulation space, such as pH 5–7, aids in candidate selection. Molecular modeling and predictive biophysical characterization for colloidal stability, such as k_D and self-interaction nanoparticle spectroscopy (SINS), have been used to predict formulation viscosity but with limited success [47–49].

Once a mAb candidate is selected for high concentration formulation development, while the intrinsic properties of the mAb cannot be changed, formulation components such as buffer, pH, salt, and other excipients can be optimized to manage solution viscosity by minimizing intermolecular interactions in the formulation.

Viscosity-Reducing Strategy

For high concentration mAb formulations, factors affecting the formulation viscosity include mAb concentration, buffer type, buffer concentration, pH, excipient type, and excipient concentration. Excipients in formulations (Table 1) usually

include but not limited to buffering agents, thermal stabilizers, surfactant, viscosity reducer, chelators, and antioxidants [19]. The concentrations of surfactants, chelators, and antioxidants are usually low and practically have minimal impact on formulation viscosity. Buffers, pH, thermal stabilizers, and viscosity reducers could have an impact on ionic and other molecular interactions in solution, resulting in pronounced effect on formulation viscosity.

Buffer and pH

Formulation pH can significantly influence mAb formulation viscosity and should be explored prior to evaluating other methods of viscosity reduction. The selection of a specific formulation buffer will be driven by the pH required for optimal formulation viscosity.

Protein surface charge profile varies at different solution pH. When the pH is close to the pI, proteins carry as much negative charge as positive charge. When pH is several units away from the pI, proteins carry a significant positive or negative charge. At different pH, protein-protein electrostatic interactions may change significantly, resulting in different solution viscosity. Examples of viscosity-pH dependence for three mAb candidates are shown in Fig. 8. For mAbs with viscosity-pH dependence like R-mAb6, modifying formulation pH could be a simple solution to manage viscosity.

Different buffer types and concentrations can impact formulation viscosity. Charge state of the different buffer species, as well as buffer concentration, may influence intermolecular interaction between solute molecule and yield different formulation viscosities at the same pH. For this reason, buffer type should be included in the pH-viscosity screening to select the optimal combination with desired viscosity.

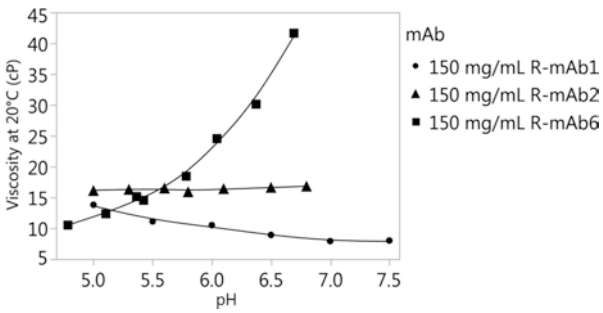

Fig. 8 Examples of viscosity-pH dependence of three mAb drug candidates at 150 mg/mL with no stabilizers or viscosity modifiers

Thermal Stabilizer

Thermal stabilizers such as polyols, for example, sucrose, trehalose, and sorbitol, are often added to mAb formulation to improve formulation stability. However, studies have shown that these molecules (trehalose, sucrose, sorbitol, glucose, fructose, xylose, and galactose) resulted in a significant increase in viscosity of high concentration mAb solutions [50]. To minimize the viscosity of high concentration formulations, lower amount of polyols, or alternative stabilizers such as arginine salt, lysine salt [51], or proline [52] (Table 1), could be considered.

Viscosity Reducer

Another strategy for controlling formulation viscosity involves the use of viscosity-reducing excipients, which include amino acids, inorganic salts, hydrophobic salts, and chaotropic reagents (Table 2).

For commercial drug product development, it is worth to mention that the use of novel viscosity reducers will require additional safety evaluations. To shorten the development timeline and to reduce the uncertainty of product safety, these novel excipients are rarely pursued for mAb drug product commercialization. When using viscosity reducers in high concentration formulations, it is also important to confirm the freedom-to-operate space as some of the viscosity reducers may be under intellectual property protection.

Table 2 List of viscosity reducers for high concentration mAb formulations

Category	Examples	Comments
Amino acid [51]	Arginine hydrochloride Arginine glutamate Histidine hydrochloride Sodium glutamate Lysine hydrochloride Proline [53]	Effective in reducing viscosity caused by protein-protein charge interactions. These excipients may also act as mAb stabilizers
Inorganic salt [51]	Sodium chloride Magnesium chloride Calcium chloride Sodium acetate Sodium sulfate Ammonium chloride	Effective in reducing viscosity caused by protein-protein charge interactions
Hydrophobic salt [54]	Procainamide-HCl Salt of camphor-10-sulfonic acid with L-arginine (CSA-Arg) [55]	Effective in reducing viscosity caused by hydrophobic protein-protein interactions
Chaotropic reagents [42]	Urea Guanidine hydrochloride	Reducing mAb solution viscosity by alteration of protein conformation
Other [56]	Imidazole Camphorsulfonic acid Taurine, theanine, sarcosine, citrulline, betaine [57]	

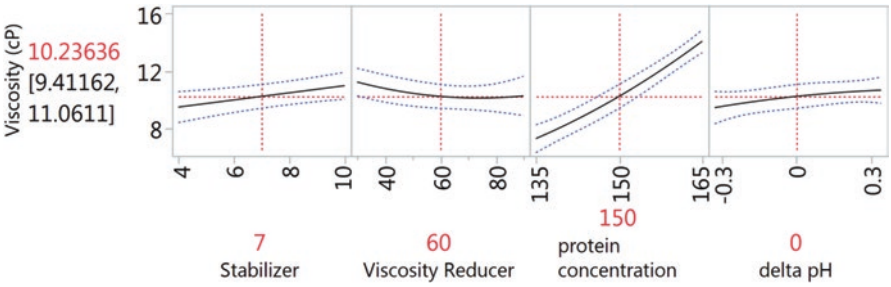

Fig. 9 A viscosity model built by a DOE study for R-mAb6. Dashed lines indicating the predicted viscosity range with 95% confidence interval

The effect of excipients on formulation viscosity could be evaluated individually or by a design-of-experiment (DOE) approach. DOE is effective in selecting formulation excipients and in optimizing excipient concentrations, protein concentration, and pH. Results from well-designed DOE studies can build an effective viscosity model in understanding the formulation viscosity with formulation composition, pH, temperature, and storage time. Figure 9 shows an example of a viscosity model built from a DOE study for R-mAb6. The model is effective in determining the target formulation composition and characterizing a formulation space.

While selection of formulation excipients is critical to manage high concentration formulation viscosity, it will also positively or negatively impact the product stability and solubility. Proper risk assessment should be performed, and all quality attributes, such as viscosity and aggregates, should be assessed together when designing studies to achieve a stable formulation with acceptable viscosity.

Alternative Strategy to Overcome High Viscosity Challenge

For some high concentration mAbs, the viscosity of the formulation may still be too high for SC injection, even after exhausting all the viscosity-reducing strategies detailed in Sect. 2.3.1. Alternative development strategies for reducing formulation viscosity include:

- Decrease the mAb concentration to achieve an acceptable viscosity, and deliver the desired dose by increasing the dose volume. For SC injections, large-volume injections [58, 59] can be achieved by using large-volume SC delivery system [60], using hyaluronidase [61], or even splitting the doses by multiple injections.
- Development of novel delivery device capable of handling high viscosity (>20–100 cP).
- Innovative formulations, such as nonaqueous suspensions and crystalline suspensions, could be considered for lowering formulation viscosity [62–65].

2.4 Increased Impurity Concentrations

Monoclonal antibodies are usually produced by Chinese hamster ovary cells. Trace amount of host cell proteins (HCP) from upstream process can be carried over to drug substance during purification process and becomes part of the drug product formulations as impurities. These HCP impurities usually present at very low concentrations (ppm), and the concentration of each individual HCP impurity may be too low to quantify. In low concentration formulations, the HCP concentrations remain low and usually do not impact formulation stability. In high concentration formulation, the HCP is co-concentrated with mAb, and the increased HCP concentration may impact the high concentration mAb formulation quality or stability.

For example, phospholipase B-like 2 (PLBL2 or PLBD2), an esterase presented as a HCP, can hydrolyze ester bond of polysorbate 20 or polysorbate 80, yielding free fatty acids and sorbitan head group [66–72]. Free fatty acids have very limited aqueous solubility and can form insoluble subvisible particulates in aqueous solutions. When trace amount of PLBL2 is co-purified with mAbs during manufacturing process, the concentration of PLBL2 increases as it is co-concentrated with mAb, leading to increased PLBL2 concentration in drug product formulations. Under 2–8 °C drug product storage condition, concentrated PLBL2 can degrade the surfactant, such as polysorbate 20 or 80, commonly presented in a mAb formulation, leading to formation of fatty acid subvisible particles [69], as well as loss of surfactant activity and in some cases the formation of protein aggregates, impacting drug product quality and stability [69, 71].

Thus, controlling HCP content during drug substance purification is particularly important for ensuring both the quality and stability of high concentration mAb formulations. Application of hydrophobic interaction chromatography in the protein purification process removes or reduces the host cell protein impurity such as PLBL2 [73]. Alternatively, if the risk of HCP impurity on mAb product quality has been identified, changes in cell line and expression system can be made to remove the expression of specific HCP [74].

3 Analytical Considerations

From drug discovery to product commercialization, different analytical methods are used for determining mAb structure and stability, formulation characterization, as well as drug substance/drug product release and stability testing. For high concentration mAb formulation development, the analytical considerations in this section focus on the methods used for formulation and drug product stability monitoring.

For the analytical methods limited by protein concentration, such as molecular variants by capillary electrophoresis, high concentration formulation samples will require a dilution step before analysis. When dilution is needed, it is important to

make sure the dilution process is well-controlled and compatible with the method. For analytical methods not limited by protein concentration, such as pH and appearance, many of the low concentration formulation analytical methods are generally applicable to high concentration formulations with minor modifications. Liquid chromatography methods, determining molecular weight species, charge variants, and protein concentration, can be applied from low to high concentration formulations with little modification as liquid chromatography has a built-in online dilution process and is capable of handling viscous solution. Modifications in injection volume and injection amount are usually sufficient. In the case of high viscosity samples, the needle withdrawing rate may need to be adjusted to ensure the accuracy of sample injection.

This section provided a few case studies in addressing analytical challenges for high concentration mAb formulations.

3.1 Protein Concentration Determination

Analytical methods in determining protein concentrations include UV spectroscopy, HPLC, intrinsic fluorescence spectroscopy, colorimetric assay, amino acid analysis, etc. For formulation characterization and stability monitoring, UV spectroscopy and HPLC are the most common methods, attributing to minimal sample preparation and generating accurate results quickly [75].

Protein concentration determination by UV detection is based on the Beer-Lambert Law, $A = \varepsilon \times l \times c$, in which A is the UV absorbance, ε is the extinction coefficient, l is the pathlength, and c is the protein concentration. For UV spectroscopy, with known pathlength and specific protein extinction coefficient, protein concentration can be measured directly ($c = A/\varepsilon l$). When using HPLC methods for protein concentration determination, a calibration curve is generated from a protein standard at the time of analysis, and the concentration of the protein is derived from the UV absorption area based on the standard curve. Between the two methods, UV spectroscopy does not require a standard or a calibration curve and can measure protein concentration directly. UV spectroscopy, therefore, is suitable for in-process monitoring as well as for product release and stability assessment.

While it is preferable to assay the mAb concentration in an undiluted sample as dilution may introduce errors and cause dissociation of some aggregates, there are challenges when directly assaying high mAb concentration formulations by UV spectroscopy.

Two types of UV spectrometers, fixed-pathlength spectrophotometers and variable-pathlength spectrophotometers, can be used to determine protein concentrations. When using fixed-pathlength spectrophotometers, protein concentration is determined from the measured UV absorbance, the pathlength, and extinction coefficient. In order to avoid UV absorbance saturation, the pathlength must be extremely narrow to measure high concentration samples. Studies have shown that using

pathlength as narrow as 0.01 cm on a conventional UV spectroscopy, mAb sample concentrations as high as 70 mg/mL can be measured accurately [76]; above this concentration, the UV absorbance will be out of the linear range, leading to underestimation of the actual mAb concentration by fixed path spectrophotometers [76]. The same concern exists with variable-pathlength spectrophotometers. Variable-pathlength UV spectrometer automatically adjusts the optical pathlength from 0 mm to 15 mm in 5-μm increments. The protein concentration is determined from the slope derived from the Beer-Lambert Law. Although the instrument manufacturers claimed that the variable-pathlength spectrophotometers are capable of measuring protein concentration over 100 mg/mL directly, studies have shown that protein concentration may still be underesimated [77]. An example of concentration underestimation using variable-pathlength UV spectrophotometers with R-mAb5 (extinction coefficient 1.39) is shown in Fig. 10.

In this example (Fig. 10), a high concentration R-mAb5 solution was diluted in a formulation buffer at 2-, 3-, 4-, and 5- fold by weight, and the concentrations of the undiluted and diluted solutions were measured in triplicates by SoloVPE variable-pathlength UV spectrometer. The theoretical concentration in x-axis was calculated by the concentration of the stock R-mAb5 solution devided by the actual dilution level. The stock R-mAb5 concentration was measured at four-fold of dilution. The higher the protein concentration, the more underestimation effect was observed in the measured concentrations. In addition, high variability was observed when measuring the high concentration sample (>200 mg/mL) directly by SoloVPE without dilution. The concentration underestimation together with the high variability make the direct measurement by variable-pathlength UV spectrophotometer unreliable for high concentration mAb drug substance and product in-process control or release.

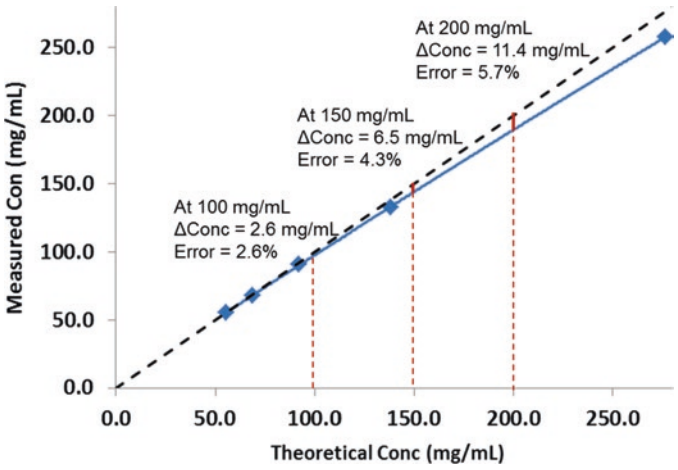

Fig. 10 Concentration underestimation for high concentration R-mAb5 formulation by variable-pathlength UV spectrophotometer

With current UV technologies, based on the study above, it is recommended to dilute the high concentration sample to lower than 100 mg/mL (such as twofold to fourfold of dilution) and measure the diluted samples by UV spectrometer (fixed-pathlength or variable-pathlength) for accurate concentration determination.

3.2 Subvisible Particulate Analysis

Subvisible particulates often present in mAb drug product formulations. Two compendial methods, light obscuration particle count test and microscopic particle count test, specified in USP <788> can be used to quantify the subvisible particulates. Light obscuration method is usually preferred when examining liquid mAb formulations for injections and infusions due to simple sample preparation and automated test procedure. When the light obscuration method cannot be used, such as with samples presenting reduced clarity or transparent particles, the microscopic method can be used for particulate counting.

In addition to the light obscuration and microscopic counting methods, flow microscopy with imaging capabilities, such as micro-flow imaging (MFI), is complementary to the two compendial methods on the characterization of subvisible particulates including the morphology, density, and transparency of the particulates.

The detection of subvisible particulates by MFI and light obscuration are both based on the refractive index difference between the subvisible particulates and the formulation solution. In high concentration mAb formulations, the refractive index of the formulation solution increases and can approach that of subvisible particulates, therefore adversely affecting the ability to detect the particulate matter. For example, the refractive index between the particulates and a mAb formulation (containing 20 mM histidine hydrochloride, 200 mM arginine hydrochloride, 0.04% polysorbate 20 at pH 6.0) decreased from 0.0623 to 0.0327 when protein concentration increased from 0 to 150 mg/mL. This resulted in undercounting of the subvisible particles in the 150 mg/mL formulation by the light obscuration method [78]. Light obscuration was more affected than flow microscopy by low contrast between the particles and the surrounding media due to detection mechanism [79].

In such cases, diluting the formulation to increase the difference in refractive indices between the particulates and solution phase can be a viable strategy for improving particulate detection. However, sample dilution may introduce artifacts, especially when the particulates are sensitive to overall protein concentration or formulation composition. For example, dilution may lead to either formation of new particulates or disruption of pre-existing particulates [79]. Therefore, the sample dilution strategy should be evaluated and confirmed by an orthogonal method that is not sensitive to refractive index or solution viscosity such as microscopy method or Coulter counter method.

When using MFI, quantitation of subvisible particles in high concentration formulations can be dependent on the particular blank solution chosen for illumination optimization. Illumination intensity needs to be adjusted before measuring each

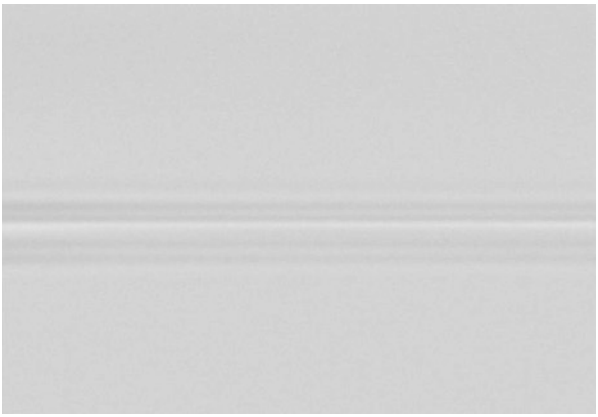

Fig. 11 Artifact from MFI subvisible particulate measurement in high concentration formulation samples when using placebo or water as blank

sample to ensure the optical setting is optimal in detecting the particulate matter. A blank solution, free of particulates with matching density, viscosity, and refractive index to the testing samples, is ideal for this step. Density and viscosity matching is important for ensuring homogenous flow-through of the sample after blank without reflux or heterogenous mixing in the flow channel. Matching refractive index is to ensure the optical setting is optimal for accurate detection of the subvisible particulates in the samples. For low protein concentration samples, typically purified water (Milli-Q or water for injection) or placebo can be used as the blank solution. This is usually not applicable for high concentration mAb formulation as using pure water or placebo does not match the density or viscosity of high concentration formulations and may result in mixing artifacts as shown in Fig. 11. Developing a surrogate blank solution matching all three properties for high concentration mAb formulation could be challenging. To minimize the artifact in subvisible particulates measurement, freshly filtered formulation matching the composition of the high concentrations samples can be used as the blank for micro-flow imaging illumination optimization.

4 Primary Container Considerations

During high concentration drug product development, it is important to integrate formulation selection with primary container selection. Different primary containers have different requirements regarding formulation viscosity and fill volume and should be evaluated early during high concentration formulation development. Commonly used primary containers for high concentration mAb formulations are made of glass or polymers and include vials, prefilled syringes (PFS), and cartridges (Table 1). Table 3 describes the characteristics of these three primary containers.

Table 3 Characteristics of commonly used primary containers for high concentration formulation drug products

Primary container	Vial	PFS	Cartridge
Delivery	Manual withdrawal to a syringe and inject	Manual injection or by autoinjectors	By injection pens or delivery device
SC delivery volume	Up to 2 mL	Up to 2 mL	Up to 10 mL (device)
Recommended SC injection time	≤10 sec	≤10 sec	Varies (10 sec–h)
Recommended formulation viscosity	≤20 cP	≤10–15 cP[a]	≤10–15 cP[a]
Convenience of use	Least convenient	Most convenient	Convenient
Cost	Low	Medium	High
Development time	Short	Medium for PFS; long for autoinjector	Long

[a]The viscosity range for PFS and cartridges is based on using 27 gauge thin-wall needle

Monoclonal antibody drug products for IV administration are usually presented in vials as vial products are versatile for both liquid and lyophilized formulation and flexible in delivering different doses, whereas drug products for SC administration use vials, PFS, and cartridges as primary container. When using vial products for SC administration, formulation in vials is withdrawn into a syringe with an appropriate-size needle for manual injection. PFS, on the other hand, does not require manual dose preparation and can be dosed directly to patients with or without a delivery device (such as an autoinjector). It enables patient self-administration and offers convenient dosing at home. The current upper limit of injection volume using PFS is about 2 mL. Dupixent 300 mg dose is delivered with a single 2 mL injection using 150 mg/mL formulation in 2.25 mL PFS. When large injection volume is desired, cartridge can be considered since the most important feature of cartridge is the capability of dosing greater than 2 mL of drug formulation. Cartridge is often developed with a compatible device, which could deliver the large volume of high concentration formulation subcutaneously in relative long period of time (>10 seconds). When PFS or cartridges are combined with delivery devices, the time for device development must be built into the overall product development.

While drug products with vials as primary container are common, PFS or cartridges have gained popularity due to the convenience of use and capability of home-based administration. The selection of PFS or cartridges is based on many factors, such as dose, patient needs, device compatibility, and market competitive landscape. When PFS or cartridge is used as primary container, it is important to balance dose concentration, dose volume, and formulation viscosity. A few examples for PFS and cartridge selection based on formulation concentration are given in Table 4.

Typically, the primary container selection starts with the understanding of the formulation viscosity and mAb concentration to enable the highest possible protein concentration with acceptable viscosity to deliver targeted doses. The formulation

Table 4 Examples of PFS/cartridge selection for given SC doses

Dose	Dose concentration (mg/ml)	Total dose volume (mL)	PFS or cartridge	Container size	Dose volume per container (mL)	No. of injections	Example
200 mg	200	1.0	PFS	1 mL	1.0	1	Cimzia
	175	1.14	PFS	1 mL	1.14	1	Kevzara
	150	1.33	PFS	2.25 mL	1.33	1	
	100	2.0	PFS	2.25 mL	2.0	1	
300 mg	200	1.5	PFS	2.25 mL	1.5	1	
	175	1.72	PFS	2.25 mL	1.72	1	
	150	2.0	PFS	2.25 mL	2.0	1	Dupixent
	100	3.0	PFS	2.25 mL	1.5	2	
			Cartridge	5 mL	3.0	1	
400 mg	200	2.0	PFS	2.25 mL	2.0	1	
	200	2.0	PFS	1 mL	1.0	2	Cimzia
	175	2.29	PFS	1 mL	1.15	2	
			Cartridge	5 mL	2.29	1	
	150	2.67	PFS	2 mL	1.33	2	
			Cartridge	5 mL	2.67	1	

viscosity against the variation of formulation composition, such as protein concentration and pH, and temperature within product specification must be fully evaluated. With the understanding of the highest possible protein concentration, selection of the primary container will be based on dose and dose volume, primary container compatibility, and device compatibility if applicable. The standard 1 mL long or 2.25 mL of PFS is usually the first option over vial or cartridge as they offer convenience for patient use. While 1 mL PFS is commonly used, several recent products, such as Siliq and Dupixent (Table 1), used 2.25 mL PFS to deliver 1–2 mL high concentration formulation with a single PFS. At any given dose, the priority is to deliver the dose with only one injection. Although not preferred, splitting one dose into two injections has been used in developing high concentration drug products. For example, Cimzia 400 mg dose is delivered using two 200 mg SC injections; Praluent 300 mg dose is administered by given two 150 mg Praluent injections consecutively at two different injection sites.

5 Summary

High concentration mAb formulations enable parenteral delivery of high drug doses in relatively small volumes. For high dose IV products, the reduced drug volume allows more facile dose preparation, whereas for SC administration, doses as high as 300–600 mg can be administered with only one or two injections. With the development of autoinjectors, high concentration SC products provide the convenient and cost-effective option for patient self-administration at home. From a logistic

perspective, high concentration formulations reduce the volume of the bulk drug substance that needs to be stored or handled during manufacturing. Practical considerations, as discussed in this chapter, should be built into high concentration formulation and product development, particularly when the formulation poses stability, viscosity, and/or analytical challenges. Each high concentration mAb formulation development may face one or more such challenges. Proper risk assessment should be performed to identify the development risks, and strategies to minimize the risks should be developed. When optimizing high concentration formulations, it is important to balance the effect of formulation composition on overall solubility, stability, and viscosity.

Quality target product profile (QTPP) has been widely used in the industry to establish the goals for the product development. For high concentration drug product development, it is crucial to understand the doses (or dose range) and product presentation requirements defined in QTPP. When target doses are clearly defined, the dose concentrations can be determined based on deliverable volume. When target doses are not defined at the time of formulation development, the formulation stability and viscosity should be characterized over a range of mAb concentrations (up to the highest possible concentration) to allow flexibility in the event of clinical dose change.

Many of the challenges encountered with analytical methods during high concentration formulation and product development can be managed by well-controlled dilutions. Other than the case studies presented in Sect. 3, it is also worth mentioning that high concentration proteins may also interfere with excipient quantitation using chromatography methods.

In addition to formulation development, many additional challenges are associated with primary container selection, device development for combination product, high concentration drug substance, and drug product manufacturing. Collectively with the experience and learnings from the marketed high concentration products, drug products with mAb concentrations ranging from 100 to 200 mg/mL mAb are achievable as evidenced by the increased number of such products in recent years. For future work in this area, development efforts in the following areas could potentially bring even higher concentration (>200 mg/mL) drug products to market: novel but safe viscosity reducers, devices for high viscosity product administration, stable crystalline protein nanosuspensions, and fill/finish facilities capable of handling highly viscous formulations (>100 cP).

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Chapter 15
Drug Product Formulation Development and Formulation Robustness Criteria for a mAb in a Pre-filled Syringe

Alavattam Sreedhara, Gregory Downing, and Karen Rutherford

1 Introduction

A majority of monoclonal antibodies (mAbs) are formulated for intravenous (IV) administration, specifically in oncology indications wherein the patient is dosed in a hospital setting or an infusion center. Since bioavailability through other routes is limited, IV dosing provides an easier alternate and has greater control during administration. The latter is particularly critical under conditions where there may be immediate adverse events and the dosing has to be monitored or stopped (such as during first in human trials). In addition, pharmaceutical development of typical IV administered drug products is faster than development of drug products administered using alternative delivery methods since many companies have significant experience in developing such formulations and potentially have a platform approach that they rely on to reduce timelines. Recently several subcutaneous (SC) products have been approved that are ready to use in a physician's office, clinic, or at home using either a pre-filled syringe (PFS), an auto-injector (AI), or patch pumps. AIs have shown to be more convenient for patients and ensure better compliance, especially while treating chronic conditions such as diabetes, rheumatoid arthritis (RA), or asthma. SC products have certain limitations during pharmaceutical development. Effective treatment using mAbs requires relatively high clinical doses, usually in the range of 5–700 mg per patient for flat doses or >1 mg/kg for weight-based dosing by IV administration. This is not a particularly challenging issue when given by IV because patients can be dosed with higher volumes (around 50–250 mL IV) over 60–90 minutes. However, SC administration has several limitations including volume per injection (usually 1–2 mL), bioavailability (typically 60–70% of IV dosing), and number of injections per dose cycle

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(preferably one or two injections). This makes development of high-concentration formulation mandatory if SC administration by PFS is called for in the target product profile (TPP). While patient convenience and compliance for SC formulations are an advantage, it adds a range of complexity to the pharmaceutical scientist developing high-concentration mAb formulations that will deliver the required efficacious dose in small volumes and in short injection times.

Quality by design (QbD) is a science- and risk-based approach to drug product (DP) development. While many of the same principles have been historically used during development, this knowledge was not always formally documented or proactively submitted to regulators. In recent years, the US Food and Drug Administration has launched an initiative for pharmaceutical quality for the twenty-first century to modernize pharmaceutical manufacturing and improve product quality. An important element of the QbD approach is the design and utility of multivariate experimental studies, especially to understand the robustness of the selected formulation. Results generated using these approaches can be used to strengthen data packages to support specifications and manufacturing ranges and hopefully simplify implementation of post-approval changes. Formulation scientists typically use a multivariate study with statistical analysis to comprehend any interactions between the formulation parameters, their ranges, and if there is any impact on protein product quality over time and temperature. Typical formulation parameters that are tested during a QbD filing for biologics and risk ranking tools have been discussed previously [1] and will not be a subject of this chapter. However, formulation and formulation robustness considerations while developing a mAb for use in a PFS will be described.

2 Formulation Considerations for a mAb in PFS

While working with high-concentration mAb formulations, the formulation scientist is typically faced with physical stability challenges such as aggregation and viscosity. These two important parameters can limit manufacturing processes and/or syringeability. For a thorough understanding of the formulation considerations for high-concentration mAb products, the reader is pointed to excellent articles by Shire et al. [2] and Whitaker et al. [3]

An important consideration at this point of development is the robustness of the formulation and a basic understanding of the interactions of various formulation parameters such as protein concentration, pH, surfactant, and any other excipients (e.g., viscosity reducing agents, etc.). A majority of chemical degradation pathways in proteins are concentration independent, and studies could be performed at lower protein concentrations. For example, selection of target formulation pH could be conducted at 1–10 mg/mL protein concentration even if the target protein concentration is >100 mg/mL. This could potentially save material during development. However, gelation, high viscosity (Fig. 1), and bi- and multi-molecular interactions leading to aggregates are directly proportional to protein concentration and need to

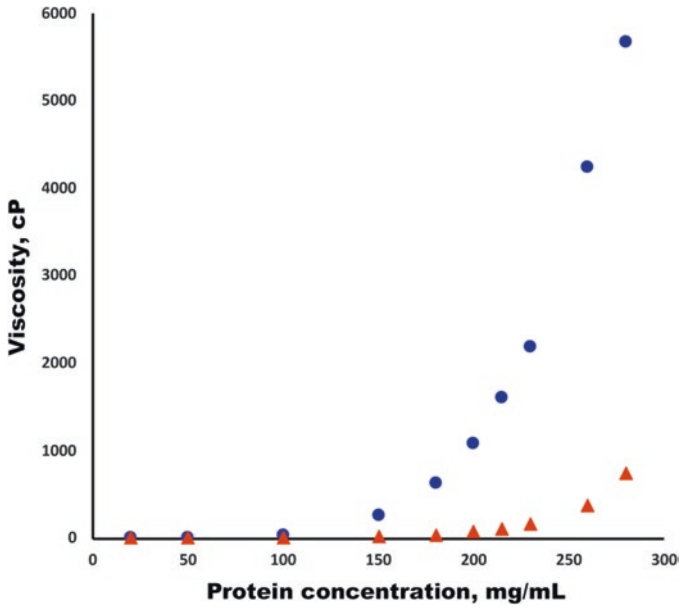

Fig. 1 The effect of mAb concentration on viscosity (20 mM His OAc, pH 5.5; closed circles); (20 mM His OAc, 200 mM L-Arg.HCl, pH 5.5; closed triangles)

be studied at the target concentrations needed for the commercial drug product since these can significantly impact syringeability and manufacturing [4]. Certain excipients, such as L-arginine hydrochloride (L-Arg.HCl), have been shown to reduce the viscosity of protein solutions (Fig. 1), through ion-screening and suppression of electrostatic interactions at concentrations ≤200 mM. L-Arg has also been shown to suppress cation-pi interactions when used at concentrations ranging from 500 to 1000 mM [5]. While L-Arg may not decrease viscosity in all cases, reducing protein concentrations to a reasonable concentration could potentially work if the product TPP allows for multiple injections. Recently, several studies to extrapolate various biophysical properties (e.g., B_{22}) at lower concentrations to predict viscosities have been reported [6]. Similarly, in silico tools are being developed to predict self-associations and viscosity of mAb solutions [7, 8]. These tools could potentially become very valuable in the near future by saving both time and resources for development of high-concentration mAb products.

While the development of high-concentration formulations that control mAb aggregation and viscosity issues is already a challenge, the formulation scientist also has to consider interactions with devices, especially when developing PFS, further complicating the study design and analysis. PFS typically have a coating of silicone oil (lubricant) that may interact with the protein or other excipients in the formulation. In addition to this, the finding that tungsten from pins that are used to make PFS with staked-in needles can cause protein aggregation raises concerns regarding potential immunogenicity [9, 10]. Developing statistical experiments

around these parameters can result in a robustness study with multiple formulations that require significant resources and time. In this chapter we present a two-pronged approach to tackle this issue: (1) design and execution of a pre-robustness study to identify worst-case formulations and (2) utilizing robustness studies on worst-case formulations to support product development in a PFS.

The design and development of the pre-filled syringe and needle safety device combination product often occur concurrently with formulation development. Device development is guided by user requirement specifications and design input requirements which inform design selection, development, and optimization of the combination product. This chapter focuses on formulation development of drug products for subcutaneous delivery; device development and topics such as control of air bubble, device validation, etc. are not covered here. However, the formulation scientist should collaborate closely with the device development team to assess compatibility of the drug product with the device.

2.1 Pre-robustness Study Design and Raw Data Management for mAbs in a PFS

Design

Following the various principles laid out by Shire et al. [2] and Whitaker et al. [3], a high-concentration formulation for mAb1 was selected using a variety of univariate studies. As mentioned before, high concentrations of mAbs tend to aggregate and have high viscosity issues. During formulation development of mAb1, special emphasis was made on selecting the right protein concentration that could meet the TPP and meet stability requirements for commercial use. Once the target formulation composition was established, the robustness of the formulation across the specification ranges needed to be demonstrated (Table 1). This ensured that manufacturing variability would not adversely impact the drug product for commercial use.

Table 1 Fractional factorial formulation design for mAb1 in PFS

Form. #	pH	[Protein]	[Surfactant]	[Buffer]/[tonicifier]
1	−	+	−	+
2	+	+	−	−
3	0	0	0	0
4	+	−	−	+
5	−	+	+	−
6	−	−	+	+
7	+	−	+	−
8	+	+	+	+
9	0	0	0	0
10	−	−	−	−

The robustness evaluation of the mAb1 drug product utilized a two-level fractional factorial experimental design. Ranges of design of experiment (DOE) factors were selected based on anticipated specifications for pH, protein concentration, and tonicity. This study also utilized factor levels wider than presumptive product specification ranges for polysorbate 20 content and buffer concentration as these components are present in low levels and might vary more than 10% from target during commercial manufacture. Composition ranges for DOE factors (Table 1) in this study include a pH range of 5.4–6.0, a protein concentration range of 110–140 mg/mL, a surfactant (polysorbate 20) range of 0.1–0.5 mg/mL, and a single factor of buffer (histidine acetate) and tonicifier (sucrose) combined, respectively, at low levels of 10 mM/160 mM or high levels of 30 mM/ 250 mM. Buffer and tonicifier components were combined into a single DOE factor since univariate composition studies showed that levels of both excipients did not have a significant impact on product stability [1]. The fractional factorial design included two center points at the target formulation. By combining these factors and utilizing a fractional factorial design for the experiment, a significant reduction in the number of formulations (18 reduced to only 10) could be achieved to characterize the robustness of the formulation. This fractional factorial design allowed for analysis of all primary effects from formulation components as well as some two-factor interactions with aliasing (Table 2). Samples were stored in PFS at various temperatures and time points (25 °C for 6 months; 2–8 °C for 24 months, etc.) and analyzed using various analytical techniques such as size exclusion chromatography (SEC), imaged capillary isoelectric focusing (ICIEF), HIAC, and PFS glide forces to detect any changes in aggregates, charge variants, subvisible particles, and injection force, respectively (Table 3).

Raw Data

Management of the raw data was a significant challenge with over 4600 averaged result values. Qualitative analysis of raw data trends as a function of storage time was performed using Origin 8 software. A thorough examination of the data for outliers, anomalous/unexpected features, and overall trends was performed to ensure data quality and consistency between testing sessions and analysts. Qualitatively, the range of measured data between formulations as a function of time may indicate if there is something to be modeled. For example, Fig. 2 shows the change in basic peak 1 in icIEF with time. Data from this study shows that pH could potentially be an important attribute and hence can be used to model further

Table 2 Summary table of fractional factorial DOE aliasing scheme for two-factor interactions

Interaction effect	Alias
pH × protein	PS-20 × tonicity
pH × PS-20	Protein × tonicity
pH × tonicity	Protein × PS-20

Table 3 Example model fitting result table

Temp (°C)	Container	Assay	Formulation parameter	Estimate	Std error	t ratio	Prob > |t|
40	DP	ICIEF %Basic1	pH	−0.39289	0.041045	−9.57	0.0107
	DP	ICIEF %Basic1	Protein (mg/mL)	−0.08232	0.041045	−2.01	0.1827
	DP	ICIEF %Basic1	PS-20 (% w/v)	−0.01654	0.041045	−0.4	0.726
	DP	ICIEF %Basic1	HisAce (mM)	0.070036	0.041045	1.71	0.2301
	DP	ICIEF %Basic1	pH ∗ Protein	0.040179	0.041045	0.98	0.4309
	DP	ICIEF %Basic1	pH ∗ PS-20	−0.04432	0.041045	−1.08	0.3931
	DP	ICIEF %Basic1	pH ∗ HisAce	0.020536	0.041045	0.5	0.6665

Model fitting results of Drug Product at 40 °C for basic variant 1 of icIEF describing each formulation parameter and the interactions of parameters specified by DOE design. "Estimate" values represent the model fit difference between center point and formulation edge along with the standard error of the estimate. The "T-ratio" is not used in this analysis. The column-labeled "Prob> t" provides the estimate of statistical significance for each formulation parameter effect

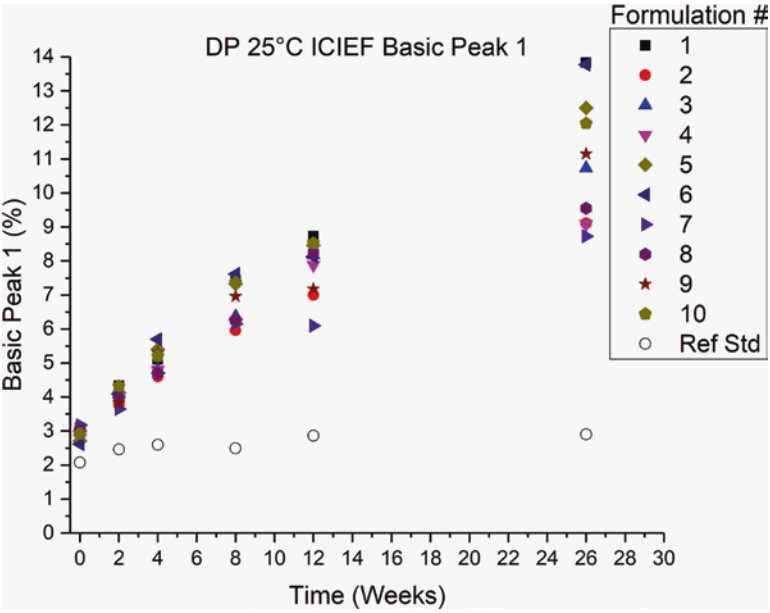

Fig. 2 Formulation performance differences in relative area percentage of icIEF basic peak 1 for mAb1 drug product stored at 25 °C over time

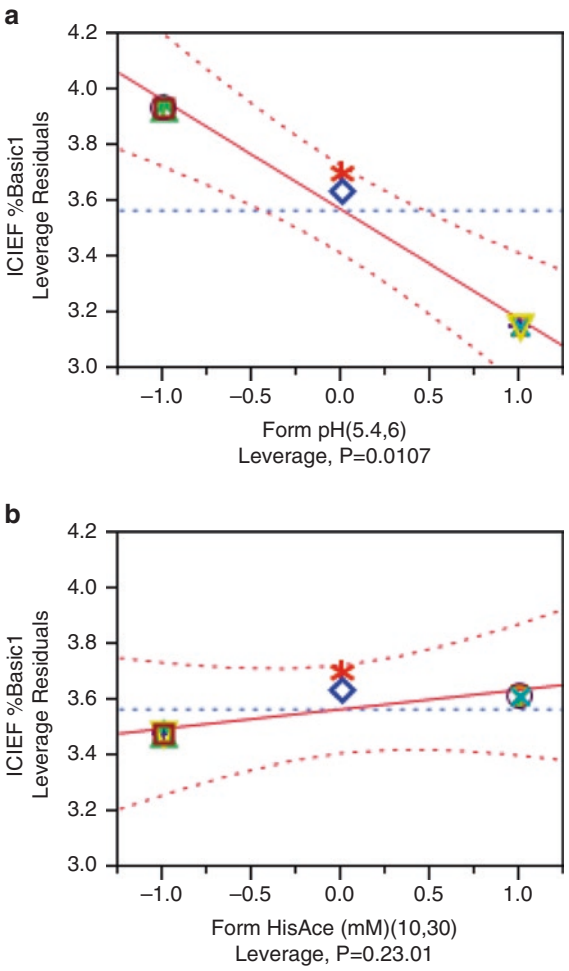

Fig. 3 JMP model fits of rates for icIEF basic peak 1 after Drug Product storage at 40 °C. Each formulation component generates a leverage plot as well as two-factor interactions based on the DOE setup. (**a**) pH demonstrates a formulation factor which impacts degradation rates. (**b**) [Buffer] does not impact degradation rates

as shown in Fig. 3a. Similarly, buffer concentration does not seem to play a role in changes to basic peak 1 and is modeled as shown in Fig. 3b. Whereas, if all formulations remain tightly grouped, then all formulations are performing similarly and the formulation is deemed robust.

Drug product syringe functional performance is an important aspect of combination product quality and was monitored at select stability time points. Small increases in break-loose force are observed over time and in a temperature dependent manner but generally remained within 1 N of the initial values. Average glide and peak glide force values showed dependence on formulation composition ranging from 4 to 6 N (data not shown) where formulation run #s 1, 2, 5, and 8 each had glide and peak glide forces greater than 5 N. The common feature between these formulations is the high protein concentration (139 mg/mL). Protein concentrations,

particularly those above 100 mg/mL, are known to significantly modify protein solution viscosity and, as consequence, syringe forces [4]. Thus, the different glide forces measured over the mAb concentration range evaluated here were expected. As a function of storage duration and temperature, average glide and peak glide forces showed little change at 5, 25, or 40 °C.

2.2 Selecting Worst-Case Formulations for a Final Robustness Study

Using JMP "Fit X by Y" analysis, graphs and attendant linear regression analysis outputs were generated for each temperature condition, formulation run, and assay output. This linear regression analysis produced 1228 rate values, along with the standard error and R^2 of slopes. The calculation of degradation rates was a necessary form of data reduction that serves a dual purpose. First, the rates for each assay output along with the standard error of the slopes can be used to qualitatively interpret the differences in formulation performance and review/confirm the trend-line observations. Second, because JMP DOE modeling procedures are only amenable for use with single values characterizing each assay output, the rate values were a required form of data reduction to leverage statistical analysis of the DOE formulation ranges.

In addition, the multivariate data also provides an opportunity to identify a formulation composition or set of compositions within the DOE space that represents a "worst-case formulation" (WCF). This was achieved through a rank ordering of the degradation rates for each quantitative assay metric (e.g., aggregates, charge variants, fragments, and turbidity). By using rank ordering, the three poorest performing formulations could be readily identified for each assay metric; each time a formulation appeared in the bottom third, the formulation would receive a tally mark. Tally marks were summed (without weighting), and the formulation(s) with the most tally marks was identified as the worst-case formulation(s). A cumulative tally of Drug Product provided the overall worst-case formulation. This exercise was conducted with the actual rate data for the ten formulations in the half-factorial DOE evaluated and was mapped onto the entire full factorial formulation model. In both procedures, formulation #6 was identified as Drug Product-WCF. Formulation #6 appeared 14 times among the fastest degrading and was also identified consistently between both approaches as the combined overall WCF of the full formulation parameter ranges. It is anticipated that this additionally reductive analysis of formulation performance may provide a useful tool to help evaluate other potential product impact scenarios. Similar analysis could potentially be used for drug substance (DS) formulations.

Once the worst-case formulations have been established using the pre-robustness DOE, the final/confirmatory robustness study could be minimized to as low as four formulations, thus decreasing resources and effort while providing valuable

Table 4 Worst-case formulation design

Form. #		pH	[Protein]	[Surfactant]	[Buffer]/[tonicifier]
1	Target	0	0	0	0
2	DP worst-case	−	−	+	+
3	DS worst-case	+	−	+	−
4	Specification bracketing	−	+	−	+

information regarding robustness of the chosen formulation in a PFS. An example of such a study with mAb1 is shown in Table 4.

The study consists of one formulation at target formulation conditions, two worst-case formulations, and a final formulation to ensure all the high and low limits of all parameters are tested. Lot-to-lot variability of excipients could also be tested into the robustness study by adding an optional fifth formulation under target conditions using different lots of excipients. Qualitative comparison of the degradation rates between formulations can be made to determine robustness of the formulation space. If all formulations have similar rates of degradation, then the worst-case formulation is comparable to target and the formulation is considered robust and in good control.

Some product quality aspects not considered in this multivariate DOE pre-robustness study design are also worth noting. PFS syringe component quality attributes were assumed to be constant and not a variable in this study as they are subject to process controls by the PFS component manufacturers as well as in-process controls for final PFS processing and filling.

Once the robustness of the formulation is established, then the formulation lead could use only the target formulation to study the impact of various PFS components on product stability as described below.

3 Stability and Compatibility with Pre-filled Syringe Container Closure System

Pre-filled syringes come in many flavors. Typically, they include, at minimum, a syringe barrel, plunger stopper, and plunger rod. The syringes can be outfitted with a staked-in needle and rigid needle shield (RNS) or a Luer lock and tip cap for assembly with a needle at administration (Fig. 4). The syringe barrel and plunger stopper are typically siliconized to allow for easy delivery of the Drug Product; however, silicone-free syringe barrels and stoppers are becoming available. Stability data collected under intended storage, relevant stress, or worst-case conditions are used to establish compatibility of the Drug Product solution with individual components of the primary packaging system. These studies assess the impact of each component on both product quality and syringe functionality.

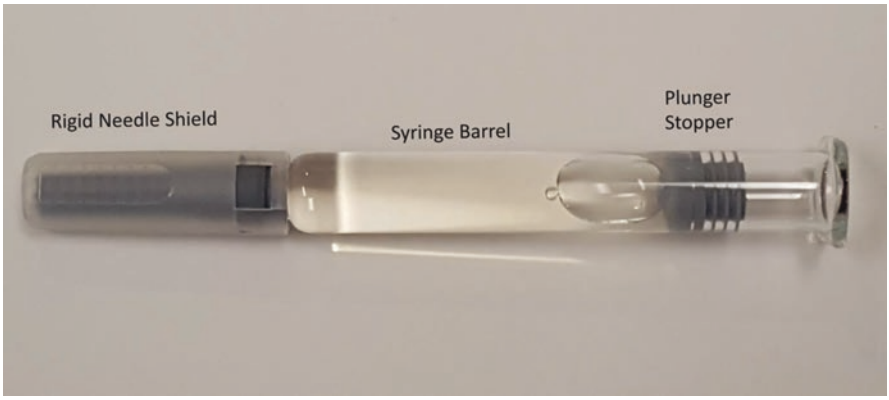

Fig. 4 Pre-filled syringe components

3.1 Drug Product Stability

Compatibility of the Drug Product solution with the PFS container closure system is demonstrated through long-term stability studies of the target formulation at the intended storage temperature as well as shorter-term stability studies under accelerated temperature conditions. These studies can combine testing for syringe functionality and product quality. Drug Product in PFS is stored in the horizontal orientation to ensure worst-case exposure to container closure components including the plunger stopper, needle and needle shield, or tip cap. The potential influence of shear forces on the Drug Product solution when extruded through the needle is evaluated through release and stability data. The Drug Product solution is extruded through the needle prior to physiochemical (including subvisible particles, protein aggregation, and fragmentation) and potency testing. In addition, RNS removal force, break-loose and glide forces, and deliverable volume are assessed at select time points.

3.2 Syringe Components

Plunger Stopper

During long-term storage, the Drug Product is in direct contact with the plunger stopper (Fig. 4). Plunger stoppers can be composed of rubber composites and may be siliconized to allow smooth gliding of the stopper throughout the length of the syringe barrel and thus influence syringe performance, specifically injection forces. Studies to assess compatibility of the Drug Product with the plunger stopper are conducted under worst-case immersion conditions for time and temperature as well as surface area to volume ratio. A study to assess compatibility of mAb1 Drug Product with the

West 4023/50 plunger stopper was performed. One mL of target Drug Product solution was filled into 2 cc glass vials containing a plunger stopper. Glass vials containing 1 mL of Drug Product solution were used as controls. Samples and controls were stored upright and protected from light for up to 3 months at 2 °C–8 °C and 25 °C and up to 1 month at 40 °C to assess the impact of worst-case contact with the plunger stopper on Drug Product quality and stability. Stability samples were assessed for impact on product quality, including appearance; visible particles; subvisible particles; pH; protein concentration; osmolality, polysorbate 20 content; size variants; and charge variants. Data from this study suggested that mAb1 formulations were compatible with this stopper, and no further evaluation was carried out. Long-term stability at intended storage temperature and time (2–8 °C, 24 months) corroborated mAb1 stability with this plunger type.

Rigid Needle Shield

During long-term storage, the Drug Product is in direct contact with the rigid needle shield (Fig. 4). A study to assess compatibility of mAb1 Drug Product with the FM27 rigid needle shield was performed. Target Drug Product solution (1 mL) was filled into 2 cc glass vials containing an entire RNS. Glass vials containing 1 mL of Drug Product solution were used as controls. Samples and controls were stored upright and protected from light for up to 3 months at 2 °C–8 °C and 25 °C and up to 1 month at 40 °C to assess the impact of worst-case contact with the RNS on Drug Product quality and stability. Stability samples were assessed for impact on product quality, including appearance; visible particles; subvisible particles; pH; protein concentration; osmolality, polysorbate 20 content; size variants; and charge variants. Long-term stability at intended storage temperature and time (2–8 °C, 24 months) corroborated mAb1 stability with this rigid needle shield.

Tungsten

Tungsten pins are used to form the needle hole in the hot glass barrel during the manufacture of the staked-in needle glass syringe barrels [9]. Due to heat and oxygen exposure, the pin surfaces corrode over time which can lead to the deposition of oxidized tungsten species on the inner syringe surface in the tip area. During Drug Product manufacturing and storage, the tungsten species could potentially leach into the Drug Product. Staked-in needle syringe barrels can be designated low tungsten. Inductively coupled plasma-mass spectrometry (ICP-MS) can be used to assess tungsten levels present in empty syringes representative of the final commercial syringe.

Worst-case studies are conducted to assess compatibility of the Drug Product with various levels of tungsten. Tungsten species are extracted from representative tungsten pins used to manufacture the staked-in needle syringes. These pins can be obtained from the syringe vendor. Stability samples were assessed for impact of tungsten on product quality, including appearance; visible particles; subvisible

Table 5 Impact of tungsten on stability of mAb1 in PFS at 2–8 °C

		Charge variants			Size variants	
Tungsten (ppm)	Time(months)	Acidic region (%)	Main peak (%)	Basic region (%)	HMWS (%)	Main peak (%)
0	0	16.8	73.2	10.0	0.9	99.1
	3	16.6	72.5	10.8	0.9	99.1
	6	16.1	74.6	9.3	0.8	99.3
0.2	0	15.8	74.3	9.9	0.9	99.1
	3	17.4	71.8	10.8	0.9	99.1
	6	15.5	74.6	9.9	0.8	99.2
2	0	16.3	72.2	11.5	0.9	99.1
	3	17.7	72.4	9.8	0.9	99.1
	6	17.2	74.1	8.7	0.8	99.2
10	0	16.7	72.4	11.0	0.9	99.1
	3	16.2	72.7	11.1	0.9	99.1
	6	15.4	74.9	9.8	0.7	99.3

HMWS high molecular weight species

particles; pH; protein concentration; osmolality, polysorbate 20 content; size variants; and charge variants. Compatibility of mAb1 with various levels of tungsten is shown in Table 5. Results indicate that exposure of mAb1 to up to 10 ppm tungsten has no impact on product quality. Compatibility of the target Drug Product formulation with residual tungsten levels in the syringe is demonstrated by the long-term Drug Product stability data.

Silicone Oil

Siliconization of PFS is essential because it allows smooth gliding of the plunger stopper throughout the length of the syringe barrel and thus influences syringe performance, specifically injection forces [11]. Siliconization is performed by spraying a predefined amount of dimethicone oil or emulsion (Ph. Eur., USP/NF) onto the inner surface of the syringe barrel. The process is typically validated and controlled to ensure that a specified level of silicone (Si) is applied to each syringe barrel. The syringe barrels used for the mAb1 Drug Product are siliconized with a specification of ≤1.0 mg Si-oil/syringe and target level of 0.3 mg Si-oil/syringe. Compatibility of the Drug Product with the target silicone oil levels in the syringe is demonstrated by the long-term Drug Product stability data.

Staked-in needle glass syringe barrels cannot be exposed to high temperatures for prolonged periods to bake in silicone (as is done for Luer-type syringes) because the adhesive used to fix the needle cannula to the syringe tip is incompatible with such conditions. Therefore, for staked-in needle syringe systems, the silicone is not "baked" to the glass surface but exists as "free" silicone. During manufacturing and long-term storage of the Drug Product, silicone oil could potentially leach into the Drug Product [12].

A silicone oil compatibility study for the mAb1 Drug Product was performed to evaluate the impact of Si-oil level on the Drug Product. Syringes were spray-siliconized with 0.2 mg Si-oil/syringe, 0.3 mg Si-oil/syringe (target), and 1.0 mg Si-oil/syringe. The low (0.2 mg Si-oil/syringe) and high (1.0 mg Si-oil/syringe) siliconization levels are considered worst-case for syringe functionality and Drug Product quality, respectively. The siliconization levels were assessed using methylisobutylketone extraction of the syringe barrels and measurement with inductively coupled plasma – optical emission spectroscopy (ICP-OES).

Drug Product solution was filled into syringes siliconized with 0.2 mg, 0.3 mg, and 1.0 mg Si-oil/syringe, and the samples were stored horizontally and protected from light for up to 24 months at 2 °C–8 °C and up to 6 months at 25 °C to assess the impact of silicone oil on Drug Product quality and syringe functionality. Stability samples were assessed for impact of silicone oil on product quality, including appearance; visible particles; subvisible particles; pH; protein concentration; osmolality, polysorbate 20 content; size variants; charge variants; and potency. Break-loose force, average glide force, and peak glide force were monitored to assess syringe functionality. Data is shown in Table 6 and indicates that storage of mAb1 in syringes siliconized with 0.2 mg–1.0 mg Si-oil/syringe has no impact on the stability of mAb1. While higher levels of subvisible particles were observed in syringes coated with 1.0 mg Si-oil/syringe, these increased counts were due to the presence of increased levels of silicone oil in the Drug Product.

An agitation study was performed to assess the impact of silicone oil on protein aggregation and particulate formation during routine processing, handling, and transportation. Filled syringes siliconized with 0.2 mg Si-oil/syringe, 0.3 mg Si-oil/syringe, and 1.0 mg Si-oil/syringe were exposed to horizontal oscillation for up to 3 days at ambient temperature and then assayed for impact to product quality, including visible particles; appearance; protein concentration; polysorbate 20 content; and size variants including aggregates. Similar to the above studies, no impact of Si-oil/syringe was noticed on the stability of mAb1 (data not shown).

4 Drug Product Processing Conditions

4.1 Ambient Light

mAb1 solution is exposed to ambient light and temperature during routine manufacturing of Drug Product PFS and assembly of the PFS into the needle safety device (NSD), from in-line sterile filtration (drug product solution leaving the storage vessel) to final packaging of the combination product. Drug product processing at various facilities is carried out typically under one of these light sources: fluorescent lamps, metal halide lamps, or light-emitting diodes (LED). A small-scale study was performed to evaluate the impact of light and inspection on product quality of mAb1 formulations. Light exposure during drug product processing,

Table 6 Impact of silicone oil on stability of mAb1 stored in PFS at 2–8 °C

Silicone oil level (mg/syr)	Time(months)	Size variants		Charge variants			Subvisible particles			
		HMWS (%)	Main peak (%)	Acidic region (%)	Main peak (%)	Basic region (%)	≥2 µm	≥5 µm	≥10 µm	≥25 µm
0.2	0	0.9	99.1	17.4	72.2	10.4	3057	604	78	3
	3	0.9	99.1	16.6	73.0	10.4	780	205	47	2
	6	0.8	99.2	16.5	73.1	10.4	1248	226	43	2
0.3	0	0.9	99.1	16.0	73.2	10.9	1607	359	77	4
	3	0.9	99.1	17.3	72.5	10.1	797	136	20	0
	6	0.8	99.2	17.5	73.9	8.7	1166	235	40	0
1.0	0	0.9	99.1	16.6	73.5	9.9	9587	1417	195	0
	3	0.9	99.1	17.5	71.6	10.9	11016	2080	280	3
	6	0.8	99.2	16.3	74.2	9.6	8582	2162	346	9

HMWS high molecular weight species

including fill, visual inspection, assembly with NSD and plunger rod, and final packaging, was estimated based on a report by Sreedhara et al. and light measurements at the filling facility [13].

The small-scale ambient light study used a 70 W fluorescent light with a canopy as the light source. mAb1 Drug Product PFS was exposed for up to 60 hours of visible light at approximately 5000 lux and ultraviolet (UV) light at approximately 0.1 W/m^2, for a total of 0.29 × 10^6 lux-hours (visible) and 4.8 W-h/m^2 (UV) simultaneously. The light box was maintained at ambient temperature. PFS samples were analyzed to assess impact of ambient light exposure to product quality, including appearance; visible particles; subvisible particles; pH; protein concentration; polysorbate 20 content; size variants; charge variants; potency; and Fc and Fab oxidation. Results indicated that exposure of mAb1 to up to 1.2 × 10^5 lux-hours of visible light had no impact on product quality; small increases in Fc oxidation were observed with exposure to greater levels of ambient light (data not shown).

4.2 Hydrogen Peroxide

Protein drug products are frequently filled in isolators that have been decontaminated using vapor phase hydrogen peroxide (VPHP). Given the rise in use of VPHP prior to filling protein products, it is imperative to understand its impact on protein product quality. Hydrogen peroxide is a known oxidant and typically oxidizes residues such as methionine [14] and sometimes tryptophan residues, especially in the presence of metal ions [15]. Hubbard and others have recently published the uptake of hydrogen peroxide and its impact of protein product quality [16].

The aeration cycles after VPHP are long, and manufacturing sites would like to optimize the cycle times in order to meet production deadlines. In order to support VPHP and aeration cycles that are amenable for commercial production, a systematic study to understand the impact of hydrogen peroxide on mAb1 formulation was carried out following the criteria laid out by Hubbard et al. mAb1 samples spiked with different levels of hydrogen peroxide were filled into syringes and stored for up to 24 months at 2–8 °C and 6 months at 25 °C. The samples were analyzed to assess impact of hydrogen peroxide to product quality, including appearance; visible particles; subvisible particles; pH; protein concentration; polysorbate 20 content; size variants; charge variants; potency; and Fc and Fab oxidation. In addition, hydrogen peroxide levels were monitored in the mAb1 samples at each time point to determine how long residual hydrogen peroxide, with the potential to impact product quality, remains in the drug product. Results from these studies are shown in Table 7 and indicate that mAb1 can tolerate up to 150 ng/mL hydrogen peroxide based on long-term stability studies.

Table 7 Stability data for mAb1 spiked with 0 ng/mL to 500 ng/mL hydrogen peroxide and stored in PFS at 2–8 °C

		Size variants		Charge variants			Oxidation	
H_2O_2 level (ng/mL)	Time(months)	HMWS	Main peak	Acidic variants	Main peak	Basic variants	Fc oxidation (%)	Fab oxidation (%)
0	0	0.9	99.1	16.9	72.2	10.9	3.1	4.6
	3	0.8	99.2	16.9	72.7	10.5	3.5	4.6
	6	1.3	98.7	16.9	71.1	12.1	3.6	5.1
75	0	0.9	99.1	15.9	74.0	10.1	3.0	4.8
	3	0.8	99.2	16.3	73.5	10.2	3.8	4.4
	6	1.2	98.9	17.0	70.7	12.4	3.9	5.0
100	0	0.9	99.1	15.8	73.5	10.7	2.9	4.8
	3	0.8	99.2	16.4	73.0	10.7	3.8	4.3
	6	1.0	99.0	16.4	71.6	12.1	3.9	5.1
150	0	0.9	99.1	15.9	73.9	10.2	3.0	4.9
	3	0.8	99.2	16.6	72.6	10.9	4.1	4.5
	6	1.0	99.0	16.4	71.9	11.8	4.3	5.2
500	0	0.9	99.1	17.1	72.6	10.3	3.2	4.5
	3	0.9	99.2	17.2	72.7	10.1	5.9	4.5
	6	0.9	99.1	16.5	71.7	11.9	6.3	5.5

HMWS high molecular weight species

5 Conclusions

Rational selection of formulation components, formulation robustness, and worst-case formulation selection can provide significant value for protein product development in pre-filled syringes. Given the confidence that the target formulation and worst-case formulations behave similarly for product quality at the end of shelf life, the above studies for compatibility with various syringe components, ambient light exposure, and VPHP with target formulations are justified and eliminate the need to perform multiple studies to support commercial specifications. On the contrary, if the worst-case formulations are found to impact any quality attribute, then it could become necessary that process parameters and process design studies be conducted with that formulation rather than the target. Similar justifications could be provided for other process development studies (e.g., recirculation, freeze/thaw, shipping validation, etc.) provided the robustness study is carried out in a systematic and rational fashion.

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Chapter 16
Development of Robust Lyophilization Process for Therapeutic Proteins: A Case Study

Ehab M. Moussa, Tong Zhu, and Feroz Jameel

1 Introduction

Biotherapeutics is a general term that describes several different therapeutic modalities that are produced by biological means. Such modalities include proteins, peptides, RNA, gene therapy, cell therapy, vaccines, etc. Among these modalities, therapeutic proteins constitute the largest class of approved products as well as new biological entities (NBEs) in development. Therapeutic proteins encompass a large number of different modalities including monoclonal antibodies, antibody-based molecules, protein conjugates, fusion proteins and enzymes.

Typically, therapeutic protein modalities are produced by very complex bioprocesses and have complex higher-order structures that render them vulnerable to chemical and / or physical instability in the liquid state. Oftentimes, such instabilities pose significant challenges to the development of a robust drug product with enough shelf life stability to support clinical and commercial operations.

One approach to overcome the instability of these complex modalities in liquid is to develop a solid dosage form. Due to the instability of proteins at high temperatures, spray drying is not commonly used for the manufacturing of solid protein drug products. To mitigate the vulnerability of proteins to high temperature, lyophilization or freeze-drying is the most commonly employed manufacturing process to produce solid dosage forms of proteins.

Drying of protein formulations by lyophilization involves three main steps. The first step is to freeze the formulation to form ice crystals and an amorphous phase

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wherein the protein is molecularly dispersed in a stabilizing excipient [1]. Excipients with tendency to crystalize may also be included to serve as bulking agents and to provide structural strength to the final dried product. The second step is called primary drying, and its purpose is to remove the ice crystals formed during freezing, by sublimation at low temperature and vacuum conditions. The final phase of the process is called secondary drying and is employed to remove the remaining bound unfrozen water by evaporation at vacuum and relatively high-temperature conditions.

One major challenge of lyophilization process development is the design of a process during which no product collapse occurs in the primary drying phase [2]. Lyophilizate collapse happens when the temperature of the sublimation interface exceeds the critical collapse temperature of the formulation. For amorphous formulations, this temperature is typically low and hence renders the lyophilization process long and less robust. The use of crystalline excipients in the formulation provides the advantage of a high eutectic melting point, which allows the use of aggressive drying conditions without compromising the robustness of the formulation with regard to varying critical process parameters.

Collectively, the main challenge for biotherapeutic modalities for which long-term stability in the liquid state is not feasible is to develop a formulation that (1) achieves stability in liquid that is sufficient to endure the stresses posed by the different manufacturing unit operations preceding lyophilization and (2) enables efficient and robust lyophilization process that is viable for commercial operations.

In this chapter, we present a case study of the lyophilization process development of a therapeutic protein aimed at enabling a fast and robust lyophilization cycle for a moderate concentration drug product.

2 Formulation Development

2.1 Sucrose-Mannitol Formulation System

The main purpose of the case study is to develop a fast, robust, and commercially viable lyophilization process for a therapeutic protein. To this end, the use of a sucrose-mannitol formulation for lyophilization was evaluated. For low to moderate protein concentrations (1–30 mg/mL), in addition to providing protection to the protein, a sucrose-mannitol formulation can provide a number of desirable manufacturing properties [3]. The crystallization of mannitol allows the product to be dried at high temperature during the primary drying phase with low risk of cake collapse due to the high eutectic melting temperature (T_{eu}) of mannitol. The high eutectic melting temperature also results in a wider lyophilization design space wherein process parameters can be safely varied with low risk of process and product failure both in the laboratory and at the commercial scale. In addition, the crystalline matrix of mannitol provides mechanical strength and elegance to the cakes, which are highly desirable quality attributes.

2.2 Formulation Selection

First, a limited study was conducted using a standard buffering agent and sucrose as formulation constants and pH and surfactant concentration as variables given that the effects thereof would be independent of sucrose concentration. The results indicated that a certain pH value and surfactant concentration are optimal for the stability of the molecule in solution.

Next, the buffering agent and strength, pH, and surfactant concentration were fixed, whereas the sucrose and mannitol concentrations were varied. In these formulations, sucrose and mannitol were used as stabilizer and bulking agent, respectively. The mannitol-to-sucrose ratio is selected so that complete crystallization of mannitol is achieved, whereas the sucrose-to-protein ratio is selected to ensure stability of the protein by maintaining sufficient hydrogen bond interactions with the molecule and minimizing the mobility of the molecule [4].

In the following studies, one optimized sucrose-mannitol formulation was compared to a benchmark amorphous sucrose formulation. In both formulations, protein concentration was fixed, but the sucrose level was less in the sucrose-mannitol formulation.

2.3 Liquid Stability in the Liquid and Characterization

Conformational Stability

The conformational stability of the protein in the two formulations was characterized by measuring the melting point (T_m) using microcalorimetry. The result showed a slight decrease in the Tm in the sucrose-mannitol formulation consistent with the theory of preferential exclusion of solutes put forward by Timasheff and coworkers [2].

Aggregate formation in the two formulations was then monitored as a function of temperature using dynamic light scattering (DLS). The analysis indicates that the protein in both formulations has similar aggregate growth profiles as a function of temperature. Oligomers seemed to form at ~65 °C and then significant aggregation is observed at ~76 °C (Fig. 1).

Chemical and Physical Stability

In order to evaluate the storage stability of the formulations in the liquid state, stability studies at 40 °C, ambient room temperature under normal laboratory lighting, and 2–8 °C were carried out. Additionally, in order to evaluate the stability of the protein against freeze-thawing, all the liquid formulations were subjected to multiple freeze-thaw cycles. For all test conditions, orthogonal analytical methods were

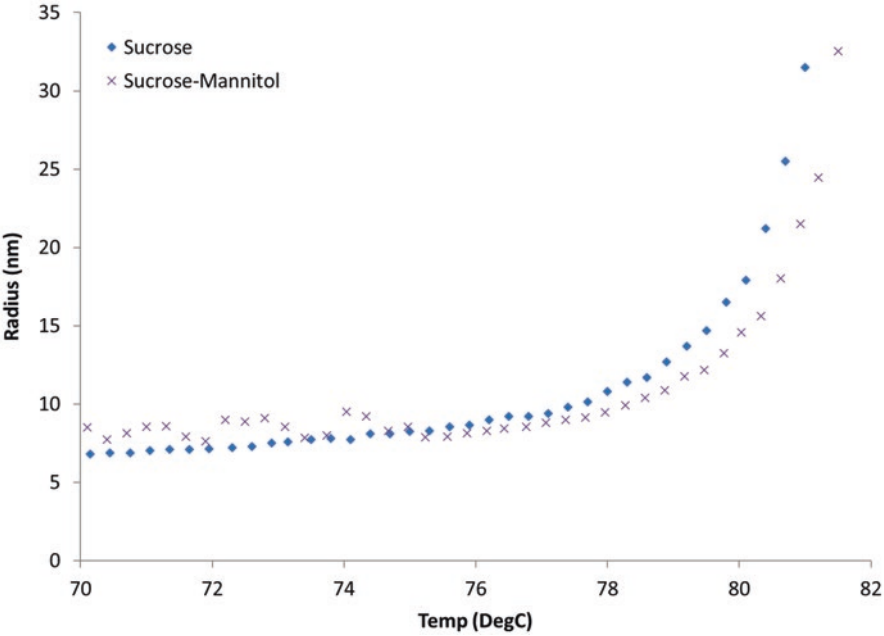

Fig. 1 Hydrodynamic radius profile as a function of temperature

employed to understand the integrity of the protein. The results of the characterization indicate that the stability profile of the two formulations was comparable at all conditions except for some differences at 40 °C/75%RH. Both formulations maintained sufficient stability profile at room temperature and refrigerated conditions, as well as freeze-thawing stability to enable successful manufacturing.

2.4 Lyophilization Process Development

Glass Transition Temperature of the Maximally Freeze Concentrate

The development of a lyophilization process typically starts with determining the critical collapse temperature of the formulation. This temperature is either directly measured using a freeze-drying microscope or indirectly determined by measuring the glass transition of the maximally of freeze-concentrated formulation solution (T_g') using differential scanning calorimetry (DSC). The T_g' of the benchmark amorphous sucrose formulation was measured using modulated DSC (Fig. 2) and was determined to be approximately −28.5 °C. The T_g' of the sucrose-mannitol formulation is determined to be about −25.8 °C (Fig. 3). Such increase in T_g' is in agreement

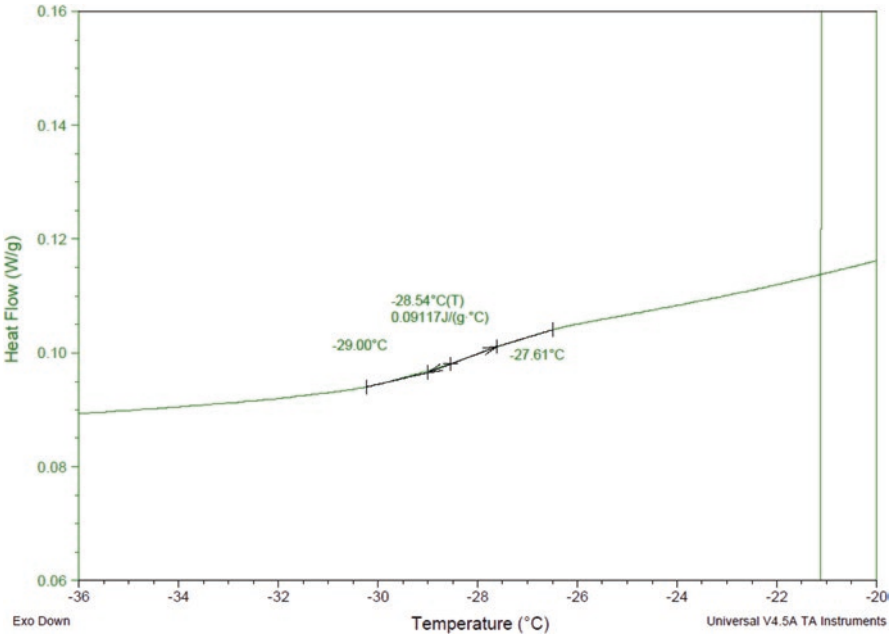

Fig. 2 T_g' of the benchmark sucrose formulation measured using modulated DSC

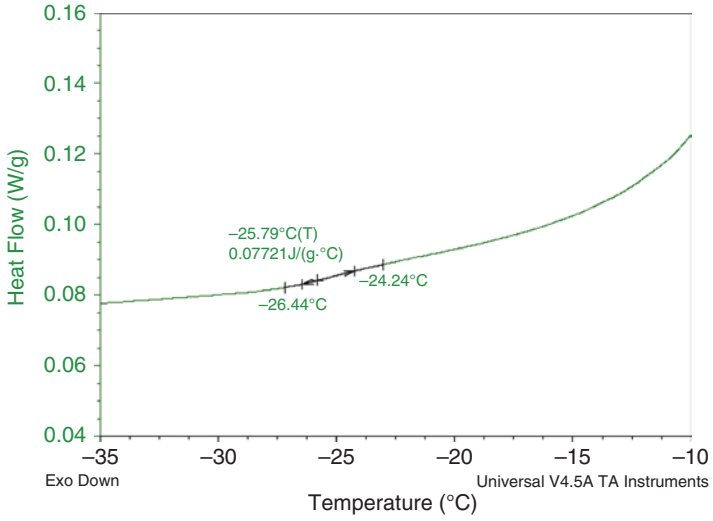

Fig. 3 T_g' of the sucrose-mannitol formulation measured using modulated DSC

with a higher protein-to-sucrose ratio in this formulation compared to the benchmark sucrose formulation.

In general, the low T_g' of sucrose formulations may pose a risk of failure upon scale-up due to cake collapse. As such, one mitigation strategy is the inclusion of mannitol in the formulation, which renders the risk of collapse very low due to the high eutectic melt temperature of mannitol crystals.

Lyophilization Process Parameters

Based on the T_g' values and eutectic melt temperature of the formulations, the following lyophilization process parameters were selected for the target lyophilization cycles for the sucrose-mannitol formulation (Table 1) as well as the sucrose formulation (Table 2) with the aim to accomplish drying below the eutectic melt or collapse temperatures, respectively, within a reasonable timeframe. The capabilities and limitation of the full-scale equipment, manufacturing environment, and procedures were also taken into consideration so that the process can be easily scaled up and transferred without having to make significant adjustments to the process parameters or equipment.

Table 1 Lyophilization cycle of the sucrose-mannitol formulation

Step	Ramp rate (°C/min)	Shelf temperature (°C)	Chamber pressure (mTorr)	Hold time (hr:min)
Loading	N/A	20	N/A	N/A
Equilibration	3	5	N/A	15
Freezing	1	−45	N/A	2:00
Annealing	1	−13	N/A	2:30
Freezing	1	−45	N/A	3:00
Primary drying	N/A	−45	100	0:30
Primary drying	1	−5	100	30:00
Secondary drying	0.25	20	100	6:00

Table 2 Lyophilization cycle of the sucrose formulation

Step	Ramp rate (°C/min)	Shelf temperature (°C)	Chamber pressure (mTorr)	Hold time (hr:min)
Loading	N/A	20	N/A	N/A
Equilibration	3	5	N/A	15
Freezing	1	−45	N/A	2:00
Annealing	1	−20	N/A	2:30
Freezing	1	−45	N/A	3:00
Primary drying	N/A	−45	100	0:30
Primary drying	1	−25	100	60:00
Secondary drying	0.25	20	100	6:00

Lyophilization Design Space

Differences in the heat and mass transfer arising from differences in equipment design, environment, and load size render the scale-up of the lyophilization process challenging. Such differences should be identified, and a link between the laboratory, clinical, and commercial scale equipment can be developed based on classical differential equations, which govern heat and mass transfer aspects of the lyophilization process. A steady-state model that best describes drying and predicts the response variables (product temperature, drying rate, and primary drying time) for a given set of independent variables (shelf temperature and chamber pressure) was developed in-house. The heat transfer coefficient was determined for laboratory lyophilizer using water sublimation tests and was predicted for manufacturing-scale lyophilizer used as described previously [5]. The model was used to develop a design space for the sucrose-mannitol formulation using mass transfer coefficients obtained from the process data of the target cycle ran on a laboratory-scale lyophilizer. The manufacturing of a laboratory batch resulted in non-collapsed cakes as confirmed visually using microcomputed tomography scanning. The product temperature profiles of the vials in the batch were in agreement with the design space.

Lyophilization Process Robustness

Design space verification was conducted using process data from a high-high (Table 3) and low-low cycles (Table 4), and product temperature profiles were within 1–2 °C of the predicted values (Fig. 4) suggesting a high predictive accuracy of the model. The results of the robustness study indicate that the lyophilizates produced under a wide range of process parameters covering conservative and aggressive conditions maintained desirable quality attributes with regard to appearance. Additionally, the product resistance profile (Fig. 4, insert) decreased as a function of the annealing temperature consistent with the expected effect thereof [6]. These results also indicate that a primary drying phase under 10 hours can be achieved using more aggressive drying conditions.

Table 3 High-high and lyophilization cycle of the sucrose-mannitol formulations

Step	Ramp rate (°C/min)	Shelf temperature (°C)	Chamber pressure (mTorr)	Hold time (hr:min)
Loading	N/A	20	N/A	N/A
Equilibration	3	5	N/A	15
Freezing	1	−45	N/A	2:00
Annealing	1	−10	N/A	2:30
Freezing	1	−45	N/A	3:00
Primary drying	N/A	−45	130	0:30
Primary drying	1	5	130	30:00
Secondary drying	0.25	20	130	6:00

Table 4 Low-low and lyophilization cycle of the sucrose-mannitol formulations

Step	Ramp rate (°C/min)	Shelf temperature (°C)	Chamber pressure (mTorr)	Hold time (hr:min)
Loading	N/A	20	N/A	N/A
Equilibration	3	5	N/A	15
Freezing	1	−45	N/A	2:00
Annealing	1	−16	N/A	2:30
Freezing	1	−45	N/A	3:00
Primary drying	N/A	−45	70	0:30
Primary drying	1	−15	70	30:00
Secondary drying	0.25	20	70	6:00

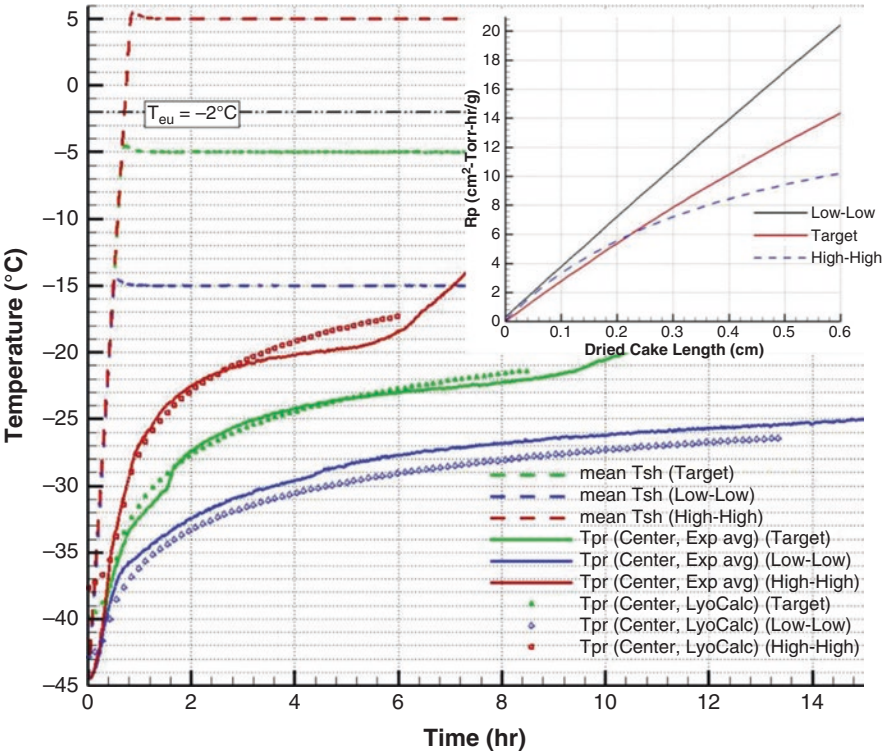

Fig. 4 Verification of the design space obtained using steady-state models. Insert: product resistance of the formulation during the primary drying phase in the target, high-high, and low-low cycles. (Reprinted with permission from Ref. [5])

Physical Properties and Stability of the Lyophilizate

Lyophilizates were characterized for their physical (reconstitution time, residual moisture content, and sub-visible particles using micro-flow imaging) and chemical properties and characteristics. Time zero data were collected, and the samples were

placed at different stability conditions. The lyophilized formulations were first characterized for reconstitution time, residual moisture content, and sub-visible particles. Vials of each the sucrose-mannitol formulations were also stored at 2–8 °C, 40 °C/75%RH or at room temperature. The degree of crystallinity of mannitol in the dried cake of the sucrose-mannitol formulation was evaluated using modulated DSC and powder X-ray diffraction (PXRD). The formation of mannitol hemihydrate during lyophilization was also investigated using PXRD.

Crystallinity and Glass Transition of the Lyophilizate of the Sucrose-Mannitol Formulation

The glass transition of the lyophilizate (T_g) of sucrose-mannitol formulation is determined to be approximately 86.7 °C (Fig. 6) consistent with the high weight ratio of the protein in the amorphous phase of the lyophilizate. The high T_g also indicates the absence of amorphous mannitol which would otherwise significantly decrease the T_g of the amorphous phase. The complete crystallization of mannitol is also confirmed by the absence of mannitol crystallization exotherm in the nonreversing heat flow signal (Fig. 5).

The physical form of the mannitol crystals was determined using PXRD (Fig. 6). The diffractogram indicates the formation of the anhydrous delta polymorph. The peak characteristics of mannitol hemihydrate were not detected indicating that the selected annealing temperature and secondary drying conditions are adequate to minimize the presence of the hemihydrate form in the lyophilizate. Residual moisture content and reconstitution time were similar in the two formulations (Fig. 7).

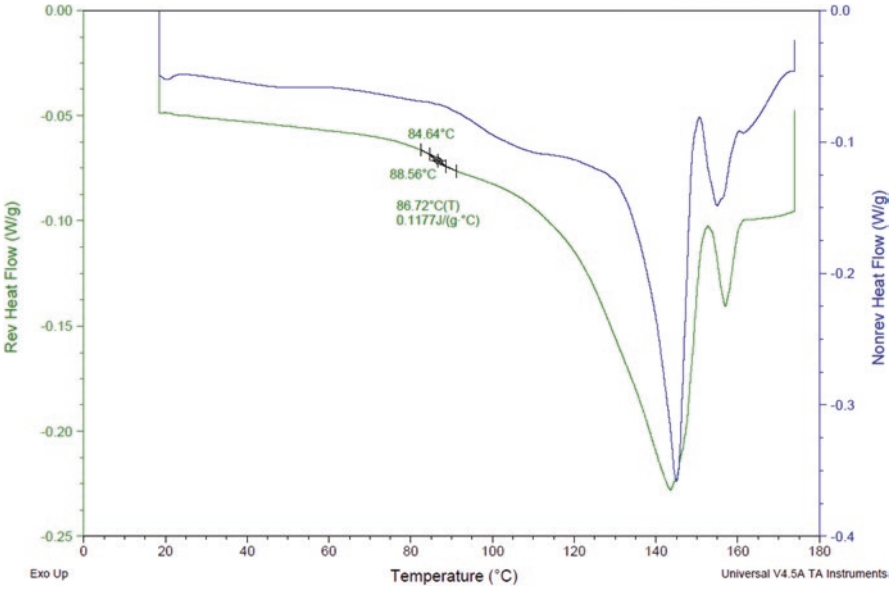

Fig. 5 Modulated DSC thermogram of the lyophilizate of the sucrose-mannitol formulation

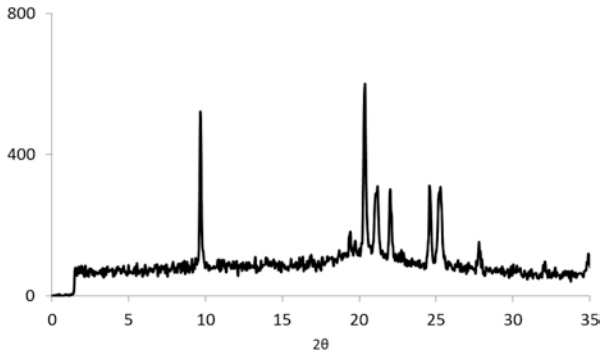

Fig. 6 Powder X-ray diffractogram of the lyophilizate of the sucrose-mannitol formulation

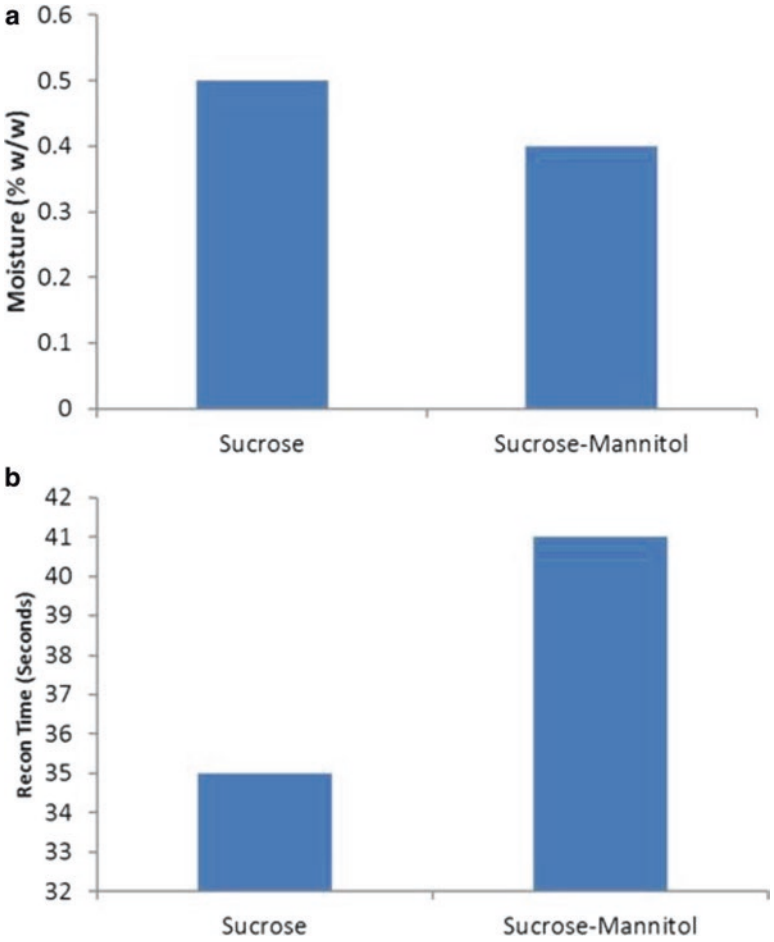

Fig. 7 Residual moisture content and reconstitution time of the lyophilizates of the benchmark sucrose and the sucrose-mannitol formulations

Sub-visible Particle Analysis

The formation of sub-visible particles was evaluated using micro-flow imaging analysis. The results show that sub-visible particle counts at ≥2 μm are higher in the sucrose-mannitol formulation; however, at the ≥2 μm size range, the particle images are too small to discern if they are protein or nonprotein particles. At the other three particle size ranges (≥5 μm, ≥10 μm, and ≥25 μm), there is no meaningful trend. The particle images were nondistinct and were primarily nonprotein particles. Taking into consideration that the formulations were prepared in the open lab environment involving multiple sample manipulations, there are no concerns related to the number of sub-visible particles after reconstitution between the two formulations (Fig. 8).

Stability in the Drug Product at Accelerated Stress Conditions

The lyophilizates were placed at accelerated stability conditions as a measure of potential long-term stability. Analytical results for the lyophilized drug product indicate that the formulations have comparable stability profile at 2–8 °C and at room temperature. Compared to the initial time point, there is no meaningful trend observed over the short-term accelerated stability conditions up to 6 months. Minimal increase in high molecular weight (HMW) species in the mannitol formulation after storage for 4 weeks at 40 °C/75%RH was observed in agreement with both water replacement and vitrification theories of protein stability in the solid state [7]. The higher sucrose level in the benchmark amorphous formulation provides more hydrogen bonding interactions with proteins as well as lower mobility in the lyophilizate [8]. Notably, the aggregation at both 25 °C/75%RH and 40 °C/75%RH in the sucrose-mannitol formulation followed standard square root of time kinetics (Fig. 9) consistent with previously published reports [9]. Additionally, a small increase in basic species at 25 °C/75%RH and 40 °C/75%RH was observed,

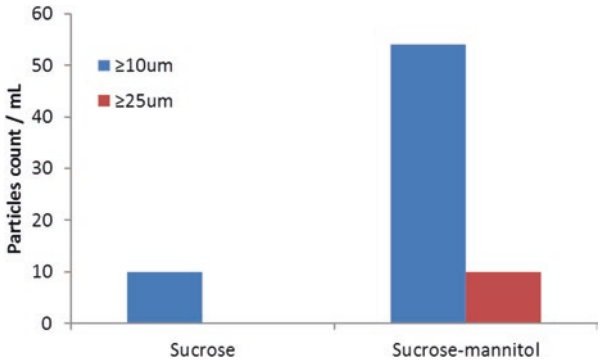

Fig. 8 Sub-visible particles in the reconstituted solution of the sucrose and the sucrose-mannitol formulations measured using micro-flow imaging

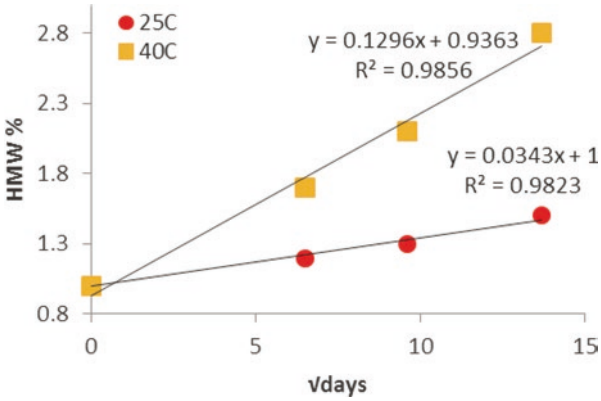

Fig. 9 Rate of formation of high molecular weight (HMW) species of the protein in the lyophilizates of the sucrose-mannitol formulation follows square root of time kinetics

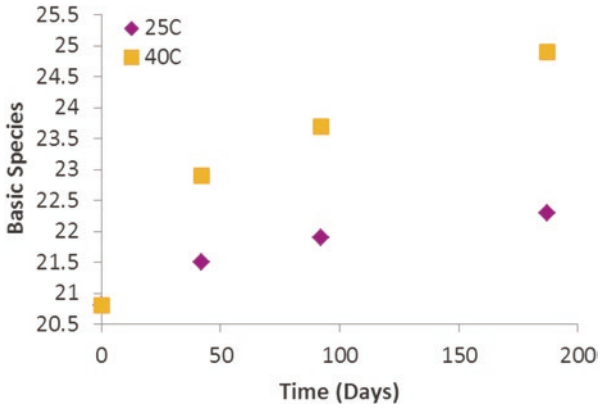

Fig. 10 Rate of formation of basic species of the protein in the lyophilizate of the sucrose-mannitol formulation follows apparent first-order rate

and followed apparent first-order rate (Fig. 10) also in agreement with literature. Taken together, the results of this investigation indicated that the low sucrose concentration is sufficient for the stability of the protein in solution and that it could be sufficient for stability of the protein in the lyophilizate.

3 Conclusion

In summary, different formulations were evaluated through a tiered approach and utilizing multiple different stress conditions in order to select a candidate formulation that has suitable manufacturability and sufficient stability profile. The sucrose-

mannitol formulation was selected because of its superior manufacturability (i.e., short lyophilization cycle and large design space of process parameters) and suitable stability profile.

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Chapter 17
Scale-Down Models for Robust Biologics Drug Product Process Development

Smeet Deshmukh and Maria O. Ogunyankin

1 Introduction

The number of biological products has significantly increased in last few decades due to their specificity and relatively low toxicity [1]. There are more than 150 of approved products [2] and many more in development. Development of commercially viable biological product is very challenging as due to their complex structures and large number of functional groups, multiple degradation pathways are possible leading to physical and chemical instability [3, 4]. A robust formulation and process development is needed to keep the biological product stable during manufacturing as well as during its shelf life at recommended storage conditions [1, 5, 6]. Drug product (DP) manufacturing of biologics involves unit operations such as freeze–thaw, pooling, mixing/dilution, pumping, and filling for ready-to-use liquid product. For lyophilized products, lyophilization is an added unit operation which is carried out after filling into vials. The focus of this chapter is on ready-to-use liquid product manufacturing either in vials or in prefilled syringes. The biologics drug products in the market and in development can be found in presentations up to 200 mg/ml [1, 7]. Challenges for biologics drug product at high concentration arise from thermal instability, viscosity, processing, and delivery to patients [8]. A typical drug product manufacturing process involves the following steps depicted in Fig. 1. Freezing of drug substance (DS) is done at drug substance manufacturing facility, but it is still an important consideration of drug product process development as it can influence critical quality attributes of the drug product.

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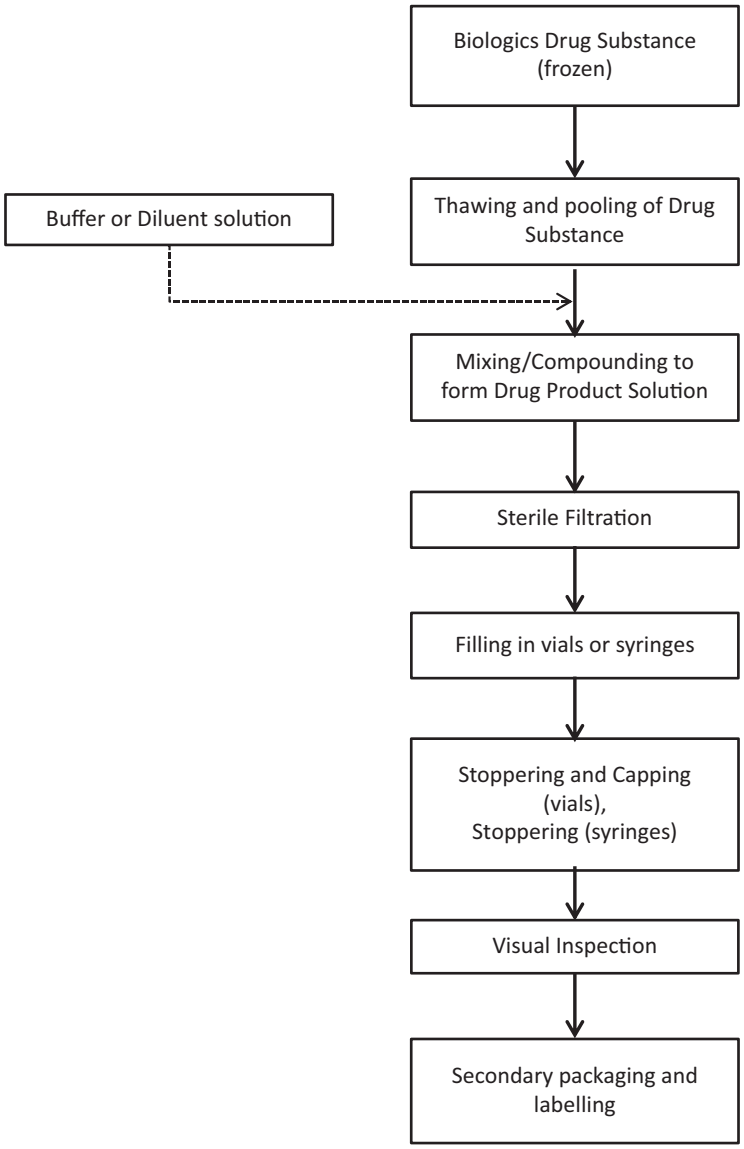

Fig. 1 Process flow diagram for biologics drug product manufacturing (ready-to-use liquid). The buffer/diluent addition step may be required if drug substance and drug product composition are not the same

The overall formulation fill/finish process (shown in Fig. 1) is the same for different modalities of liquid drug product such as monoclonal antibodies, bispecific antibodies, probody, nanobodies, PEGylated proteins, Fc fusion proteins, etc. Based on the modality, structure, and hydrophobicity of the molecules, the impact of stresses applied during these unit operations vastly varies. The formulation approaches to stabilize these modalities also significantly differ from each other.

Different formulation components pose unique challenges to certain stresses such as freeze–thaw, metal, and light. Hence, fundamental understanding of the unit operations as well as the application of modeling tools to guide, evaluate, and quantify some of the stresses involved during processing is very advantageous.

2 Process Development Using QbD Approach

The International Conference on Harmonization (ICH) Q8 (R2) defines QD as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management." The main philosophy behind is that it is not just about meeting the end-product quality based on the set specification but also understanding how the different formulation and manufacturing variables would impact the quality. Hence, a proper understanding of impact of different process variables on product quality and the control strategy to adjust these variables during manufacturing is critical. The important aspect of process development by QbD is developing the manufacturing process, risk assessment based on identified critical quality attributes (CQA), outlining the design space, and defining a control strategy for a product to remain in the design space.

Phase appropriate risk assessment is needed to be done from early stage of development. More robust risk assessment needs to be in place once the phase of development progresses. The process risk assessment with an understanding of the impact of each process parameter on critical quality attributes should be carried out. The risk assessment tools can be simplified, and a stepwise procedure can be used. Initial risk assessment, minipiloting tools (using volume as low as 10–20 mL) can be utilized to understand the critical stress that can affect the product quality. The stresses can be then looked in perspective of the unit operations and where applicable can help define presumptive critical process parameters (pCpp). This is a key input for the formulation development as well as in the DP manufacturing site selection process. For the later stage of development when the site has been selected, the use of scale-down models, closely mimicking the equipment at scale, helps evaluate facility fit as well as defines design space for critical parameters for different unit operations. Based on the impact of critical quality attributes (CQAs), the level of process parameters needs to be defined. When there is more than one parameter for a given unit operations with medium and high risk, a design of experiments (DoE) should be considered to define the levels of the parameters or in other words, the design space.

2.1 Process Risk Description for Drug Product Manufacturing

Understanding the stress a molecule encounters during development and manufacturing is a vital risk assessment step that enables application of appropriate mitigation strategies. The most common stresses seen during the DP manufacturing

process are shear stress and interfacial stresses. Molecules are exposed to air–liquid interface during pooling, mixing, pumping, and filling operations, and they are exposed to other interfaces such as ice–water interface and oil–water interfaces during freeze–thaw and delivery/storage in syringes, respectively. As the solution goes through pooling, filtration, mixing, pumping, filling, and injection, the molecules undergo different level of shear stresses. Along with these stresses, there is light stress as well as metal-induced oxidation stress seen during processing.

The use of mechanistic models as well as characterization during processing helps correlate process parameters to each stress. Quantification of each of the stresses applied can be done with certain assumptions for each operation. The advantage of this approach is that these stresses can be applied independent of scale and unit operation and hence can be carried out with small amount of material. The knowledge that it provides is very rich, and it also forms as a bridge to the studies done with scale-down models as well as at-scale.

3 Small-Scale/Minipiloting Tools

During drug product manufacturing and handling, multiple unit operations such as filtration [9, 10], filling, mixing, and shipping/handling [11] can lead to protein aggregation. During processing, these operations impose various stresses on the protein, [6] due to turbulent flow conditions and/or multiple passes through pumps [12, 13], valves, and "pinch" points [14]. Exposure to these stress conditions can lead to protein instability.

The key mechanical drivers that lead to protein aggregation include shear [15–20], cavitation [10, 11, 20, 21], and/or interfacial stress [23–29], and these effects are often combined [19, 22] in a given unit operations. Some of the studies conventionally done include convoluted stresses, making it hard to quantify the risk level and define control strategy. One such example is agitation study with drug product in vials/syringes, which involves placing the drug product on orbital shaker at certain rpm and length of time. This study is helpful in rank-ordering formulations, but it is difficult to de-couple the impact coming from interfacial stress and shear stress. This had led to the development of some of minipiloting tools described below to allow for the isolation of interfacial and shear stress [30]. The other important point to note is on the level of stresses used for these studies and should be significantly different from forced degradation study levels. The objective of forced degradation study is to monitor the molecular liabilities and identify the degradation pathways. The impact of these degradation products on the product quality is studied to define critical quality attributes. However, the stress study done as a part of process risk assessment is to apply stresses relevant to manufacturing and storage conditions which may yield degradation products significantly lower levels than seen in the forced degradation study. This section focuses on four major stresses seen during manufacturing and the studies done to assess their individual impact.

3.1 Shear Stress Minipiloting Tool

The shear stress small-scale tool simulates the shear experienced by the protein during processing using a small-scale high pressure pump connected to stainless steel tubing. Passivated tubing is used in order to avoid or minimize leaching of metal ions into the formulation. The tool allows for the isolation of shear stress by modulating the flow rate, tube length, and tube diameter. The shear applied to the sample is characterized by shear rate and total shear. The shear rate is calculated using Eq. (1), and the total shear is the product of the shear rate and the residence time (total shear = $\gamma X t_{\text{shear exposure}}$).

$$\gamma = \frac{8v}{d} \rightarrow \frac{4Q}{\pi r^3} \tag{1}$$

where γ is the shear rate, v is the linear fluid velocity, d is the inside diameter of the tube, where the linear fluid velocity v is related to the volumetric flow rate, Q, by $v = Q/A$ where A is the cross-sectional area of the tube and $A = \pi r^2$ and r is the radius of the tube.

The tool offers the flexibility to customize different combinations of shear rate and total shear. Thus, the first step of the workflow is to estimate the shear for a given process. The shear rate and total shear during filling operations are estimated from fundamental fluid transport correlations for the given drug product properties and filling time requirements. The total shear and shear rates that the molecule will experience through the different components of the filler (i.e., tubing, needle, orifice, etc.) can be calculated. The estimated shear rates for different filling technologies in the different components are between 3 K and 100 K s^{-1} and total shear is between 5 K and 10 K.

In the case of mixing, computational fluid dynamics (CFD) simulations are used to calculate the shear and total shear. Typical mixing shear rates are between 60 and 200 s^{-1} depending on the mixing technology and drug product properties. The total shear for mixing is about 1–2 K. For filling operations, the shear rate is high, as the fluid quickly moves through the tubing, but the residence time is low. The reverse is true for mixing. Once the shear rate and total shear are determined for a process, the small-scale shear tool is set up to recreate the same levels of shear.

In this *case study*, the tool is applied to assess the impact on aggregation of shear stress in a 100 mg/ml protein formulation (histidine base) during mixing and filling processes. The quality attributes evaluated were high molecular weight species (HMWS %) and particulate matter. Since the tool allows for the customization of the shear stress applied, three conditions were evaluated, one for mixing and two for filling. The shear experimental conditions are summarized in Table 1. The objective is to cover the operational space of mixing and filling, as well as to evaluate harsher conditions/worst-case scenario to map the design space. A sample that was not subjected to shear stress was used as a control (held at room temperature, protected from light, while the experiment was being conducted). In addition, the samples were placed on stability to evaluate the impact over time.

Figure 2 shows the subvisible particle data for the different shear stress conditions. It is clear that at the highest shear condition evaluated (100 Ks^{-1} shear rate and 10 K total shear), the particle formation at ≥10 μm and ≥25 μm size increases considerably. The same trend is observed after 1 month at 25 °C. Moreover, this data identifies the risk of particle formation on the protein formulation associated

Table 1 Shear stress experimental conditions

Experiment	Shear rate (1/s)	Total shear
Mixing shear		
Design space	500	2000
Filling shear		
Pumping operational space	100,000	7500
Pumping, design space	100,000	10,000

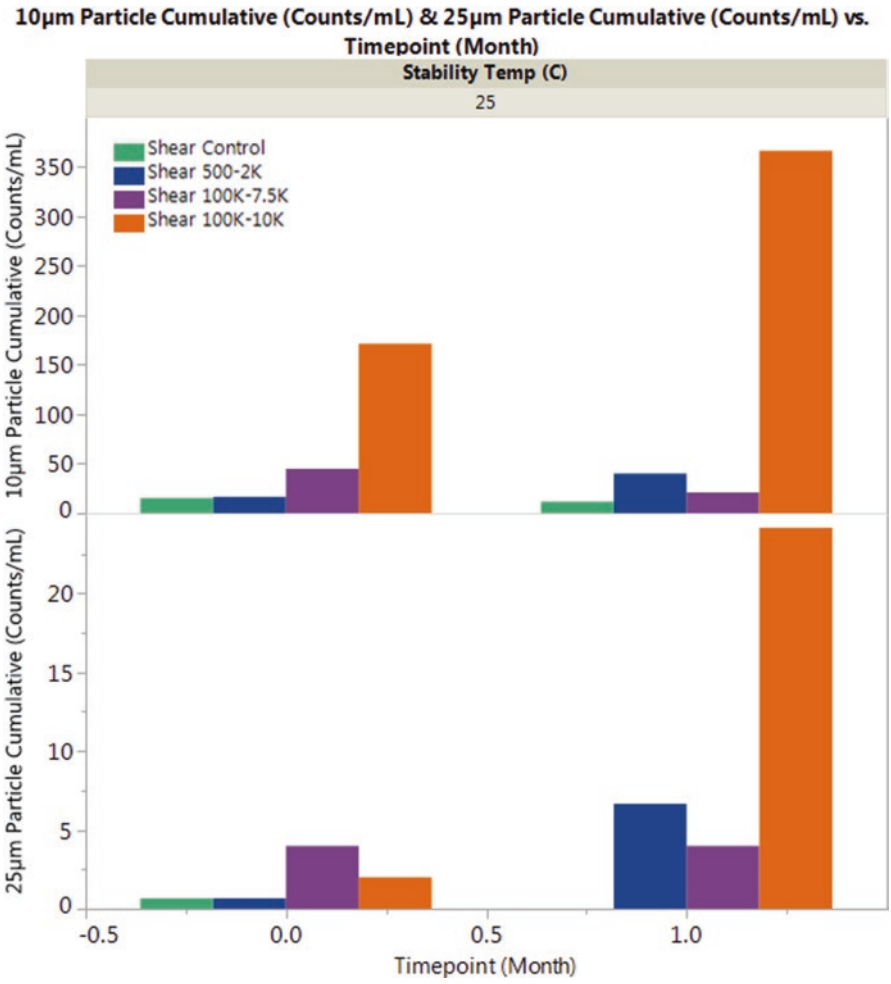

Fig. 2 Shear stress conditions and subvisible particles (>10 µm and >25 µm/ml) measured via HIAC after protein formulation have been exposed to different shear stresses and placed at 25 °C for 1 month

to the total shear instead of the shear rate. The total shear represents the residence time that the molecule is subjected to shear stress. This finding is relevant when excursions happen during manufacturing. The small-scale tool supported the determination of the threshold of total shear when particle formation becomes a problem.

It was possible to make a recommendation to the manufacturing process parameters to operate at a total shear not higher than 7.5 K. There was no change in %HMWS from the control sample after being stressed (data not shown). Additional case studies can be found in the journal article [30].

3.2 *Interfacial Stress Minipiloting Tool*

Interfacial stress is present during filling and mixing operations, mainly when bubbles are formed during the process. The objective of the small-scale tool is to provide a risk assessment of the impact of the interfacial stress on the protein formulation. To correlate the "typical" exposure of interfacial stress present in different operations, filling experiments were performed under different conditions. The filling experiments are representative of commercial filling, as well as worst-case scenarios aimed to maximize the interfacial stress per volume of fill. Each fill experiment was recorded with a high-speed camera in order to evaluate and quantify the bubble generation and surface area of exposure. An image processing analysis "Hough circle transform" was performed to measure an average bubble diameter and surface area. Figure 3 shows an example of the characterization of vials filled under worst-case filling conditions. From the image analysis, it was seen that the majority of the bubbles had a mean bubble diameter of 1.54 mm and 1.42 mm with a total surface area of 7510 mm^2 and 8090 mm^2, respectively.

To provide a controlled generation of interfacial stress in the sample, the small-scale interfacial stress tool is applied. This tool allows the user to explore the impact of interfacial stress encountered during development on key quality attributes.

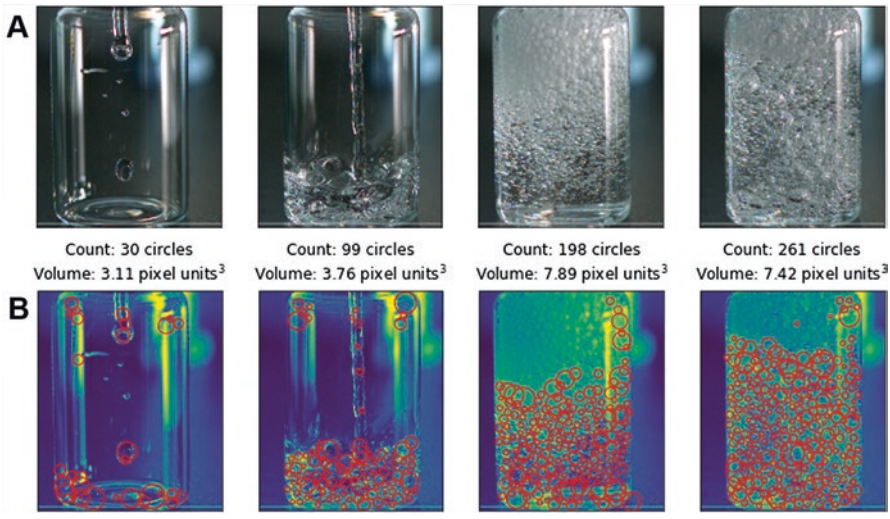

Fig. 3 High-speed camera captured images of a 20 R vial (**A**) filled with a protein solution using a peristaltic pump operating at 350 rpm with a 1.6-mm internal diameter (ID) needle. (**B**) Hough circle transform process analysis was performed to measure average bubble diameter and surface area (This figure is best viewed in color)

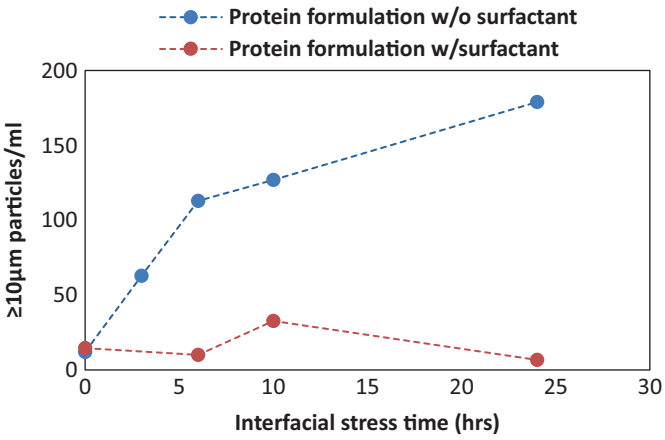

Fig. 4 Subvisible particle per mL in formulations with and without surfactant after exposure to different intervals of interfacial stress, as measured by MFI. The lines are meant as a guide

Table 2 Different formulations of Ab1 (50 mg/mL) used to investigate the impact of interfacial stress

Formulation 1	Phosphate/sugar/amino acid A/pH 6.5
Formulation 2	Histidine/sugar/amino acid A/pH 6.0
Formulation 3	Histidine/pH 6.0
Formulation 4	Phosphate/pH 6.5
Formulation 5	Histidine/amino acid A/pH 6.0

The interfacial stress is applied by generating uniform bubbles when recirculating air from the headspace of a vial in a closed loop. The rate and total number of bubbles applied to each samples were determined based on the image analysis that was performed to measure bubble generation during actual filling operations. For example, after 1 hour of exposure, the total surface area of the bubbles is ~7200 mm^2, which is in the similar range of the surface area obtained by analyzing the images of the filling process. Based on the correlation of the image analysis characterization and experience with the different operations in biologics drug product development, an experimental workflow was developed. The sample is subjected to interfacial stress for 24 hours with collection time points at 0 hour (control), 1 hour, 3 hours, 6 hours, 10 hours, and 24 hours; the collection time points allow evaluation of the impact (if any) of the interfacial stress over time. The 24-hour exposure time not only allows coverage of the normal operational-space time frame for filling and mixing but also helps map the design space and/or the potential worst-case scenario.

In this *case study*, a formulation with 50 mg/ml protein concentration with and without surfactant was subjected to interfacial stress to assess the impact on aggregation.

The sample was stressed for 24 hrs with collection time points at 3, 6, 10, and 24 hrs. Figure 4 shows the clear impact that interfacial stress has in particle formation in the protein formulation without surfactant. The data stresses the need of surfactant during mixing and filling operations.

Another *case study* is the application of the tool in the formulation screening. Four different formulations were prepared and subjected to interfacial stress via minipiloting tool to identify which one mitigates better the impact of interfacial stress. Table 2 shows the components of the different formulations after buffer

exchange for a given Fc fusion protein. The first four are the same that were used in a large-scale TFF process. As shown in Table 2, none of the formulations have surfactant since the objective is to understand the impact of the interfacial stress on the molecule and the ability of the different formulations to mitigate such impact. This information serves in the formulation screening process.

After stressing the samples in the small-scale tool following the previously described workflow (collection times 1, 3, 6, 10, and 24 hr), the analytical characterization techniques showed the data in Fig. 5.

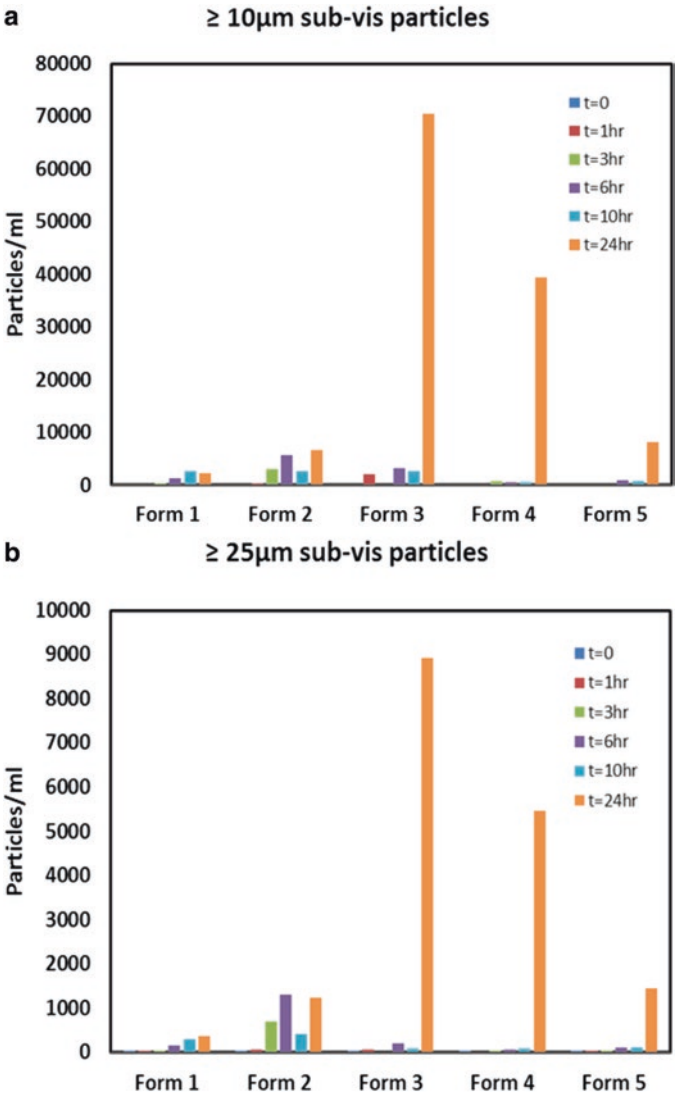

Fig. 5 (**a**, **b**) Particles per mL in formulations of Ab1 after exposure to different intervals of interfacial stress, as measured by MFI. (**c**) Temperature of unfolding (T_{PU}) for different formulations of Ab1, after exposure to interfacial stress for 24 hours, as measured by DSF with Sypro orange. (**d**) HMWS in different formulations of Ab1 at T0 and after 24 hours of interfacial stress

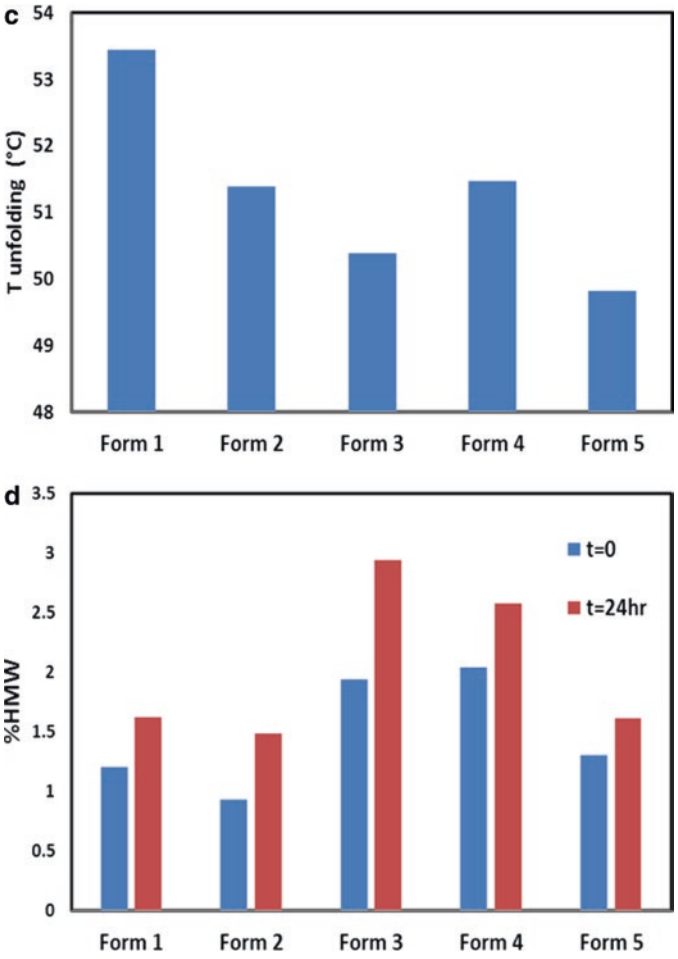

Fig. 5 (continued)

Figure 5 shows the temperature of protein unfolding (T_{PU}) and allows rank ordering of these formulations (most to least thermally stable): Formulation 1 > Formulation 2 ≅ Formulation 4 > Formulation 3 > Formulation 5. Figure 4 also shows the levels of HMWS and particulate formation following interfacial stress for 24 hours. Formulations containing sugar and amino acid A (Formulations 1 and 2) presented lower HMWS and/or particle formation. Without sugar or amino acid, formation of HMWS and particles was lower in phosphate buffer (Formulation 4) than in histidine buffer (Formulation 3).

From the formulation screening perspective, the interfacial stress tool assessment shows that for the histidine-based formulations (Formulations 2, 3, and 5), the presence of sugar and amino acid A provides the best thermal and aggregation stability for the molecule after subjecting to interfacial stress. Also, this provides evidence that the least stable formulation is the one that does not have any sugar or amino acid A. The same trend applies for the phosphate-based formulations

(Formulations 1 and 4), where the formulation that has sugar and amino acid A provides better mitigation of the impact of interfacial stress to the molecule. Based on the interfacial stress tool, the overall best formulations were Formulations 1 and 2; this result correlates with the extensive formulation screening that was done for Ab1 at normal scale. The interfacial stress tool assessment served as an efficient tool to screen formulations that will better perform under TFF at large scale and under other interfacial stress conditions, such as DP processing, shipping, etc. Additional information of the application of the tool can be found in the journal article by Ogunyankin et al. [30].

3.3 Light Stress Study

Photostability characteristics of new drug substance and drug product is an integral part of the stress test for new DS and DP as indicated in ICH Q1B [31]. Photostability under these ICH conditions is usually carried out using total light exposure of 200 W h/m^2 of UV light and 1.2×10^6 lux-hours of visible light. The UV exposure per ICH Q1B guidelines is high enough to degrade most of the biologics, and there are main evidences in literature showing UV light-induced oxidation [32–34]. The biologics manufacturing is done in GMP processing areas under ambient conditions that are equipped with metal halides, LED, or fluorescent lights. It is therefore important to carry out studies that are done to understand the impact of ambient light exposure during manufacturing. This can be done by carrying the studies on drug product in the laminar hood under ambient light for 5–10 days. It is important to identify how the total exposure as well as the irradiance spectra used in these studies correlates to those during manufacturing process. The light mapping of the manufacturing sites is critical to quantify the total illuminance as well as the UV quotient/wavelength of the lights using spectroradiometer for range of 190–850 nm. The light exposure can significantly vary across sites based on the source of light used, the room design, and canopies/plastic casing used over these lights [35]. Certain types of fluorescent lights have higher UV quotients at wavelengths <400 nm [36] and would impact the product quality specifically in terms of oxidation differently [37]. For the case study shown in the published work [35], it can be seen that the UV exposure of about 5–10 Wh/m^2 can also lead to significant increase in aggregates. The light stress risk assessment should be done based on the manufacturing site information; hence, for early development studies, when the site has not been selected, it is good to be carried out at the conditions reflecting the worst case of the manufacturing options available for the molecule. The commonly used hoods without any modification may not be able to mimic the light conditions as the light type as well as canopies may not be exactly the same. Hence, customized light box or modification of photochamber needs to be implemented to generate different level illuminance and spectrum with the use of filters [38]. This helps understand the sensitivity of molecules to the realistic light stress, and based on the acceptable ambient light exposure concluded from these studies, the control strategy of setting limits on the ambient conditions that the molecule should be subjected during manufacturing, inspection, and packaging.

3.4 Metal-Induced Degradation Study

During manufacturing and storage of biologics, there is possibility of metal ions leaching into the products coming from different sources such as stainless steel metal tanks, glass vials, prefilled syringes, water, excipients, and residual from cell culture media. The amount of ions in the product is dependent on the different variables such as formulation component, pH of the solution, and contact surface area. Iron, chromium, and nickel are the most abundant metal ions present followed by copper, molybdenum, barium, and tungsten (residual coming from prefilled syringe forming process) [39, 40]. These transition metals lead to generation of radials by redox reactions with molecular oxygen and organic and inorganic substrates [41]. The major mechanism of oxygen activation by metal ions involves Fenton/Haber Weiss chemistry and auto-oxidation generating reactive oxygen species (ROS) leading to protein oxidation. The oxidation can also lead to physical instability as the oxidative modification of proteins leads to intermolecular associations [42]. To test the sensitivity of biologics product to metal stress in early phase of development, metal spiking studies with metal ions mentioned above can be carried out. The levels of the metal ions are usually in parts per billion (ppb) levels, and relevant levels should be used to distinguish this from forced degradation studies to understand degradation that can happen during manufacturing and storage. The quantification of the metal ions concentration of different drug substance and drug product batches should be done using ICP-MS. For a given DS and DP manufacturing sites, this can help determine the range of metal ions that can be found in the drug product in certain buffer system to inform the spiking studies. For later stage of development, exposure to stainless steel coupons with contact surface area similar to the one expected for highest acceptable fill volume in the tank can be carried out. In the published work by Zhou et al. [39], it was found that the buffer species and pH impact the capacity of metal ions to leach. Metal chelators such as EDTA and DTPA are usually used in the formulation for molecules sensitive to metal-induced degradation [43]. The concentration of metal chelators needs to be optimized as they can facilitate the leaching of metal ions in the product as seen in the work by Zhou et al. Hence, it is important to study the impact of each formulation composition during the exposure to metal to help design the formulation as well as exposure times for optimum product stability.

4 Scale-Down Models for Each Unit Operations

This section describes in detail the scale-down model that can be used for each unit operations. The utility of each scale-down model and the information and conclusion that can be drawn at the end of the study using the model are described below. The guiding principle for the development of the scale-down model is either based on CFD, fundamental transport phenomena, or characterization of the process using surrogate systems. The scale-down models usually require only about 100 ml to 1 L, making them highly efficient in the replacement of multiple at-scale runs.

4.1 Scale-Down Freeze–Thaw Study

Frozen bulk drug substance (BDS) is the most common storage condition for drug substance as it allows providing longer shelf life to build in flexibility in supply chain. However, freezing of biologics can cause aggregation which could be triggered by various factors such as pH changes, ice–water interface-induced denaturation, protein cryoconcentration, and crystallization of excipients [44]. Also, there are different types of container that have been used around the industry such as cryovessel, bottles, carboys, and bags with different material of construction. For freezing, the process parameters such as freezing rate, freezing temperature, fill volume, type, and size of container would all impact the stability of the protein [45]. During the process of freezing, there are multiple processes that take place during phase transition like cryoconcentration, solute redistribution, formation of ice interface, pH change, eutectic crystallization based on the drug substance, excipient concentration, and excipient type in the drug substance [46]. When small-scale system, e.g., 30- or 100-mL bags, are used to mimic 6-, 12-, or 16-L bags, the freezing time required in these small systems is substantially faster than the real scale even when the slowest freezing cycle is used. Also, the cryoconcentration seen in small size bags may not be representative of the ones seen in the large-scale bags. The cryoconcentration is one of the stresses that can contribute to protein aggregation as the cryoconcentration is also maintained after the thawing if the thaw process is static in nature. There are studies in literature showing the cryoconcentration as a function of the freezing cycles for different freeze containers such as published work by Lashmar et al. and Kolhe et al. [47, 48]. For instance, slow freezing rates can result in cryoconcentration, in which proteins and excipients form concentration gradients near the freeze front and are excluded from the ice–liquid interface. Slow freezing rates can result in pH shifts and phase separation, which can cause protein structural damage. Exposure to concentrated solutes due to water crystallization can also result in a loss of a protein's thermodynamic stability, leading to unfolding events and eventually causing aggregation. The storage of bulk protein at subzero temperatures can be performed in an uncontrolled or controlled rate. A controlled freeze and thaw rate results in reproducible temperature profiles between scales which uncontrolled rate cannot produce. Uncontrolled rate freeze and thawing of bulk protein at high concentration (i.e., > 50 g/L) in conventional storage containers (i.e., ≥1 L PETG bottles) can lead to higher fold of cryoconcentration (up to 4x fold) as shown in other work by Kolhe et al. [49]. It is seen when the freezing cycles are slow as slow freezing cycles allow the pure crystals to grow slowly without trapping solutes. Combination of studies needed to be done for proper risk assessment in bags as well as in vials to understand the impact of freeze–thaw on the protein stability. The studies in the vials can be done such that freeze and thaw at higher concentration can be evaluated to understand the risk associated with protein stability at higher concentration of solutes. During the early formulation screening and selection, it is important to keep in consideration the factors such as pH change that can happen during freezing (e.g., phosphate buffer pH change in frozen state) [50], excipient crystallization at frozen temperature such as trehalose [51, 52]. These considerations along with some initial freeze–thaw studies at concentration (1/3 or 1/4×) and (3× and 4×) of target concentration in a vial could

help give early risk assessment to freeze–thaw process specifically for high-concentration DS. Thawing can be done under different conditions such as uncontrolled ambient temperature or refrigerated conditions or controlled thaw at these temperatures. The impact of different thawing conditions on protein quality helps define the thawing procedure for clinical and commercial manufacturing.

The scale-down version of the containers can be subjected to different temperature profiles for freezing/thawing (as shown in Fig. 6) and also to different number of freeze–thaw cycles, e.g., 30-ml Celsius bags can be used for 6-, 12-, and 16-L Celsius bag, and the impact on critical quality attributes can be evaluated. The different rates can be generated using controlled system such as Sartorius Controlled S3 system or using lyophilizer. In the published work by Desai et al. [53], it was

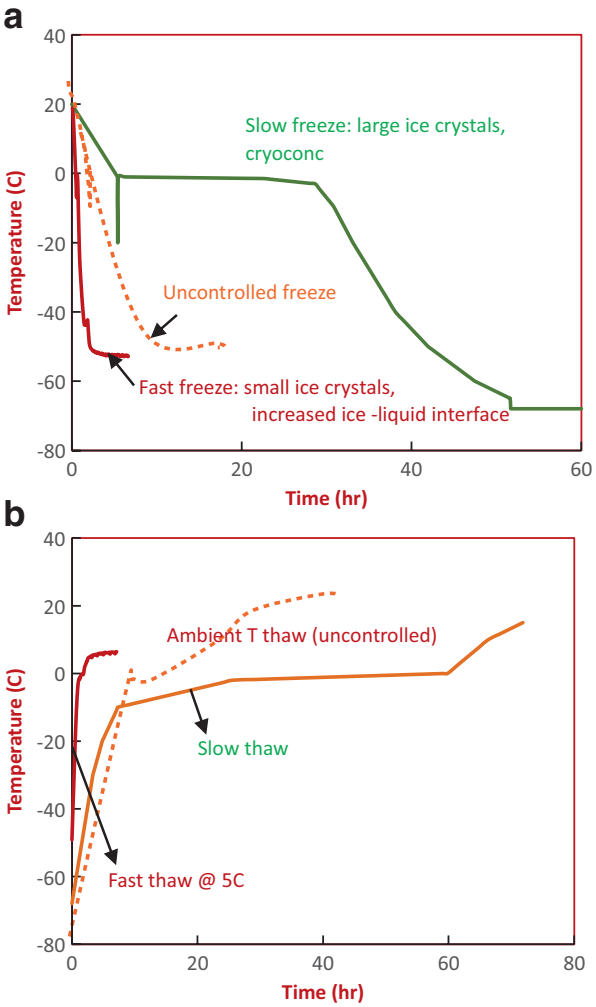

Fig. 6 (**a**) Temperature profile of freezing with different freezing rate. (**b**) Temperature profile of thawing with different thawing rates that can be used for evaluation of scale-down freezing container

shown that 30-ml bags could predict the changes in quality attributes seen at-scale. There was no significant impact on quality attributes seen for different rates for the first freeze–thaw cycles, but the differences are evident after multiple cycles. It was shown that for the given IgG1 system studied, the slow thawing after 2–3 cycles increased aggregation as the slow thawing leads to glycine crystallization due to extended exposure at low temperature due to slow rate, consistent with some discussion earlier in this section. The thaw rate and time in this case would be considered critical process parameter, and the control strategy needs to be in place to achieve it within the limits set. The use of controlled system for freeze–thaw in the manufacturing process could form the control strategy for the example discussed above.

4.2 Scale-Down Mixing Model

Mixing is an essential unit operation in the manufacturing of biologics needed to achieve the homogeneity of pooled drug substance or to dilute the drug substance and convert it to the drug product [54]. There are various factors that can impact the efficiency of mixing process as well as the product quality, such as fill volume, solution properties of the mixing components (density and viscosity), solution temperature, impeller, and tank geometry [55]. Scaling up from smaller tank size to larger tank size or even from one large tank to another is not simple as the tank aspect ratio, impeller design/aspect ratio in relation to the tank, and impeller placement/location in the tank can vary. Hence, CFD modeling can be utilized to model the various scenarios for a given tank geometry/impeller design via a systematic approach where the simulations are carried out at different levels of parameters such as fill volume, impeller speed, and incoming liquid addition rates [56]. The flow and velocity field simulated over different condition can then be processed to find the integral surface and volume shear rate during mixing [57]. There are two ways that CFD modeling along with small-scale version of the tank could be helpful in defining the design space for the mixing process: (i) mixing time validation using CFD and small-scale experimental study and (ii) shear rate impact on the product quality. Case study discussed below includes experimental addition by adding 0.1–1 M NaCl solution to the mixing tank at different conditions and using conductivity probe measuring the conductivity over time at frequent intervals (milliseconds). The experimental mixing time is defined as the time reached to achieve 99% of the expected final concentration. The mixing time is measured at different location, the worst being close to the top of the tank. Tracer transport CFD simulation is done on the tank with same parameters for prediction of mixing time using approach similar to one shown by Dongjin et al. [58]. In Fig. 7a, the comparison of experimental vs CFD mixing time in 250-ml tank is shown demonstrating the predictability of CFD simulation. Additional confidence level could be built in prediction of mixing time by estimating the scale-up factor between the small-scale tank and large-scale tank using scaling parameter such as tip speed. Figure 7b shows the approach used between 250-ml and 200-L tank simulation at different tip speed. The scaling factor of 5 is being estimated between the tanks. The design space for mixing time parameter at large scale should take this into account.

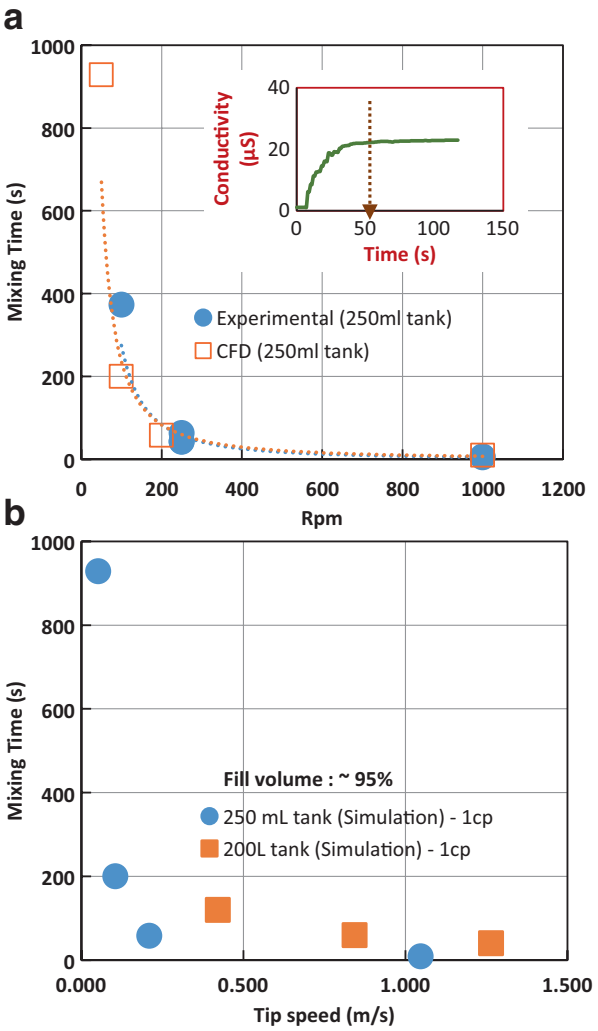

Fig. 7 (**a**) Comparison of mixing time estimation via experimentation and CFD in scale-down 250-ml tank with a fill volume of 200 ml. Insert shows the saline spiking study with conductivity measurement used to estimate mixing time experimentally. (**b**) Mixing time as function of tip speed when 250 ml and 200 L are compared to estimate scale-up factor between the two tank sizes

For shear rate impact study, the mixing experiments are carried in small-scale tank at shear rates comparable to ones expected at large scale (assuming worst case of low fill volume and highest rpm) for largest mixing time defined by operating range. The product quality of pooled drug substance as well as final diluted drug product solution is characterized by stability indicating assays. These experiments can then be done at higher shear rates (2× to 5×) of the expected shear rates, and the

product quality is tested again. If there is no impact on product quality seen, then this would help expand the proven acceptable ranges for rpm, mixing time, etc.

Theoretically, this can be kept repeating till edge of failure is seen defining the total maximum shear that the product can be exposed too. The critical aspect of using any modeling tool is validation at scale, hence whenever possible sampling to be done on clinical batches to help get an estimate of mixing time at scale. The same could be done for shear rates for process development/engineering run batches. With enough confidence demonstrated in the scale-down mixing models by validation studies, future process development or engineering run batches at scale can be minimized or avoided all together.

4.3 Scale-Down Filtration Study

Filtration is a commonly used unit operation in the pharmaceutical industry that serves different purposes such as removal of particulate matter, clarification, and sterilization of a solution. In the manufacturing of biologics, different factors such as the characteristics of the filter membranes, formulation components, and process parameters can impact the quality of the product after filtration. One major disadvantage that could be encountered during filtration is the adsorption of the active ingredient and/or excipients of the formulation to the filter membrane [59, 60]. The application of scale-down filtration systems allows for the identification of potential risks of adsorption during processing along with potential mitigation strategies.

In this *case study*, we show the application of a scale-down filtration system to identify potential processing risks associated with adsorption of surfactant and protein to sterilizing filters. The aim is to test filters commonly used during drug product manufacturing and provide an associated mitigation strategy by defining the filter flush volume (i.e., volume of protein solution and/or number of vials necessary to be rejected at the beginning of the filling operation due to low protein and/or surfactant concentration).

The scale-down filtration tool is composed by a reservoir tank connected to a stack of filters in series, normally a 0.45-μm clarifying filter and two 0.22-μm sterilizing filter (the set of filters should be selected based on the ones used at large-scale system, e.g., Millipak 20 can be used as scale-down filter for Millipak 200). The total surface area of the filters is 52.75 cm^2.

To perform the experiments, 70 ml of a protein (monoclonal antibody) formulation histidine based with 0.5 mg/ml of surfactant was added to the reservoir tank connected upstream of the filtration tool. The reservoir tank was sealed and connected to a nitrogen supply to be pressurized and begin the filtration. Filtered samples were collected in 2-mL increments for 40 mL of filtration volume. All collected filtration samples were tested for protein concentration via A280 UV-Vis absorption. The average concentration as a function of scale-down filter flush volume is shown in Fig. 8. The protein concentration remained unchanged before and after filtration, showing low risk of protein adsorption to the filter membrane.

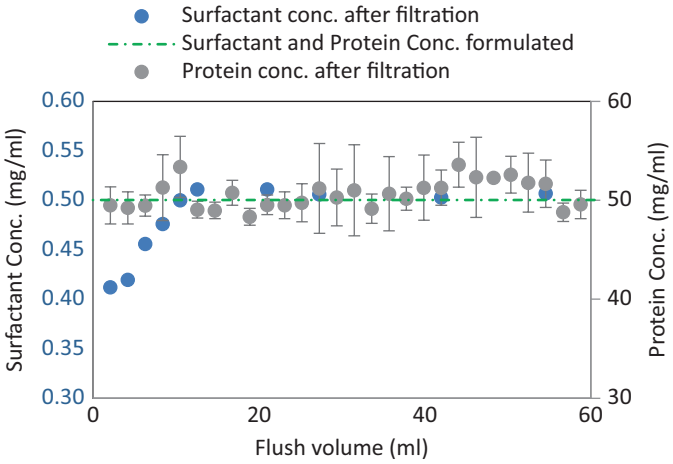

Fig. 8 Figure markers represent the average protein and surfactant measured after the filtration as a function of the collected volume

The surfactant concentration was measured using a bis-ANS fluorescence-based method [61]; the data clearly demonstrates surfactant adsorption for the first few samples (Fig. 8). The minimum saturation point (MSP) for surfactant adsorption was determined when the surfactant concentration reached the formulated concentration (0.5 mg/ml). The MSP scale-down filter flush volume was found to be 10 mL.

The amount of surfactant removed from the system by the sterilizing filters was 0.5 mg and was calculated from the data in Fig. 8 using Eq. 2.

$$Surfactant\ removed = \sum_{n=1}^{20} Vn\left(\left[Surf\right] formulated - \left[Surf\right] measured\right) \tag{2}$$

where n is the sample number, V_n is the *nth* sample volume (mL), *[Surf] formulated* is the initial formulation concentration of surfactant (mg/mL), and $[Surf]_{measured}$ is the measured concentration of surfactant (mg/mL) for each nth measurement.

The filter surface coverage (Γ) across all three filters in series was 0.01 mg/cm^2 calculated from the total surfactant removed as shown in Eq. 3.

$$\Gamma_{surfactant} = \frac{Surfactant\ removed}{SA_{filter}} \tag{3}$$

The main goal of applying the scale-down filtration tools is to identify the risk of protein and surfactant adsorption to the filters, and use this information to develop potential mitigation strategies for the scale-up process. The results summarized above clearly demonstrate that filter adsorption represents a processing risk for the protein formulation evaluated.

In order to develop a general mitigation strategy, two filter flush volume approaches for manufacturing are evaluated.

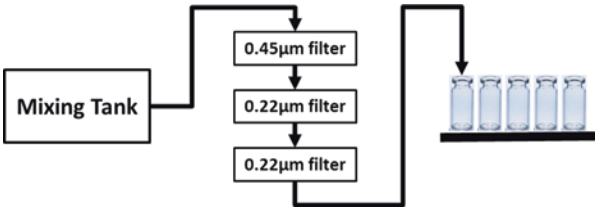

Fig. 9 Schematic of direct filtration and filling manufacturing for mitigation strategy 1

Table 3 Estimation of filter flush volume at scale based on direct vial filling after filtration

Scale	Filter surface area (cm^2)	Flush volume (mL)
Scale-down skid – Lab	52.75	10
Manufacturing	3000	569

The *first strategy* for estimating a filter flush volume to avoid vials with low surfactant levels involves a manufacturing line where the product is filtered through all three filters before being filled into the vials. See the schematic representation in Fig. 9. This manufacturing strategy represents the worst-case scenario for filter flush volume as the product is exposed to the greatest amount of filter surface area before being filled into the product vials.

The filter volume at scale can be estimated based on the ratio of total surface areas (*SA*) and scale-down filter flush volume (*V*), as seen in Eq. 4. Applying Eq. 4, the filter flush volume at scale based on direct vial filling after filtration which in this case study was around 500 mL, as summarized in Table 3. Dividing the flush volume by the vial fill volume for the protein formulation evaluated, it is possible to determine the number of vials that need to be rejected at the start of manufacturing to achieve filter saturation and reduce the risk of out of specification product vials. In this case, 30 vials need to be rejected following this approach.

$$V_{Manf} = V_{Lab} \frac{SA_{Manf}}{SA_{Lab}} \tag{4}$$

The *second strategy* for estimating filter flush volumes involves a manufacturing line where the entire product volume is filtered through a set of 0.45-µm and 0.22-µm filters into a filling tank, followed by filtration through a final 0.22-µm sterilizing filter into a secondary buffer vessel, and finally into the product vessel. A schematic of this manufacturing line is shown in Fig. 10.

This approach estimates the material loss on the filter and its impact on the concentration within the buffer vessel. If the buffer vessel is sized appropriately, the surfactant concentration will be minimally impacted. The concentration of surfactant concentration as a function of buffer vessel size (see Table 4) can be estimated based on the equilibrium surface coverage calculated from the scale-down system (0.01 mg/cm^2) and the manufacturing filter surface area (1000 cm^2).

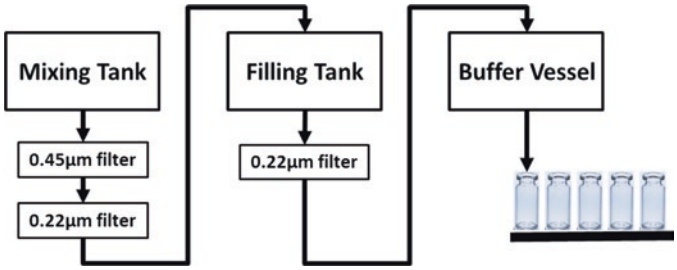

Fig. 10 Schematic of using a secondary buffer vessel filling manufacturing for mitigation strategy 2

Table 4 Estimation of secondary buffer vessel concentration as a function of vessel volume

Buffer vessel volume (ml) (manufacturing)	Surfactant concentration (mg/ml)
100	0.41
250	0.46
500	0.48
1000	0.49
2000	0.5

The results in Table 4 demonstrate that normalizing the material loss via moderate volume pooling in the buffer vessel can generate in-specification bulk concentrations without any need for filter flushing or vial rejection. This type of approach can help minimize material waste by reducing the need for filter flush volumes, thereby saving drug product.

Overall, the scale-down filtration systems allow for the fast evaluation of potential risks during drug processing prior to scale-up manufacturing.

4.4 Scale-Down Pumping and Filling Study

For filling the drug product solution in the final container or vials, the solution is transferred using pumps and filling needle. There are different types of pumps that can be used which include peristaltic pump, positive displacement (rotary piston pump), and time pressure pump. The use of rotary piston pumps has decreased in last few years because of various observations across industry and literature of particle generation during filling [62, 63]. Some of the syringes filling lines though are still integrated with rotary piston pumps. The shear rate for different pumps varies and falls in the order of 3 K to 100 K s^{-1} depending on the pump type and pumping/filling parameters. The piston pump and time pressure pumps have been used for their reliability and accuracy, but they do require regular maintenance and disassembly for cleaning and sterilizing. For example, cross-contamination between batches is a big concern for rotary piston pumps, and hence, multiple piston sets

need to be used for each product. Peristaltic pumps use single-use tubing which eliminates cross-contamination as the only part that is in contact with product is the tubing. The new pulsation-free peristaltic pumps, with multiple rollers, offer accuracy for filling, and choice of different tube sizing allows the filling over a wide range of volumes. The filling accuracy is low for highly viscous liquids as by design the pumps are calibrated to provide no more than approximately 1.3 bar of pressure [64]. Time pressure pumps comprise a pressurized product vessel and an orifice with a pinch valve that is opened for certain amount of time to dispense a given volume of product and is capable to handle viscous products.

If minipiloting tool for shear stress discussed in above sections is used, there would be some information of which pump types would be better fit for a given drug product. All of these pumps are available as a single unit for small-scale studies. Design of experiment approach could be taken to carry out the study to understand the impact of pump speed, tubing diameter, pump acceleration, orifice size, needle diameter, and needle insertion depth. This would help understand the product quality impact across the entire design space. The ranges can be stretched beyond the normal operating range of a given site to test the proven acceptable range for given pump type. Tighter control on pump parameters in terms of needle location and needle reversing path is needed to avoid foaming and splashing during the fill especially for molecules which are sensitive to interfacial stress. The interfacial stress sensitivity derived from minipiloting tool discussed in the above section would help understand if tighter controls of parameters are needed. For viscous product fill, it is important to take into consideration the needle insertion depth, the stop time between filling, the total filling time, and the needle suck back at the end of each fill. There can be crusting and drying of proteins that can take place. There is a case study shown in Hanslip et al. [65], where the variation in the suck-back pump speed and volume helps reduce the drying and the filling needle clogging for viscous liquids by altering the liquid front from a droplet or meniscus at the needle tip to a certain point inside the needle.

5 Robust Tech Transfer to Manufacturing Site

In conclusion, case studies discussed above show that combination of small-scale studies and scale-down models can help in robust tech transfer and manufacturing. The use of small-scale studies early in the development helps provide risk assessment, mitigation strategy, and critical information for site selection process. After the site selection, scale-down models can then be used to evaluate specific risk and control strategy for each unit operation independently as a part of facility-fit process. An integrated approach can also be taken, where the biologics drug substance is passed through scale-down models of each unit operation in a sequence similar to the actual manufacturing process. For this approach, the studies can be carried out at the limits of normal operating ranges (NOR) as well as proven acceptable ranges (PAR) for a given site/unit operations. The product quality at the end of the

manufacturing process as well on storage can then be evaluated to validate the operating ranges. The other advantage of this approach is its utility to evaluate the impact of variation in DS quality on final drug product quality after manufacturing and storage. This is very difficult to carry out at manufacturing scale as this would involve multiple at-scale engineering batches and need for large amount of different DS material. There are two outcomes that can come from this evaluation: (1) modification of process parameter ranges for DS and DP and (2) modification of specification of DS defining the allowed variation in DS quality. This demonstrates a holistic process development approach where quality at each stage of manufacturing is taken into consideration to ensure consistent final drug product quality.

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Chapter 18
Design of Clinical In-Use Studies

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1 Introduction

In clinical development, the safety and efficacy of parenteral biologics are typically assessed over a broad range of clinical doses. During this phase, it is common to develop the drug product at a fixed-strength formulation in a liquid or a lyophilized dosage form and, subsequently, conduct product administration through delivery routes such as intravenous (IV) infusion or subcutaneous (SC) injection [1, 2].

Biopharmaceutical companies control the manufacture and quality of the clinical trial materials as per the well-established regulatory guidelines [3]. However, during in-use, which refers to the steps involved from aseptic dose solution preparation to its administration, further manipulation of the drug product is generally necessary by the end-users in hospital pharmacies. For these operations, several quality and

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regulatory documents are available to provide general guidance [4–15], and amongst these, USP<797> Pharmaceutical Compounding-Sterile Preparation is frequently recognized and referenced as the standard.

From drug product development perspective, however, it is well known that the in-use procedures and administration components can impact the physicochemical and microbiological stability of biological parenteral products [16–20]. Therefore, it becomes imperative for the drug manufacturer to define product-handling procedures that ensure the quality of the dose solution administered to a patient.

Regulatory guidelines require manufactures to demonstrate compatibility of drugs with diluents and administration devices or components, and to file this information as part of the regulatory filings. As an example, the ICH Q8 guideline states "The compatibility of the drug product with reconstitution diluents (e.g., precipitation, stability) should be addressed to provide appropriate and supportive information for the labelling. This information should cover the recommended in-use shelf life, at the recommended storage temperature and at the likely extremes of concentration. Similarly, admixture or dilution of products prior to administration (e.g., product added to large volume infusion containers) might need to be addressed."

Clinical in-use (CIU) studies refer to evaluations performed by pharmaceutical scientists in a laboratory setting to assess the impact of the intended product in-use steps on the physicochemical and microbiological qualities of the dose solution administered to a patient. The results for these studies guide the pharmacy manual that provides instructions on how to prepare, handle, and administer the various dose solutions prepared by the end-users. During the course of development, these studies are continually refined to align with the clinical study protocol and pharmacy practices. For example, during first-in-human clinical studies, in-use studies are designed to provide flexibility to allow evaluation of a broad range of clinical doses, whereas for later stage clinical trials, after the dose is defined, the focus of these studies is to ensure the defined in-use procedure is robust and convenient.

For commercial products, dose administration instructions are provided as part of the product Dosage and Administration section of labeling as per 21 CFR 201.57(c)(3); specific requirements are described in the Guidance for Industry document titled "Dosage and Administration Section of Labeling for Human Prescription Drug and Biological Products—Content and Format" (US Department of Health and Human Services and Drug Administration 2010) [21].

In this chapter, clinical in-use testing approaches are primarily laid out for parenteral protein products administered intravenously. The discussion is geared towards products in early clinical development. In addition, as recent advances in antibody engineering have resulted in a new generation of complex biologics that often present challenging physiochemical properties (e.g., bispecific antibodies, antibody-drug conjugates (ADCs) etc.), the recommendations are presented to encompass a range of protein formats [22]. In Part I of the chapter, an overview of the technical and practical considerations in the design of clinical in-use studies is presented along with specific study design recommendations, and in Part II, several case studies highlighting in-use stability challenges, and the solutions implemented, are discussed.

2 Design of Clinical In-Use Studies: Technical and Practical Considerations

In the following sections, we discuss the key technical and practical considerations to guide the design of clinical in-use studies for protein biologics administered intravenously. These considerations are illustrated in Fig. 1 for a lyophilized intravenously administered product. For quick reference, a summary of these considerations is provided in Table 2.

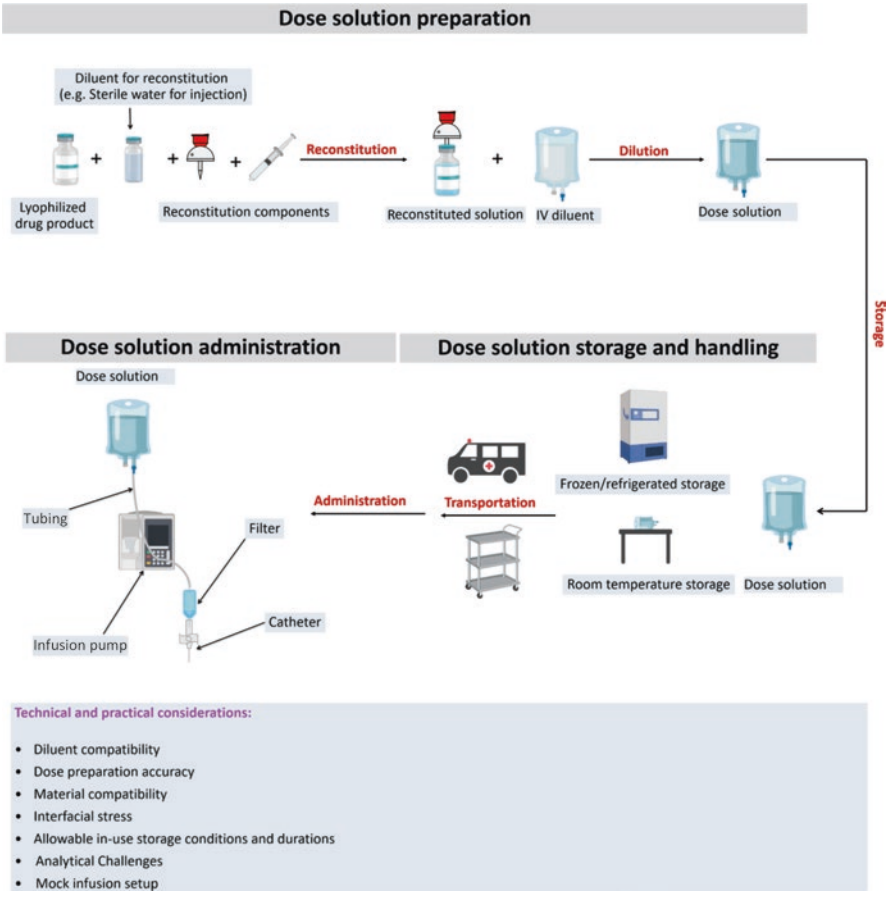

Fig. 1 Dose solution preparation, handling, and administration: an example for a lyophilized IV product

2.1 Diluent Compatibility

To prepare a dose solution suitable for patient administration, dilution of the drug product is often required at hospital pharmacies. Many diluents are available for parenteral administration [23], with 0.9% Sodium Chloride Injection (normal saline), 5% Dextrose Injection (D5W), and Lactated Ringer's Injection being the most commonly used iso-osmotic IV diluents. The dilution step alters key product formulation factors, such as pH, ionic strength, and excipient concentration, which may impact protein stability [24–28]. For example, the use of 5% dextrose solution is prohibited for several admix products [29, 30]. Therefore, the effect of protein dilution in the diluent matrix of interest must be assessed during in-use studies [31–34].

For operational flexibility, it is generally advisable to screen and enable multiple diluent options that are acceptable for patient dosing. It is important to design the study to cover the worst-case in-use scenario conditions, for example, it is a good practice to minimally test stability of the lowest and the highest dose solution concentrations that bracket the planned clinical dose range, and assess the worst-case storage duration at applicable storage temperatures. For example, if the anticipated clinical dose range for a phase 1 study is between 0.1 mg/kg and 1.0 mg/kg, assuming a patient weight range of 40–120 kg, the dose required is between 4 mg and 120 mg. If a 50 mL IV bag is planned for clinical administration, the nominal dose solution concentration range in the IV bag will be between 0.08 mg/mL and 2.4 mg/mL. The IV bag overfill practices vary considerably across manufacturers; therefore, the range of approximately 0.06–2.75 mg/mL (target ± 15%) may be selected to ensure stability [35]. Additionally, to avoid confounding interactions from the administration components such as IV bags, preparation of dose solution in inert glass container is recommended as a study control at this stage.

2.2 Dose Preparation Accuracy

For patient safety, accuracy of the dose solutions prepared by end-user is of critical importance. The dose solution preparation procedures that appear to be simple and straightforward in a laboratory, using pipettes, micro-syringes, and glass containers, are often not feasible in hospitals and clinics, where access to sterile commodities is a must for patient safety.

The dose solution preparation practices used for product reconstitution, solution withdrawal and transfer (e.g., when performing serial dilutions), vary considerably across clinical sites, which can impact dosing accuracy [19, 36–40]. Procedurally, these are commonly carried out using disposable syringes, empty IV bags, stopcock devices, and closed system transfer devices (CSTDs) [41]. The dead volume of the components used by the end-users could impact dose preparation accuracy. For example, variability in dose solution concentration has been reported based on the size and graduation of the syringes used during the dose solution preparation process. Therefore, an assessment of the dose solution preparation accuracy using realistic scenarios is important during in-use studies.

In some cases, it may be necessary to adjust the protein concentration in the formulation and/or the drug product fill configuration to achieve an acceptable dose solution preparation procedure; therefore, in-use requirements should be evaluated early on during formulation development. For example, if the anticipated clinical dose range for a phase 1 study is between 0.1 mg/kg and 1.0 mg/kg, assuming a patient weight range of 40–120 kg, the dose required is between 4 mg and 120 mg. Transfer of low-volume solutions (e.g., <0.1 mL) using the standard 1 cc syringes is generally not practical; therefore, to avoid multiple dilution steps, a 20–50 mg/mL formulation may be more suitable than a 100 mg/mL formulation.

2.3 Material Compatibility

During product in-use, the dosing solution inevitably comes in contact with various administration components. These components are comprised of materials different from the drug product primary packaging and sourced from manufacturers all over the world, which can impact product quality.

Material incompatibility due to solid-liquid interfacial stress may be linked to multiple factors including properties of the protein (e.g., size, charge, pI, Gibbs free energy of unfolding), formulation composition (e.g., pH, excipient concentration), diluent properties (e.g., pH, ionic strength, charge), and contact material properties (e.g., charge, hydrophobicity, morphology, roughness, flexibility) [42, 43]; additional factors such as the contact duration, storage temperature, shear stress conditions, etc. may also be important [44]. Incompatibility could also arise from component leachables [42, 45]. Numerous examples are reported in literature, and in most cases, the solid-liquid interface-induced instability manifests in the form of protein adsorption, aggregation, particulate formation, or loss of protein activity [46–51].

A list of the most frequently used administration components and their fluid contact material, a database generated through in-house inquiries across global clinical sites, is shown in Table 1. As it is not practical to test each component in a laboratory setting, the concept of designing clinical in-use studies that test for the contact

Table 1 Commonly used components for IV administration at clinical sites

Components	Manufacturer/brand example	Commonly reported fluid contact materials for in-use components[a]
Syringe	BD/Plastipak™ B.Braun/ Omnifix®	Polypropylene (PP) Polyethylene (PE) Polystyrene (PS)
Sterile needle	BD/PrecisionGlide®	Stainless steel PP
Spike	B.Braun/Mini-Spike® Codan/Chemoprotect®	Styrene acrylonitrile (SAN) Acrylonitrile butadiene styrene (ABS) Acrylonitrile-vinylchloride copolymer (AVC)

(continued)

Table 1 (continued)

Components	Manufacturer/brand example	Commonly reported fluid contact materials for in-use components[a]
Closed system transfer device (CSTD)	BD/PhaSeal™ BD/Texium™ ICU Medical/ChemLock™ ICU Medical/ChemClave™ B. Braun/OnGuard® Equashield/Equashield®	Methylmethacrylate acrylonitrile butadiene styrene (MABS) Acrylic Silicon Polyvinyl chloride (PVC) ABS Polycarbonate (PC) PP Thermoplastic elastomer (TPE) Stainless steel Polytetrafluorethylene (PTFE) PE
0.9% normal saline IV bag	B.Braun/PAB® B.Braun/EXCEL® Fresenius Kabi/freeflex® ICU Medical/Flexible container ICU Medical/VisIV Flexible container	Polyolefin (PO) PVC PP
5% dextrose IV bag	B.Braun/PAB® B.Braun/EXCEL® Fresenius Kabi/freeflex® ICU Medical/Flexible container ICU Medical/VisIV™ Flexible container	PO PVC PP
Empty IV bag	Baxter/IntraVia™ ICU Medical/Flexible container ICU Medical/EVA infusion bags Metrix/Secure™ EVA container	PO PVC Ethyl vinyl acetate (EVA)
Sterile peripheral intravenous catheter	BD/Insyte™ Autoguard™ B.Braun/Introcan Safety®	Polyurethane (PUR) Fluorinated ethylene propylene (FEP)
Sterile IV tubing	ICU Medical/Primary PlumSet™ B.Braun/Cyto-Set® Codan/Cyto-Ad Z® Fresenius-Kabi Baxter/Interlink®	ABS PVC PUR PE Polyethersulfone (PES) PP Polyamide (PA) PS
Sterile in-line or add-on filter	Codan/Star® PALL/Posidyne® ELD Terumo/Terufusion® BD Extension set B.Braun/Sterifix® B.Braun/Intrapur®Plus	PES PA PSF (polysulfone)
Sterile stopcock	B.Braun/Discofix® BD/Connecta™	PC PA PP

[a]Material information obtained from manufacturer/product catalog/technical data sheet

Table 2 Design of clinical in-use study: key considerations and recommendations for intravenously administered parenteral products

In-use procedure	Technical and practical considerations	Study input information	Study design recommendations
Dose solution preparation	Diluent compatibility Dose preparation accuracy Material compatibility Interfacial stress Allowable in-use storage conditions and durations Analytical challenges Mock infusion setup	Drug product formulation Administration route Clinical dose range Infusion volume range Patient weight range Preferred diluent Components used for dose solution preparation	Test lowest and highest dose solution concentrations; evaluation of intermediate dose solution concentration recommended Prepare dose solutions using procedures used by pharmacists; test for reproducibility and accuracy Evaluate worst-case dose solution fill volume Test multiple diluents Pull samples to assess change from T0 Include replicates. Include appropriate study controls (e.g., diluent blank, dose solution in glass vial)
Dose solution storage, handling, and transportation		Preferred hold-time for operational flexibility Transportation mode for prepared dose	Test to cover worst-case room temperature and refrigerated storage conditions Include interfacial stress assessment: Mixing/inversion, shaking Pull appropriate samples to assess change from T0 and draw stability trends Include replicates. Include appropriate study controls (e.g., diluent blank, dose solution in glass vial)
Dose solution administration		Infusion system setup Components used for infusion Infusion duration range Infusion flow rate range Line prime and flush requirements	Conduct mock infusion study using applicable administration setup(s). Include components to maximize coverage of material types Test to cover worst-case infusion duration (slowest flow rate) and worst-case shear stress (fastest flow rate) Test to cover solution hold-up in an infusion line Test with and without the use of in-line filter Test the impact of line flush procedure, if applicable Pull appropriate samples to assess change from start of infusion process Include replicates. Include appropriate study controls (e.g., diluent blank)

material compatibility rather than individual component is commonly implemented. If the drug product solution is compatible with the evaluated material type, the use of all commercially available materials in that category may be allowed.

Similar to diluent compatibility studies, the material compatibility assessments should be designed to cover the worst-case in-use scenario conditions. The evaluation should include worst-case simulation for the infusion line as well. For example, due to dosing interruptions, the dose solution could be held in the infusion line for durations longer than anticipated.

Comprehensive evaluations should also be performed to assess filter compatibility where issues due to protein adsorption, filter particle shedding, and leachables have been reported [52–55]. Generating data both with and without the use of an in-line filter with the commonly used 0.2 μm and 1.2 μm filter sizes is recommended. Due to patient safety concerns, for risk mitigation, the use of filter is recommended, and use is mandated if particles are observed [56–58].

It is worth noting that the use of some materials such as bis(2-ethylhexyl) phthalate (DEHP), a plasticizer that is added to make the PVC bags flexible, may be restricted in certain countries due to increasing concerns regarding its adverse impact on human health [59, 60]. DEHP can leach from PVC containers in the presence of surfactants (e.g., polysorbate 20 and polysorbate 80) under ambient conditions [61]. Alternatives to DEHP include PVC bags with tri-2-ethylhexyl trimellitate (TOTM) or PVC container that is lined with polyethylene (PE). Testing of multiple IV bag material types is therefore recommended for operational flexibility.

If incompatibilities are observed, the use of materials in the clinical study must be restricted [62]. In such cases, evaluation of alternative compatible materials, or IV diluents, or more novel in-use stabilization approaches may be necessary; the case studies presented in this chapter highlight some of these approaches.

2.4 Air-Liquid Interfacial Stress

Air-liquid interface is introduced during various in-use manipulation steps: swirling, inversion, or shaking of the vials during or after reconstitution, inversion or shaking of IV bags during dose solution preparation, shaking of dose solution during transportation, pump infusion stress, etc. [5, 7]. Interfacial stress due to the air-liquid interface is known to destabilize proteins and induce aggregation/particulate formation [63–67]. Therefore, surfactants such as polysorbate 20 and polysorbate 80 are commonly added to protein formulations to protect proteins from interfacial stresses [68–70]. Several studies have reported that to effectively suppress interfacial stress-induced instability, concentration of surfactant above a certain threshold is necessary [47, 71, 72]. During product in-use, as the surfactant concentration is reduced in the diluted dose solution, evaluation of the involved interfacial stress factors is important.

Recommendations for testing air-liquid interface stress in the in-use studies include testing the worst-case fill volume in the intended infusion container

(e.g., use half-filled IV bags), evaluating the effect of agitation and shaking stress (e.g., include steps such as inversion of the bags end-over-end several times, test the impact of shaking bags on an orbital shaker for several minutes), and testing the minimum and maximum flow rates that bracket the planned infusion duration in the clinical protocols.

2.5 Allowable In-Use Storage Conditions and Durations: Physicochemical and Microbiological Considerations

For operational convenience, it is important to provide adequate hold time duration of all solutions prepared during product in-use. The hold time duration for in-use solutions is dependent on both the dose solution stability findings based on physicochemical testing and the microbiological considerations.

In-use studies should incorporate testing at all relevant storage conditions, for example, refrigerated and room temperature conditions. Stability evaluation of dose solution in contact with the administration components at multiple time points is recommended to allow adequate trending of data. As an example, for a product requiring several hours of infusion at room temperature, to obtain a robust data set, it may be appropriate to generate in-use storage data for dose solution in IV bags through 24 hours at 2–8 °C and 30 °C/75% RH, which covers the worst-case climate zone 4 [13, 14].

For a lyophilized drug product, stability of the reconstituted solution must be tested, for example, through 24-hour exposure at room temperature/room light conditions [9]. A mock infusion study should additionally be performed to assess product quality impact through the end of the administration.

Microbiological consideration is an important factor in defining the storage conditions and durations for compounded sterile preparations. The maximum allowed beyond-use dating is provided by USP <797> based on risk level assessment, accounting for factors such as the sterile compounding environment, the number for drug product units, as well as entries into a sterile container or package.

USP<797> has strict requirements on the facility and environment for compounded sterile preparations, which may not always be possible to control at clinical sites. As per the EMEA guidelines [17], unless dose solution preparation has taken place under controlled and validated aseptic conditions, regardless of the physiochemical stability, immediate product use is recommended for a nonpreserved product due to the potential for microbial contamination.

If the product is not used immediately, the in-use storage conditions and durations should normally not exceed 24 hours at 2–8 °C (from the withdrawal of the drug product until completion of drug product administration) [17]. Within this 24-hour duration, the drug product may be handled at room temperature for a defined duration (e.g., 4 hours). If longer storage at room temperature is required [34], further testing, e.g., microbial challenge testing, may be required unless otherwise justified [20, 73].

2.6 Analytical Challenges

Dose solution characterization should be carried out using a broad range of analytical methods to assess the physical, chemical stability and biological activity of the drug product. Ideally, the test methods used are the same as those used for drug product release. However, one of the common challenges with in-use stability studies is that the available release methods may not be suitable, and method modification or new method development is required. For example, it is common for the protein concentration post-dilution to be outside the validated range of the analytical method or be below the assay quantification limit [51]. The IV diluent matrix and/or the leachables from the administration components may also affect method performance [74–76]. Therefore, method assessment in the dose solution matrix and protein concentration range of interest is critical prior to conducting these studies [77–79].

To avoid experimental artifacts, it is also strongly recommended that sample handling procedures for the collected in-use samples be defined through appropriate experimentation prior to study start. This is an important evaluation that often impacts the overall testing strategy for in-use studies. For example, unlike the drug product, in-use samples may not be stable against freeze-thaw stress, may have limited 2–8 °C storage stability, and may not withstand shipment stress across different testing labs. "Real-time" testing is often necessary for in-use stability samples. Appropriate diluent and formulation buffer controls should also be included to measure the background signal [54]. Furthermore, testing of minimally duplicate samples is recommended to generate a robust data set.

Test methods that evaluate physical degradation of the protein must be included during in-use studies (e.g., pH, osmolality, appearance, protein concentration, aggregation, particulates). Due to the short in-use exposure time, while chemical degradation is generally not a common occurrence, testing of all key product quality attributes is recommended [31].

While the drug product release criteria are not intended for sterile dose preparations, where applicable, these could be used to assess stability trends (e.g., aggregation, fragmentation, charge isoforms, or potency) [8]. Due to product dilution, for certain attributes, stability assessment is based on the change from starting value (e.g., pH, protein concentration appearance, and osmolality). For protein concentration, the commonly used limits are 90–100% of the target dose [19, 80]. For subvisible particles, while acceptance criteria guidelines are not clearly defined, a common practice is to monitor degradation trend and report particulate results on a per mL basis. For samples collected postinfusion, if particle counts meet the USP <788> specification limits, results are deemed acceptable (infusion volume $\leq$ 100 mL: small volume parenteral (SVP) specification, infusion volume > 100 mL: large volume parenteral (LVP) specification).

2.7 Mock Infusion Setup

Many infusion setup options exist for IV delivery, which are highly dependent on the drug and the patient [81–83]. Other important factors may include the pump propulsion mechanism, dose solution properties, volume of fluid administered, manifolds used, dead volume, etc. For the purpose of clinical in-use studies, to simulate the representative components and shear-stress conditions, an understanding of the infusion setup at the clinical sites is important and evaluation of the intended pump system is recommended. Large-volume infusion pump-driven systems generally utilize an IV bag with capacity of 50–1000 mL, while syringe pump-driven infusion systems utilize a syringe with capacity of 60 mL or lower.

In phase 1 studies, to enable dosing over a broad range of doses, the use of a syringe pump may be necessary. Selection of the infusion setup is generally dictated by the lowest stable dose solution concentration in IV bags. For example, if the lowest stable dose solution concentration is such that infusion of <25 mL solution is necessary, the use of a syringe pump setup may be necessary. Based on our experience, the lowest feasible infusion volume and flow rate with a syringe pump requires careful assessment through discussions with clinical sites. For example, the use of Y-ports, or a piggyback setup, may be necessary to enable low-volume infusions to keep the vein open to maintain flow continuity, which may impact the in-use study design [51, 84]. Based on system dead volume and related drug recovery considerations, a line flush procedure may also be necessary, which may need evaluation due to the further dilution of drug.

2.8 Clinical In-Use Study Approach

A staged approach may be taken to study the individual components of a clinical in-use study (e.g., diluent compatibility, dose preparation accuracy, material compatibility, interfacial stress, infusion stress, etc.). This approach is helpful to define the intended in-use process. A subsequent confirmatory study, with full simulation of the worst-case in-use process, is recommended to ensure the established procedure is robust; an example of this approach is shown in Fig. 2 (Table 2).

3 Clinical In-Use Case Studies for Varied Biological Modalities

This section highlights the CIU challenges commonly encountered for protein therapeutics by presenting case studies of several biological modalities. Table 3 summarizes the key CIU challenges encountered in these case studies and the strategies implemented to overcome those challenges.

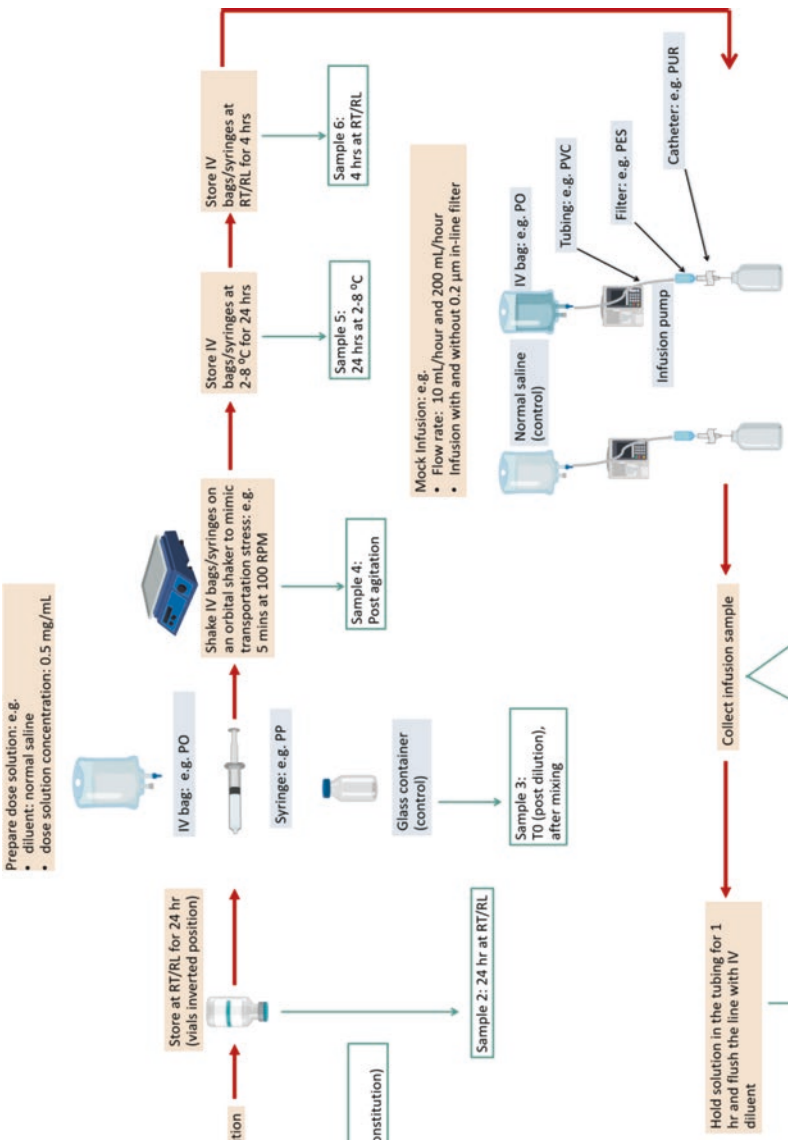

Fig. 2 Clinical in-use study design example for a lyophilized IV product (IV diluent: 0.9% normal saline; dose solution concentration: 0.5 mg/mL; infusion system: large-volume pump, polyolefin bags, and PES in-line filter; infusion flow rate range: 10–200 mL/hr)

Table 3 Clinical in-use (CIU) case studies: stability challenge and solution implemented

Case study	Key CIU challenge	Solution implemented
Case study 1: Analytical challenges	Antibody-drug conjugate: Established analytical methods not suitable for dose solution testing due to low protein concentration and matrix interference	Adapted the sample preparation procedure to enable analytical testing of dose solution
Case study 2: Protein adsorption to in-line filter	Antibody-drug conjugate: Poor drug recovery due to adsorption to an in-line filter	Implemented an administration approach that no longer uses in-line filters for low-volume dose solutions. Subvisible particulates were tested to ensure the implemented approach was feasible
Case study 3: Excipient dilution	Monoclonal antibody: Physical instability (particulates) due to surfactant dilution	Increased surfactant concentration in drug product formulation to enable dosing over a 20-fold clinical dose range
Case study 4: Interfacial stress	Bispecific protein: Interfacial stress induced physical instability (aggregation) for dose solution diluted in normal saline	Normal saline was not compatible; enabled administration using 5% dextrose as an alternative diluent
Case study 5: Syringe pump administration	Bispecific protein: Physical instability (aggregation) in commonly used IV bags at low protein concentrations	Enabled syringe pump administration for dose solutions ≤2.0 mg/mL; IV bags used for >2.0 mg/mL dose solutions
Case study 6: Ultra-low dose administration	Recombinant protein: Extensive protein adsorption to administration components at low dose solution concentrations	Developed a customized IV dose solution stabilizer to inhibit protein adsorption at low dose solution concentration

3.1 Case Study 1: Analytical Challenges

Analytical methods designed to evaluate drug substance or drug products may not always be ideal for analyzing dose solutions. Low drug concentration and dose solution matrix often challenge the established analytical methods. For one of the ADC drug products in development, the starting clinical dose was submilligram per kilogram body weight. To enable the administration of such low clinical dose with adequate volume for IV administration (~5 mL), the drug product was diluted to ~20 μg/mL in 0.9% normal saline. Analysis of this dose solution was challenging for most of the commonly used analytical methods (Size exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC), and imaged capillary isoelectric focusing (IcIEF)) due to low drug concentration and interference from dose solution components (e.g., NaCl).

To overcome these issues, dose solutions are processed using centrifugal filtration and buffer exchange to increase the protein concentration in the samples and reduce the presence of interfering components such as sodium chloride. The analytical methods with modified sample preparation procedure showed comparable results to drug product stability data generated using validated methods.

Figure 3a shows representative SEC chromatograms comparing the validated method and the method with the modified sample processing procedure. The high molecular weight (HMW) species, monomer, and low molecular weight (LMW) species peaks and their retention times are comparable to that typically observed for drug product samples at validated method nominal concentrations. Similar results were observed for HIC and IcIEF (Fig. 3b, c). The sample processing step effectively eliminated the diluent component, NaCl, which was interfering with HIC and IcIEF and generated the results similar to that of validated methods. Overall, this approach is effective in achieving comparable results as validated methods. While the current case study focused on three analytical methods, the approach can be applied to other commonly used analytical methods (ion exchange chromatography, capillary electrophoresis, and bioassays) with similar challenges.

3.2 Case Study 2: Protein Adsorption to In-Line Filters

In-line filters are one of the key components of infusion administration systems. These are often used to ensure the delivery of sterile and particle-free drug solution; however, they have to be compatible with the drug in-use. Protein adsorption to in-line filters, especially for low protein concentration dose solutions, is a widely reported incompatibility. Besheer et al. [53] recently discussed the protein adsorption to in-line filters for a series of commercially available filters. They concluded that the protein adsorption to in-line filter membranes is more dependent on the filter material and dose solution composition and less dependent on the protein properties. Similar protein adsorption issue was encountered for one of the ADC products in development that required low protein concentration dose solution (20 μg/mL) administration. In an effort to enable the administration of low concentration ADC dose solution, we studied the adsorption behavior of ADC drugs to polyethersulfone (PES) filters. As expected, the results (Fig. 4) showed significant protein adsorption to in-line filters and that large volume of low concentration dose solution is required for complete filter saturation.

An alternative approach to eliminate in-line filters for low-volume applications was tested to avoid excessive drug product wastage due to high prime volume requirements. Following common product handling and preparation techniques, the presence of subvisible particles had to be evaluated to ensure that the number of particles administered intravenously is within the regulatory limits [85]. Table 4 shows that the subvisible particles per container in postinfusion without in-line filter solution compared to particles per container in the IV bag preinfusion are within

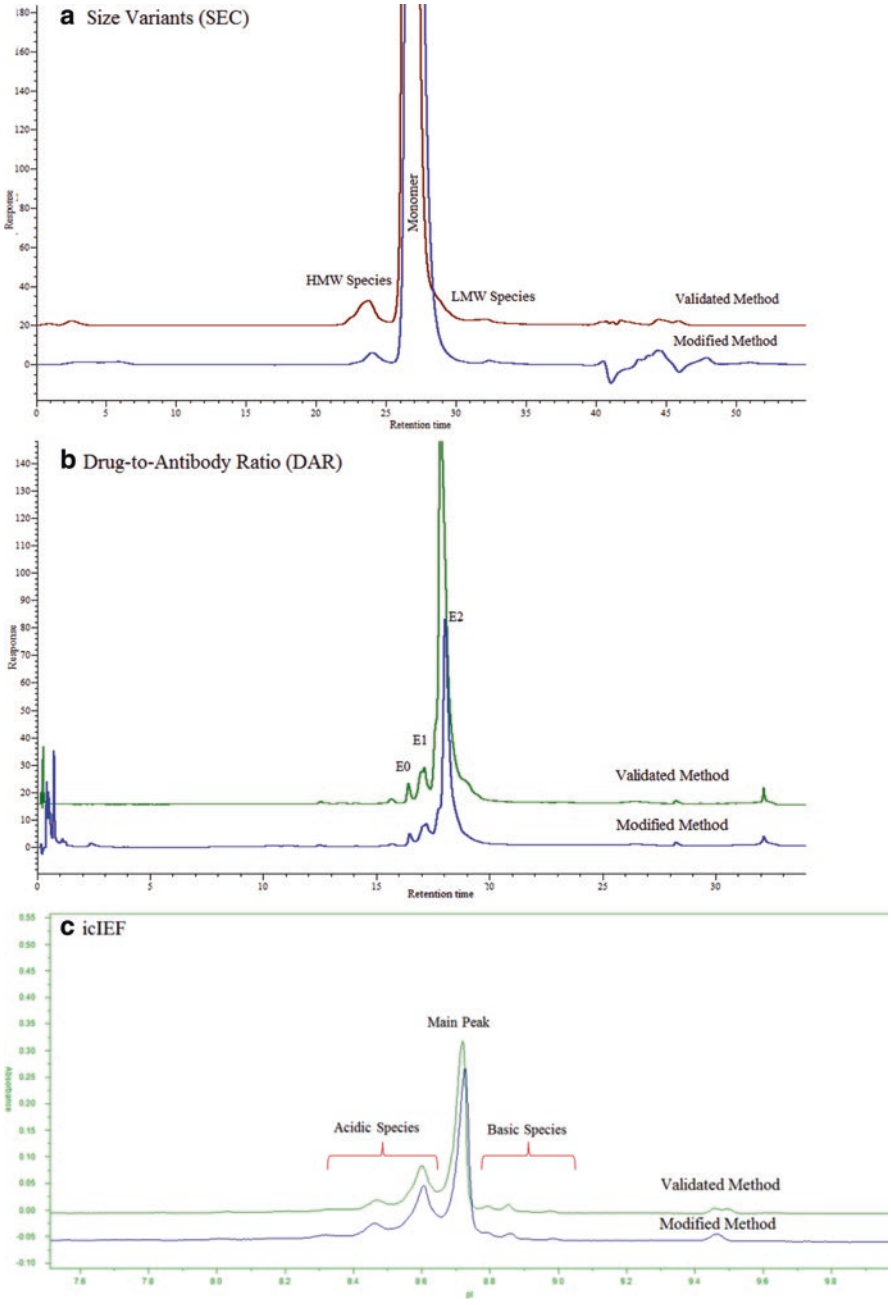

Fig. 3 Comparison of the modified method and validated method for various analytical methods

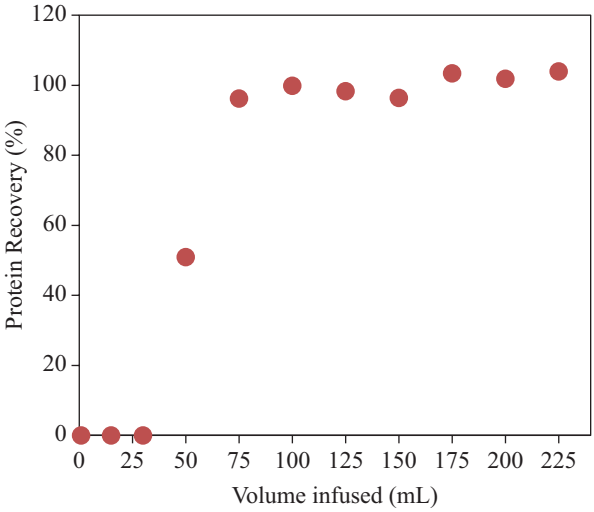

Fig. 4 ADC dose solution (20 μg/mL) infusion using 0.22 μm in-line filter

Table 4 Subvisible particle counts for pre- and postinfusion samples without in-line filter. USP<788> Requirements for small volume parenterals, ≥10 μm = ≤ 6000 particles/container; ≥25 μm = ≤ 600 particles/container

	Particles/container	
Condition	≥10 μm	≥25 μm
Preinfusion	100	0
Postinfusion without filter	200	0

regulatory requirements for IV administrated solution. Based on this data, we implemented the approach that allowed the use of infusion bags without an in-line filter for low range dose cohorts.

3.3 Case Study 3: Excipient Dilution

Biologic drug products are often supplied as concentrated protein formulations requiring dilution in IV fluids prior to administration. This common practice dilutes the stabilizing excipients in addition to the drug in the product. If the excipient levels are insufficient after dilution, it may adversely impact the physical stability of the protein such as formation of aggregates and particulates [47, 86].

This case study is related to an antibody product in preclinical development. For phase 1 studies, a 20-fold clinical dose range of 0.5–10 mg/kg was anticipated. To support this phase 1 clinical dose range, an in-use evaluation was performed

Table 5 Visible and subvisible particle counts results postinfusion for 0.5 mg/mL dose solutions prepared in normal saline in polyolefin IV bags from drug product formulations containing 0.02–0.08% w/v polysorbate 80

% Polysorbate 80 (w/v)	Postinfusion, without an in-line filter			Postinfusion, with in-line PES filter		
	Visible Particles	Subvisible particles (counts/mL)		Visible Particles	Subvisible particles (counts/mL)	
		≥10 μm	≥25 μm		≥10 μm	≥25 μm
0.02	TMVP	86	4	TMVP	103	7
0.05	EFVP	25	1	EFVP	12	0
0.08	EFVP	11	0	EFVP	6	0

TMVP too many visible particles, *EFVP* essentially free of visible particles

early on in development. The objective was to assess if the platform formulation containing 50 mg/mL protein in histidine buffer with sucrose and 0.02% (w/v) polysorbate 80 is acceptable.

During the in-use evaluation, to enable IV pump administration of the lowest 0.5 mg/kg dose, the product was diluted 100-fold with 0.9% normal saline to 0.5 mg/mL. The prepared dose solution was stored in polyolefin (PO) IV bag, and subsequently, a mock infusion was conducted, both with and without an in-line PES filter.

The visible and subvisible particulate results obtained for the samples collected postinfusion are shown in Table 5. The results indicate that the in-line filter was not effective in removing particles; many visible particles were observed, and additionally, the ≥10 μm/mL particle counts were assessed to be high for the planned 100 mL infusion volume (exceeded the USP <787> limit for small volume parenteral products [85]). In previous studies, dose solutions prepared at 0.5 mg/mL in the formulation buffer did not show the observed particulate issue (data not shown), and therefore, it was suspected that the product instability was linked to inadequate surfactant level in the dosing solution. The in-use experiment was repeated by preparing the 0.5 mg/mL dose solution from product formulations with higher levels of the polysorbate 80 (PS80) surfactant, 0.05% (w/v) and 0.08% (w/v) PS80, and particulate results obtained are shown in Table 5. A clear trend indicating a drop in subvisible and visible particles is observed with increase in PS80 concentration in the product formulation. For dose solutions prepared from DP formulation with 0.08% (w/v) PS80, visible and subvisible particles were acceptable for samples infused both with and without the in-line filter. These findings suggest that PS80 concentration above a critical threshold may be necessary for robust dose solution stability for certain proteins.

In this example, because the clinical in-use evaluation was done early on in development, it was feasible to increase the concentration of PS80 in the product formulation to ensure dose solution stability. Very often, clinical in-use evaluations are done much later in development and addressing potential in-use stability issues,

when there is limited flexibility to modify CMC processes, can be challenging. It is recommended that the impact of excipient dilution on dose solution stability be assessed early on during formulation development. With this approach, a comprehensive product stabilization strategy that spans across product manufacturing, storage, and end-user can be enabled.

3.4 Case Study 4: Interfacial Stress

In this case study, a bispecific protein (Mwt ~ 200 KDa) was formulated at a concentration of 25 mg/mL in histidine buffer with sucrose and contained 0.03% (w/v) polysorbate 80. To assess its in-use stability, an evaluation was conducted by preparing dose solutions in 0.9% normal saline (NS) in the protein concentration range of 0.05 mg/mL to 2.0 mg/mL, which was deemed adequate for the intended clinical dose range.

The dose solutions were prepared in polyvinyl chloride (PVC) and polyolefin (PO) IV bags, and also in glass vials as study controls. The SEC results for soluble aggregates for samples pulled from the bags immediately after preparation (initial time point, T0) are shown in Fig. 5. The aggregate level remained stable in the control glass vial samples; however, the aggregate level was distinctly higher for samples stored in IV bags, in particular for dose solutions stored in PO bags. Subvisible particle results by micro-flow imaging (MFI) also showed a similar trend; ≥2 μm subvisible particle counts were higher in PO IV bags relative to the PVC IV bags and glass vial controls. The ≥10 μm and ≥ 25 μm particle counts were also high and exceeded the USP <787> limit for small volume parenteral products (data not shown).

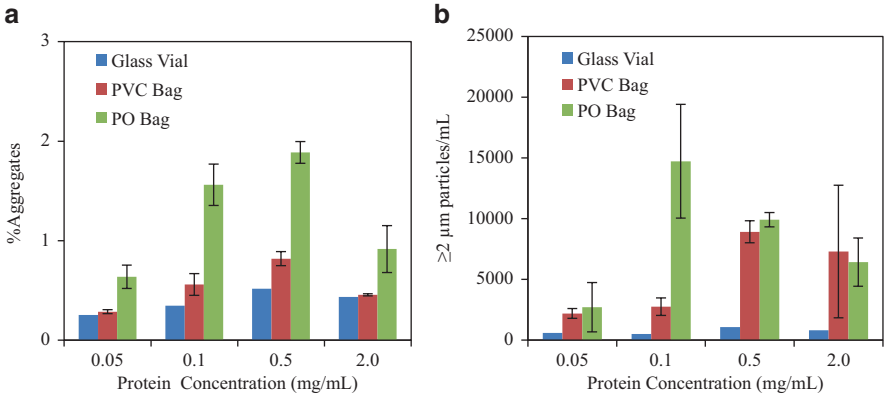

Fig. 5 Dose solution stability in 0.9% normal saline postpreparation at T0. (**a**) %Aggregates by SEC and (**b**) ≥2 μm/mL particle counts by MFI. The IV bags were partially filled to assess the worst-case fill configuration: 25 mL fill in 50 mL bags. The fill volume in the 10R glass vial controls was 10 mL

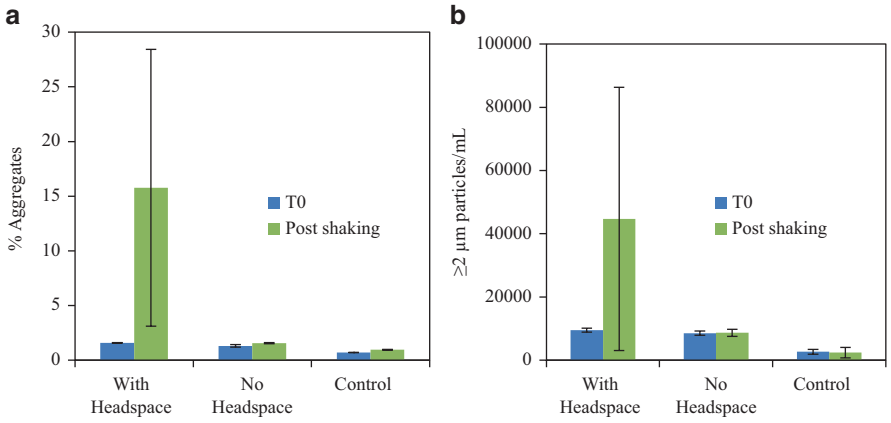

Fig. 6 Dose solution stability in 0.9% normal saline in PO IV bags postshaking (15 minutes at 100 RPM). (**a**) %Aggregates by SEC and (**b**) ≥2 µm particle counts by MFI. The bags with headspace were partially filled with 25 mL solution, and air was withdrawn from the IV bags with no headspace. The fill volume in the 10R glass vial controls was 10 mL

Additional experiments performed showed that the observed physical instability was primarily induced by interfacial stress. As shown in Fig. 6, IV bags subjected to shaking stress conditions were highly prone to aggregate and particle formation for the partially filled bags (with headspace). By increasing the surfactant concentration in the formulation, this instability could be minimized (data not shown). However, the level of surfactant required in the drug product was very high (>0.4% (w/v)), and therefore, unlike the previous case study, this approach was not practical to implement.

Interestingly, no clear trend concerning the protein concentration was apparent in these experiments. It is speculated that due to the sensitivity of the protein to interfacial stress, sample handling differences across the various dose preparations may have confounded the concentration dependence trend. Nevertheless, these results support that the dose solutions in normal saline did not demonstrate robust stability, and therefore, additional diluents were screened.

The in-use experiment was repeated by preparing the dose solution in 5% dextrose, another commonly used IV diluent. In this matrix, no adverse aggregate or subvisible particle trends were observed in the evaluated protein concentration range (Fig. 7). Experiments performed under shaking stress conditions also showed robust stability (Fig. 8). Based on the results of this study, dose preparation instructions mandated the use of 5% dextrose for this protein. It was also recommended that as clinical development continues, based on the recommended phase 2 dose, additional studies be conducted to assess the feasibility of using normal saline at higher protein concentrations. The results of this study also indicate that incorporating studies that allow assessment of interfacial stresses during in-use studies is important.

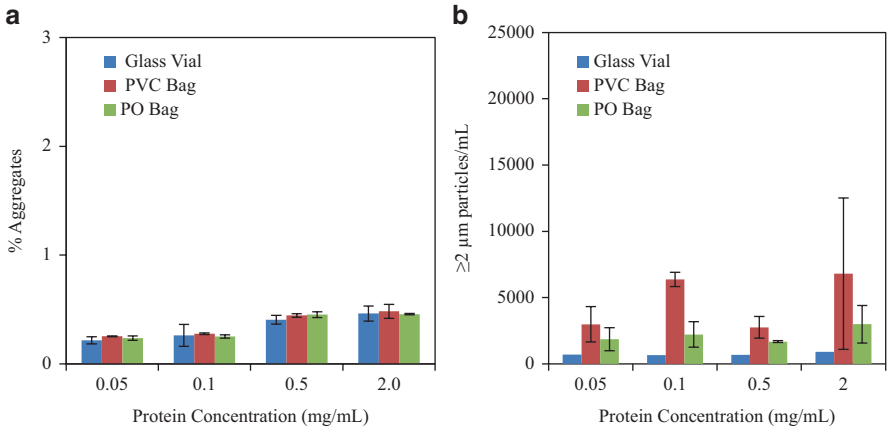

Fig. 7 Dose solution stability in 5% dextrose postpreparation at T0. (**a**) %Aggregates by SEC and (**b**) ≥2 µm particle counts by MFI. The IV bags were partially filled to assess the worst-case fill configuration: 25 mL fill in 50 mL bags. The fill volume in the 10R glass vial controls was 10 mL

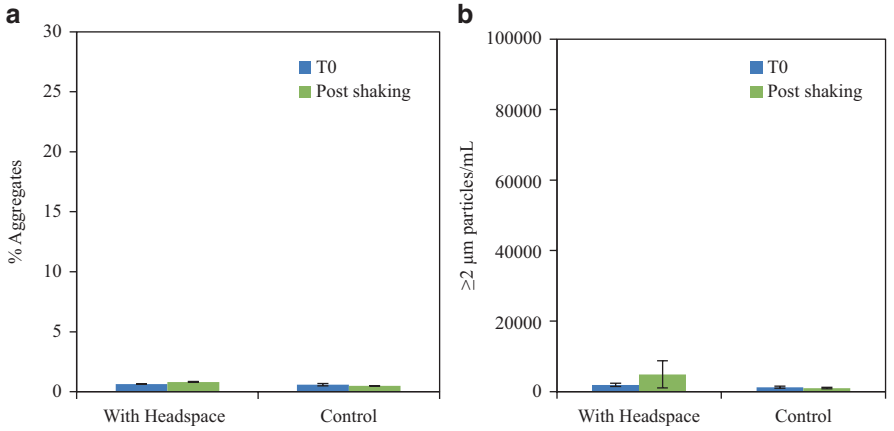

Fig. 8 Dose solution stability in 5% dextrose in PO IV bags postshaking (15 minutes at 100 RPM). (**a**) %Aggregates by SEC and (**b**) ≥2 µm/mL particle counts by MFI. The bags with headspace were partially filled with 25 mL solution. The fill volume in the 10R glass vial controls was 10 mL

3.5 Case Study 5: Syringe Pump Administration

In this case study, the bispecific protein (Mwt ~ 160 KD) was known to be highly hydrophobic and susceptible to aggregation, and therefore, instability due to previously presented challenges such as excipient dilution and interfacial stresses were anticipated. The product was formulated at a protein concentration of 4 mg/mL in phosphate buffer with sucrose and contained 0.03% (w/v) PS80.

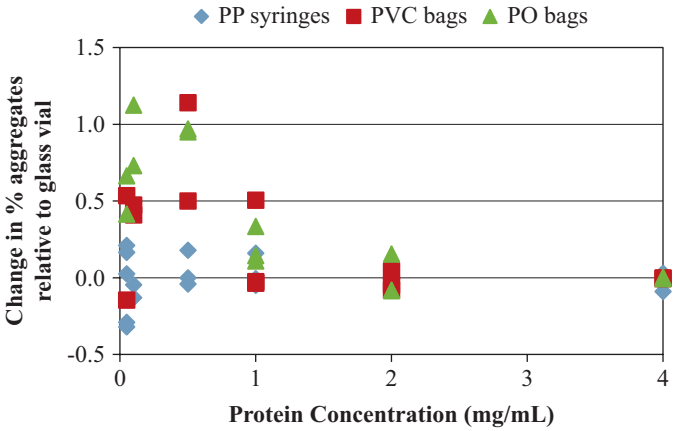

Fig. 9 % Aggregates change by SEC at T0 for dose solutions prepared in 0.9% normal saline in the concentration range of 0.05 mg/mL to 4.0 mg/mL. Dose solutions were prepared in IV bags (≥25 mL fill in 50 mL bags) and syringes (≥3 mL fill in 30 mL syringes)

Given the inherent protein instability, an extensive in-use stability evaluation was undertaken early on in the program. Dose solutions were prepared in 0.9% normal saline in the concentration range of 0.05 mg/mL and 4.0 mg/mL (undiluted), in both syringes and IV bags, to cover the anticipated clinical dose range. Subsequently, a mock infusion was performed after the solutions were held overnight at 2–8 °C followed by an additional 4 hours at room temperature.

The samples collected during the course of the study showed that aggregation was the only product quality attribute adversely impacted. The change in aggregate level relative to the glass vial control sample, which showed no change in aggregate levels, is shown in Fig. 9.

While all dose solutions prepared in syringes showed acceptable aggregate levels, at concentrations below 2.0 mg/mL, the solutions held in both PVC and PO bags showed significant increase in aggregate levels, along with high variability. Increase in PS80 concentration in the product formulation, or the fill volume in the bags to reduce the headspace, did not fully resolve the observed instability (data not shown). Robust stability was also not observed with use of 5% dextrose, and therefore, this option was not viable (data not shown).

Additional samples pulled during the course of the study after storage and postinfusion indicated that the infusion process did not further contribute to the instability; the samples held in syringes were stable postinfusion for all evaluated concentrations. These results suggest that the interfacial stress in the IV bags likely destabilized the protein, leading to aggregation.

Based on these study results, the use of a syringe administration system was mandated for dose cohorts requiring dose solutions <2.0 mg/mL. This solution, although not ideal from an operational perspective, enabled the exploration of a broad clinical dose range in the phase 1 study. It was also recommended that as

development progressed, further optimization of the formulation composition, or development of a customized IV stabilizer solution, be explored to alleviate the operational limitations.

3.6 *Case Study 6: Ultra-Low Dose Administration*

In this case study, the first-in-human clinical dose for a protein drug product was below 1 μg/kg, which required product dilution to concentration below 1 μg/mL. At these ultra-low concentrations, significant drug adsorption to dose preparation and administration components was anticipated, potentially leading to unacceptable drug recovery.

To evaluate this concern, we studied the adsorption behavior of the protein to the commonly exposed surfaces during the course of dose solution preparation and handling. One of the first challenges encountered was the lack of suitable analytical methods to quantify the protein at such low concentrations. This required significant method development efforts that lead to the use of a reversed-phase high-performance liquid chromatography (RP-HPLC) method to measure the protein concentration below 1 μg/mL.

Figure 10 shows the protein concentration results as a function of time for the 1 μg/mL dose solution in 0.9% normal saline held in borosilicate glass vials and polypropylene (PP) syringes. Not surprisingly, significant adsorption (>80% protein loss) to glass and PP surfaces was observed in the absence of PS80. Given the poor protein recovery, the experiment was repeated by preparing the 1 μg/mL dose solution in 0.9% normal saline that was formulated with 0.01% PS80. In the presence of the surfactant, the extent of adsorption was significantly reduced in PP syringes;

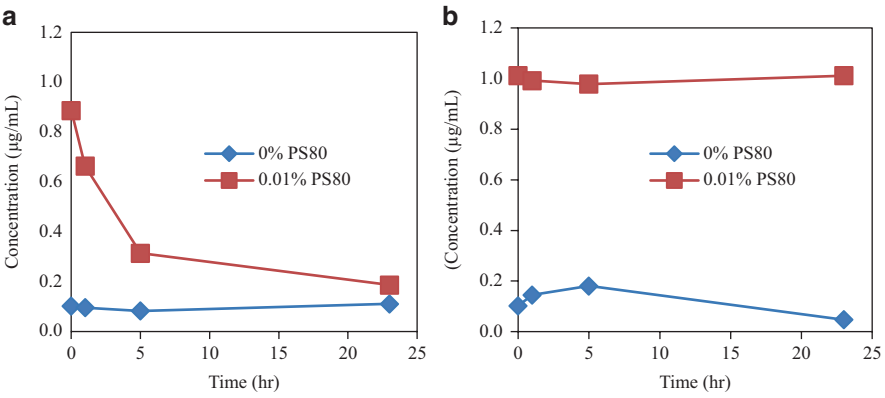

Fig. 10 Protein concentration measured as a function of PS80 level and time in glass vials (**a**) and syringes (**b**) at room temperature and room light. The solutions were prepared at 1 μg/mL protein concentration in 0.9% NaCl

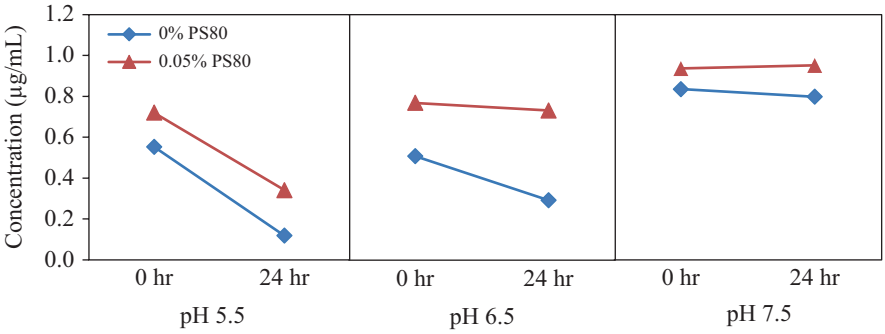

Fig. 11 Protein concentration (by RP-HPLC) as a function of IV diluent pH, PS80 concentration, and storage time. The solutions were prepared at 1 µg/mL protein concentration in phosphate-buffered saline in glass vials and held at ambient room temperature for up to 24 hours

however, results were not acceptable in a glass vial and a time-dependent adsorption behavior was observed.

Protein adsorption to borosilicate glass surfaces has been reported to follow two main pathways: (i) hydrophilic adsorption driven by ionic interaction of silanol groups with protein molecules and (ii) hydrophobic adsorption driven by siloxane groups [87]. These pathways can potentially be influenced by the formulation pH and surfactant concentration, and therefore, additional experiments were conducted to study the effect of these two factors. The results from these evaluations are shown in Fig. 11. It is evident that the adsorption of this protein to glass surface is dependent on both pH and surfactant concentration, with pH being the dominant factor. At pH 5.5 and 6.5, significant protein adsorption was observed independent of the surfactant concentration. At pH 7.5, protein adsorption trends were markedly improved, and in the presence of the surfactant, the minimal adsorption (<5%) was observed as a function of time.

Therefore, to enable low dose administration of this protein, it was important to control the diluent pH and surfactant, which required the development and manufacturing of a customized IV dose solution stabilizer (IVDSS). For early-stage clinical trials, the strategy taken was to dilute the drug product using the supplied IVDSS vials, and administer to the patients.

A similar approach was recently proposed by Zheng et al. [32] showing the use of a stabilizing vehicle containing PS80. The authors proposed to add the PS80-containing vehicle to saline before drug product addition to maintain sufficient surfactant level to prevent protein particulate formation. Further, a commercial antibody product, BLINCYTO® [34], implemented a similar approach. In this case, the drug product is supplied with an IV solution stabilizer that must be added to the IV bag prior to adding the drug product to coat the surfaces with surfactant and prevent adhesion of antibody to IV bag.

For proteins that are highly susceptible to adsorption such as with ultra-low dose regimens, development of customized IVDSS may provide an attractive solution.

This is a complex CMC approach requiring additional development and manufacturing. Furthermore, to manage the operational complexity of this approach, early engagement with the Drug Supply and Clinical Operations groups is important.

4 Conclusion

This chapter presents the current knowledge and considerations regarding the design of clinical in-use studies that are critical to enable parenteral drug product administration. Manipulation of drug product solutions during its end use is unavoidable for parenteral products; therefore, to ensure the safety, efficacy, and quality of the dosing solution administered to a patient, design of robust clinical in-use studies is imperative during the course of product development.

Given the increasing complexity of parenteral biologicals and global clinical trials with varying pharmacy practices, the importance of clinical in-use valuations should not be underestimated. While the regulatory and pharmacopeial requirements play an indisputable role in the design of these studies, an understanding of the technical and practical considerations is important for pharmaceutical scientists to simulate the real-world conditions used by the end-users to prepare, handle, and administer the product solution at clinical sites.

Unfortunately, the available literature on in-use stability challenges during drug product development is limited. We hope that the general study design recommendations and case studies presented in this chapter highlight the commonly observed stability issues for parenteral IV protein products and serve as a practical guide for conducting in-use studies. With the understanding of potential stresses impacting product quality, and continuous learning of the end-user practices and requirements, drug manufacturers can develop robust pharmacy manual guidelines to ensure safe delivery of the parenteral biologics.

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Chapter 19
Design of a Bulk Freeze-Thaw Process for Biologics

Feroz Jameel, Tong Zhu, and Brittney J. Mills

1 Introduction

Therapeutic proteins pose marginal shelf life in aqueous solution because of their inherent physical and/or chemical instability causing them to fall short of the desired pharmaceutical stability of 4–5 years, encompassing 2–3 years of storage stability as a drug substance and another ≥2 years as a drug product. The bulk drug substance can be stored and shipped in the liquid state, as a lyophilized powder, or in frozen form, with each method having its own merits and limitations. For instance, protein stored in the liquid state can be dispensed conveniently as needed, but requires more diligence in order to avoid instability, microbial growth, and proteolytic degradation. In contrast, lyophilized powders enable long-term storage of protein with very little threat of degradation, but their production involves a lengthy process of drying and reconstitution. Once frozen, proteins are relatively stable, but the freezing and thawing processes may damage proteins, and maintaining temperature control for frozen products during shipping and storage at the clinical site is more cumbersome than for liquids. Hybrid processes can be envisioned, as well. A relatively new technique called "cryogranulation" is being explored as an alternative means of storage for bulk drug substance [1]. This technique involves the creation of free-flowing, frozen granules by exposing solution or slurries to a cryogenic material such as liquid nitrogen. Hence, storing the drug substance as frozen liquid could be successfully used to enhance the pharmaceutical stability/shelf life. There are several advantages to storing in the frozen state:

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1. The rates of many of the common reactions leading to physical and/or chemical degradation are slowed.
2. Risk of microbial growth over time can be minimized.
3. Elimination of interface-driven denaturation due to agitation and foaming during transport.
4. Increased flexibility in the manufacturing processes, such as introducing a hold step for pooling batches of intermediates and storing final bulk drug substance before fill/finish operations.

Although frozen storage offers several advantages over dry powder and liquid storage and is regarded as the safest and most reliable storage method, it is not free from introducing process-related liabilities on protein stability. Biomolecules are known to undergo multiple pathways of freezing-induced denaturation including cryoconcentration, ice surface-induced denaturation, and cold denaturation [2–11]. Understanding of these denaturation processes and their impact on the protein integrity and liabilities early on will help in the design of the formulation and the freeze-thaw process. Sensitivity of the protein to these freezing-induced denaturation processes can be addressed through a well-designed formulation and/or a thorough freeze-thaw process that includes either controlled rate or uncontrolled rate freeze-thaw technology [12]. Both technologies have their benefits and limitations, and selection of one of them should be based on the properties of the protein molecule. As evident from the literature, [13] the freezing-induced denaturation processes are scale-dependent. Due to these scale-related factors and the lack of material at the early stage of product development that is required to perform full-scale evaluations, it is imperative to develop scale-down models that are representative of large-scale freeze-thaw stresses.

As there is substantial interdependency between formulation and processes, it is necessary to select the storage method early on so that the formulation and processes can be designed and developed to be in alignment with each other. The technology selection process should involve logistics and technical feasibility assessments in addition to cost analysis based on market projections. Once the decision on the choice of bulk storage and shipping technology is made, the development of the process should progress alongside formulation development. This chapter is focused on discussing the physics of the freezing process and the challenges and considerations during the development, characterization, and scale-up of each storage technology, with emphasis on the most commonly used method, cryopreservation.

2 Physics of the Freezing and Crystallization Process

The physicochemical changes and the thermal events that take place during the process of freezing and crystallization depend on the composition of the solution. Figure 1 depicts the freezing profile of two solutions: pure water (ABCDE) and a

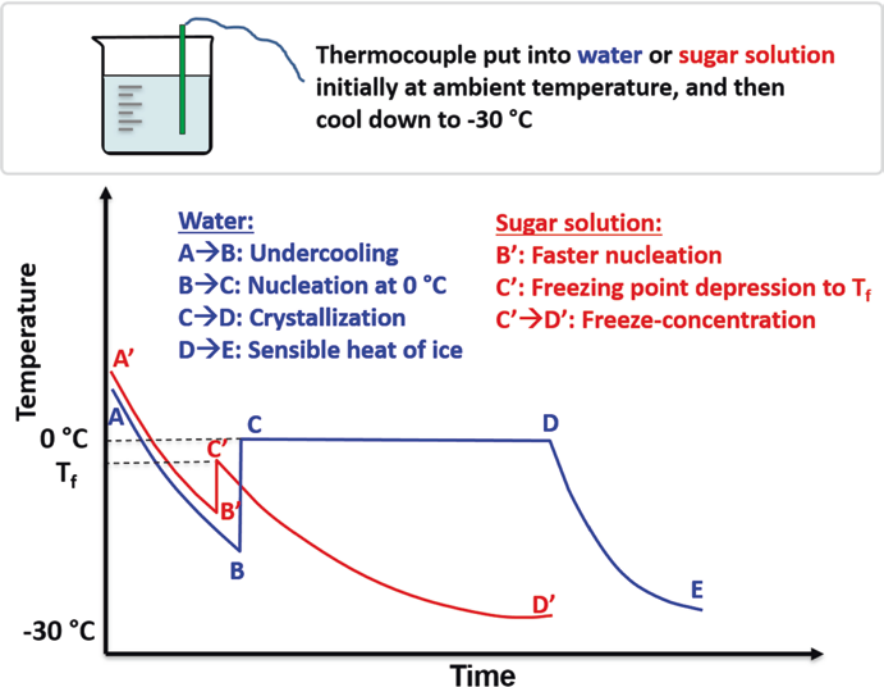

Fig. 1 Freezing profiles of pure water (ABCDE) and a sucrose solution (A'B'C'D')

sucrose aqueous solution (A'B'C'D'). In pure water, upon lowering the temperature from point A, the nucleation or critical mass of nuclei is not formed until point B. Once the nuclei are formed, the crystallization process starts. Since crystallization is an exothermic reaction, the latent heat of fusion is given out, and the temperature rises from B to C. The event shown as the solution progresses from point A to B is described as the degree of supercooling the water undergoes prior to nucleation. This degree of supercooling is dependent upon the cooling rates employed, and the purity of water (free of particles which serve as nucleation sites), with the lower number of free particles leading to a higher degree of supercooling. Point C, which corresponds to 0 °C, is the equilibrium freezing point of pure water, and at this point, the water continues to crystallize until point D. Once point D is achieved, all of the water is converted into ice, and because the crystallization process is complete and no heat is given out, the temperature starts dropping to the set point, E. The freezing time is usually defined as the time from the onset of nucleation to the end of the crystal growth phase. The size of ice crystals formed during crystallization (from C to D) is dependent upon the degree of supercooling: faster cooling rates lead to higher degrees of supercooling and smaller ice crystal size (Fig. 2) [14].

A different freezing behavior is expected once a solute is added to pure water. A solute-containing solution is governed by Roult's law which relates vapor pressure of the solution to that of the pure solvent based upon solute concentration. Figure 1

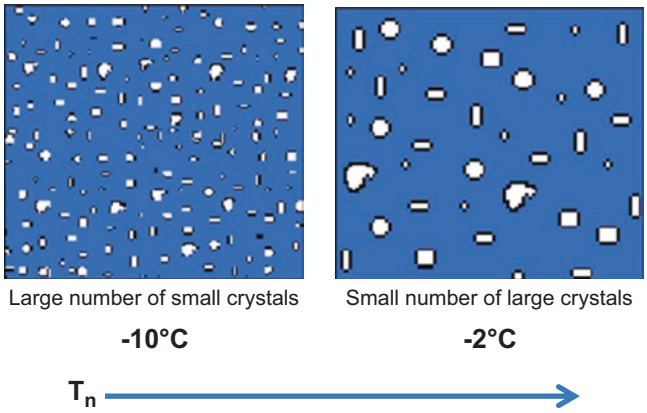

Fig. 2 Effect of freezing rate on the morphology of ice

shows the key differences that exist between pure water and a sucrose solution. First, B' is not the same as B in terms of temperature; a sucrose solution nucleates earlier than B because of the presence of sucrose molecules, which act as nuclei. Secondly, C', the freezing point temperature, is not as high as C, due to the initial freezing point depression caused by the presence of sucrose. Both of these events are dependent on the concentration of solutes in the solution. Additionally, in aqueous solutions containing solutes, a phenomenon called cryoconcentration is observed [4, 11]. As the water starts converting to ice upon cooling, the freezing front moves forward leaving behind the solutes to concentrate, resulting in pockets rich of solutes. These pockets rich in solutes further depress the freezing point of free water, and this phenomenon continues (C'D') with the cooling, leaving some residual unfrozen water, regardless of how low the cooling temperature is set. Also, as cryoconcentration increases, the viscosity of the free water increases, which decreases the mobility and diffusion properties of the system and inhibits the crystallization process.

The cryoconcentration process establishes the freezing curve as shown in Fig. 3. This curve can be used to predict the amount of ice at any given temperature, which in turn, is a function of the freezing point depression caused by the concentration of solutes in the solution.

2.1 Recrystallization of Ice/Ostwald Ripening

Ostwald ripening is a phenomenon where the bigger ice crystals become larger at the expense of the smaller ice crystals during warming and cooling. Smaller ice crystals are unstable and tend to melt upon temperature fluctuations due to the cycling of the freezers and/or the automatic defrosts. As a result of the smaller ice crystals melting, the amount of unfrozen water in the freeze concentrate phase

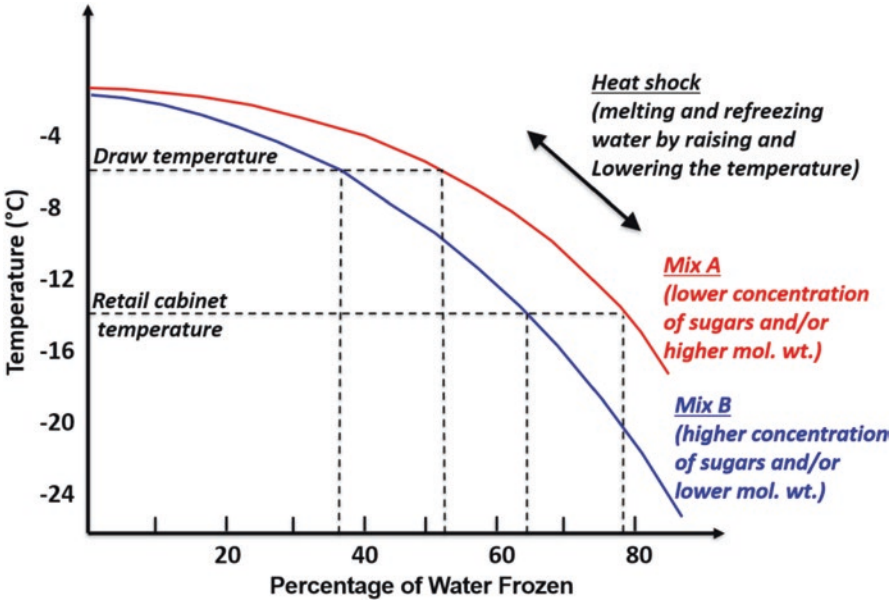

Fig. 3 Freezing curves of mixtures. (Adapted from Ref. [15])

increases, which will refreeze upon a decrease in temperature, but does not renucleate. Instead of forming new ice crystals, they get deposited on the surface of existing larger crystals so the net result is that the total number of crystals diminishes and the mean crystal size increases (Fig. 4). This advantage of this phenomenon is utilized in freeze-drying to achieve homogeneity in ice crystal size and favor larger ice crystals to facilitate faster drying.

2.2 Formation of the Glassy Phase in Frozen Systems

Upon lowering the cooling temperature, the water starts to form ice through a two-step crystallization process: nucleation followed by propagation. As the temperature continues to decrease, water is converted into ice, resulting in the concentration of the solutes in the free, unfrozen water. An equilibrium freezing temperature exists for each ice/unfrozen phase ratio, which is a function of the solute concentration. Fig. 5 depicts the equilibrium thermodynamic process modelled on a phase diagram as an equilibrium freezing (liquidus) curve, which goes from the melting temperature (T_m) of pure water (0 °C) to the eutectic temperature (T_e) of the solute. T_e is the point at which the solute has been freeze-concentrated to its saturation concentration. If the solutes reach supersaturation, then crystallizable excipients such as mannitol or glycine will crystallize and precipitate. The other solutes will remain amorphous, and when the critical solute-dependent concentration is reached, the

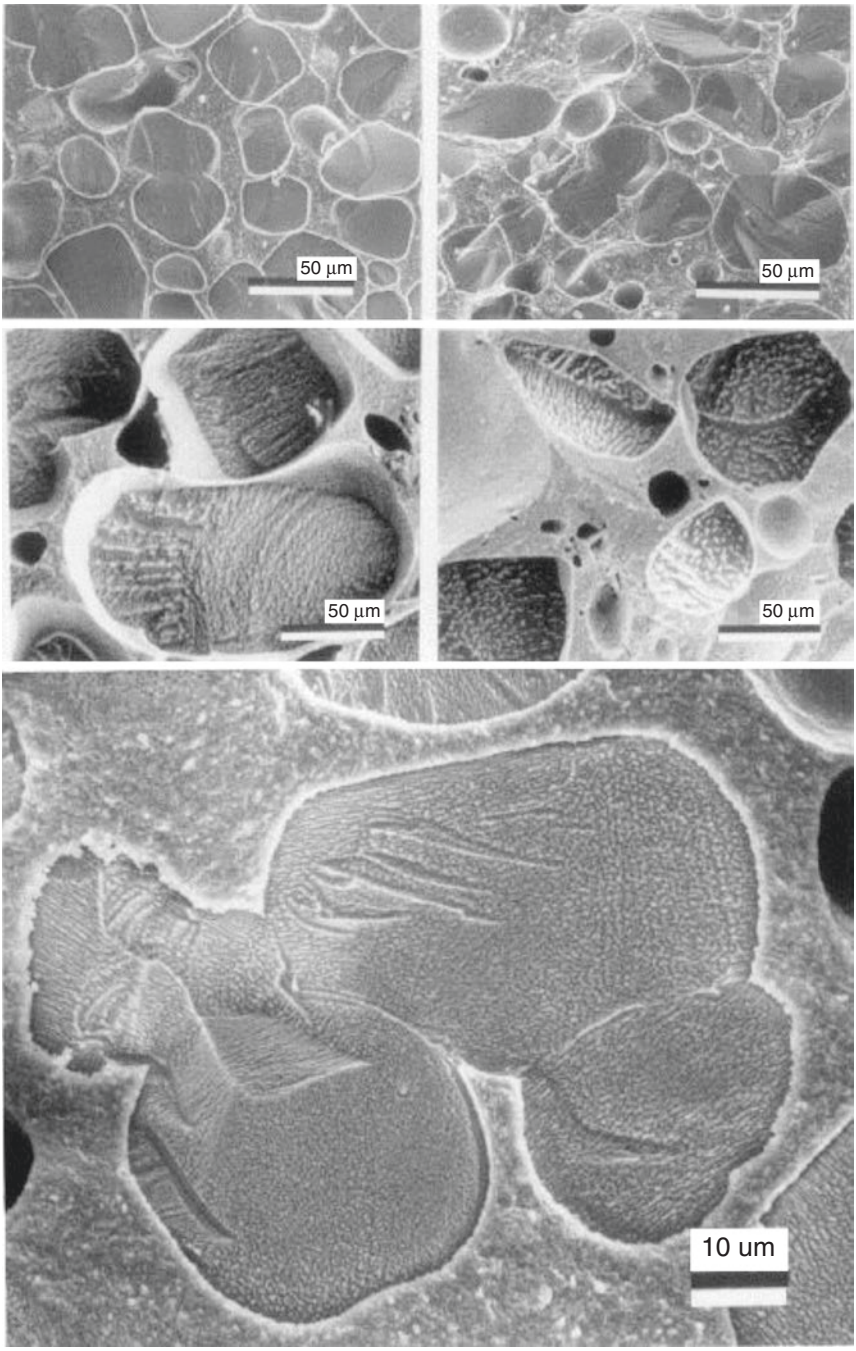

Fig. 4 Cryo-scanning electron micrograph images illustrating the effect of temperature fluctuations on crystal size. The top panel shows images before the temperature fluctuation, the middle panel illustrates the tremendous increase in crystal size that has occurred after heat shock, and the bottom panel shows an example of accretion, where crystals fuse as they grow. (Adapted from the work of A. Flores and H. D. Goff. https://www.uoguelph.ca/foodscience/book-page/temperature-fluctuations-and-ice-recrystallization)

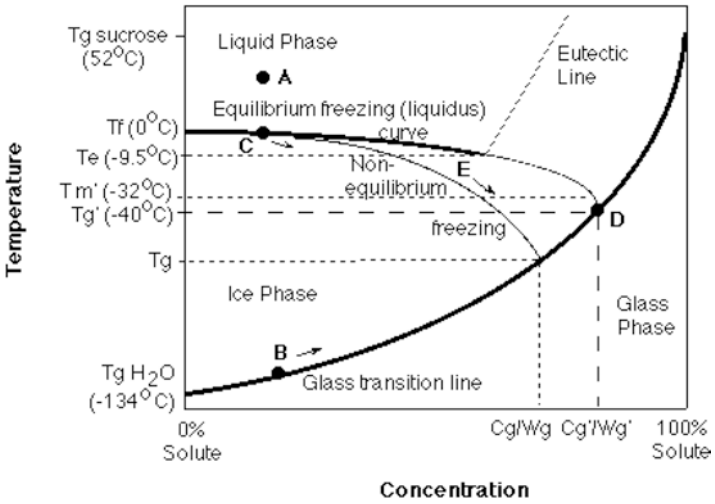

Fig. 5 Phase diagram of equilibrium freezing for binary sucrose-water system. (Adapted from Ref. [17])

unfrozen amorphous freeze concentrate exhibits restricted mobility. At this point, the physical state of the system changes from viscoelastic liquid to an amorphous solid phase called "glass" [16]. The temperature at which this occurs is called the glass transition temperature of maximally freeze-concentrated systems ($T_{g'}$), and the corresponding unfrozen water and amorphous solutes concentrations are termed $W_{g'}$ and $C_{g'}$, respectively (Fig. 5).

A glass is defined as a non-equilibrium, metastable, amorphous, disordered solid of extremely high viscosity (e.g., viscosity coefficient ranging from 10^{10} to 10^{14} Pa.s.) as a function of temperature and concentration. The glass transition curve extends from the glass transition temperature (T_g) of pure water (−134 °C) to the T_g of pure solute. The equilibrium phase diagram and the kinetically derived state diagram can be modelled together to form a supplemented state diagram. The supplemented state diagram illustrating the solid/liquid coexistence boundaries and glass transition profile for a binary sucrose/water system is shown in Fig. 5. Below and to the right of the glass transition line, the solution exists in the amorphous glass state, with or without ice present, depending on the temperature and freezing path followed. On the other hand, above and to the left of the glass transition line, the solution is in the liquid state, with or without ice, depending on the temperature.

Point A in Fig. 5 depicts the initial concentration of 20% of sucrose at room temperature, and point B depicts the initial glass transition temperature (T_g) of the 20% sucrose solution (if the solution could be undercooled to this temperature without ice formation). Upon slowly cooling the sucrose solution, nucleation and subsequent crystallization begin at point C. This occurs after some degree of supercooling due to the presence of sucrose, which initiates the freeze concentration process following the water removal as ice. As ice crystallization proceeds, the continual

increase in solute concentration (removal of water) further depresses the equilibrium freezing point of the unfrozen water phase in a manner which follows the liquidus curve (shown as path C). The increased concentration results in the glass transition line being moved up with a rapid increase in viscosity (path B), thus improving the T_g of the unfrozen water phase.

Co-crystallization of solute at the T_e is unlikely to happen as sucrose is not a crystallizable excipient, and thus freeze concentration continues past T_e into a non-equilibrium state because the solute becomes supersaturated. When a critical, solute-dependent concentration is reached, the unfrozen liquid exhibits very restricted mobility, and the physical state of the unfrozen water phase changes from a viscoelastic liquid to a brittle, amorphous solid glass.

At the $T_{g'}$, the supersaturated solute takes on solid properties because of reduced molecular motion, which is responsible for the tremendous reduction in translational, and not rotational, mobility. It is this intrinsically low mobility below $T_{g'}$ that dictates that protein products to be stored frozen below their $T_{g'}$.

Warming from the glassy state to temperatures above the $T_{g'}$ provides tremendous increases in mobility and diffusion, not only from the effects of the amorphous to viscous liquid transition but also from increased dilution due to the melting of small ice crystals that occurs almost simultaneously ($T_{g'} = T_{m'}$). The time scale of molecular rearrangement continually changes as the T_g is approached. Therefore, some enhanced stability at temperatures above $T_{g'}$ can be gained by minimizing the delta T between the storage temperature and $T_{g'}$, which can be achieved either by reducing the storage temperatures or enhancing the $T_{g'}$ through freezing methods or formulation. Hence, knowledge of the glass transition temperature provides a clear indication of molecular diffusion and reactivity and, therefore, shelf-stability.

3 Bulk Storage Options

Continual increases in the yields of purified proteins due to process efficiencies are necessitating improvements in safe and efficient storage methods, as well as flexibility in pooling and shipping bulk drug substance. The bulk drug substance can be stored and shipped in the liquid state, as a dry powder, or in a frozen form, with each method having its own merits and limitations as depicted in Fig. 6.

For instance, protein stored in the liquid state can be dispensed conveniently as needed, but requires more diligence in order to avoid instability, microbial growth, and proteolytic degradation. In contrast, dry powders enable long-term storage of protein with very little threat of degradation, but their production involves a lengthy process of drying and reconstitution. Once frozen, proteins are relatively stable, but the freezing and thawing processes may damage proteins through three modes of denaturation depicted in Fig. 7. Also, the cold cycle chain for frozen products could be expensive and more cumbersome than for liquids.

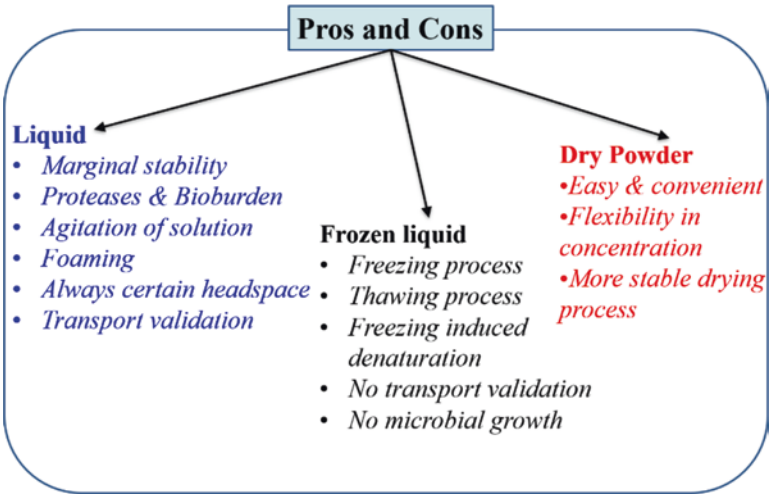

Fig. 6 Pros and cons of various options of bulk drug substance storage and shipment

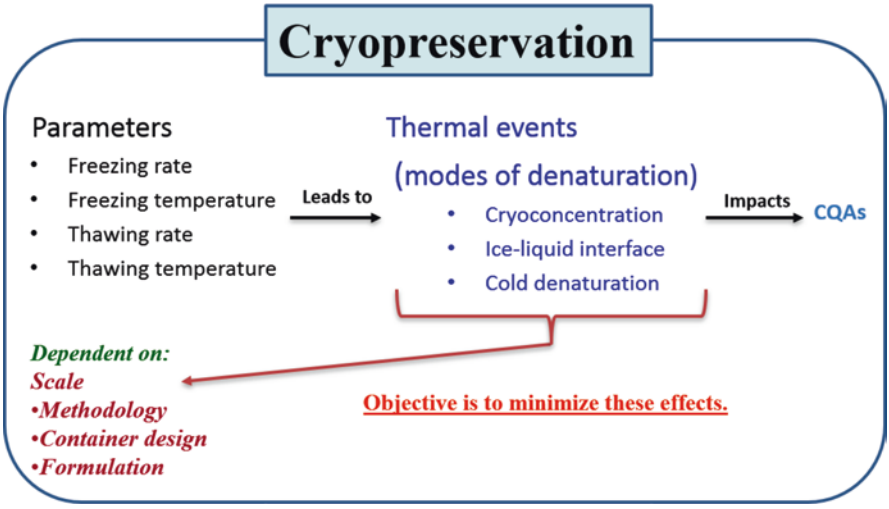

Fig. 7 Potential modes of protein denaturation during the freezing process

4 Cryopreservation: Impact of Freezing Process on Protein Solutions and Modes of Denaturation

Biological solutions are often stored frozen to enhance the storage stability, but the process of freezing can damage the biologic if it is not fully understood and designed properly. The freezing process can denature the protein through three mechanisms: (1) cryoconcentration, (2) ice surface denaturation, and (3) cold denaturation.

4.1 Cryoconcentration

The objective of freezing is to lower the temperature to a point that the solution is completely solidified, thereby arresting reactions that lead to degradation of the protein in the liquid state. As the solution is cooled, the liquid may supercool to a temperature well below the equilibrium freezing temperature, particularly in the case of vials and small containers. With sufficient supercooling, nucleation of ice proceeds rapidly, and the system freezes quickly. During the freezing of bulk solution in large-scale containers, freezing occurs slowly, and as the liquid water converts to ice, the protein and formulation excipients are progressively concentrated in the regions between the ice crystals.

After the initial ice nucleation and crystallization, the product cools with continuous conversion of water to ice. As this occurs, the amount of water in the remaining liquid phase decreases, and the concentration of the solute in the remaining solution increases. This freeze concentration effect results in an increase in protein concentration, which dramatically increases the probability of molecular collisions. The bimolecular collisions between protein molecules can lead to denaturation of the protein through aggregation. For example, although a reduction in temperature from 5°C to −40°C would reduce the rate constant significantly, the increase in the concentration factor due to the increase in concentration has a more significant impact, thus resulting in a net increase in reaction rate. If excipients such as ionic salts and buffer species are present in the formulation, they will also concentrate during the freezing process. For example, during the freezing process, a formulation containing 0.15 M NaCl will increase to 6 M NaCl before it forms eutectic with ice. Exposure of protein to high ionic strengths could contribute to the instability of the native conformation [18]. In addition, the effect of freezing on buffer choice must be considered. Buffers are included in the formulations to help maintain a stable pH. However, during the freezing process, decreases in solubility with a simultaneous increase in concentration can cause selective crystallization of the buffer component and result in dramatic pH shifts. The classic example is the sodium phosphate buffer system. It shows a dramatic decrease in pH of about four units due to the crystallization of the basic component. On the other hand, the potassium phosphate system shows an increase in pH upon freezing [19].

Mitigation Strategies for Cryoconcentration Effects

1. The ice front velocity should be higher than the diffusion rate of solutes so that the protein molecules/solutes become entrapped by the freezing front. This can be achieved through the combination of shorter freezing path lengths and efficient external heat transfer.
2. Increase the temperature differential between the heat transfer fluid and the product, which shortens freezing path lengths.
3. Minimize the product residence time within the cryoconcentrated stage.

4. Control the freezing rate within known limits.
5. Use small-scale containers for efficient heat transfer and rapid liquid-to-solid phase transition.
6. Use of controlled rate technology.
7. Optimize freezing rate to avoid back-diffusion of solutes into the liquid bulk.
8. Do not mix while freezing. Mix during thawing and aim for uniform melting with mixing.

4.2 Ice-Liquid Surface Denaturation

Through phosphorescence lifetime decay of tryptophan residues, it was demonstrated that freezing of aqueous solutions of proteins causes perturbation or loosening of the native fold due to denaturation at the ice-liquid interface, which often results in the loss of secondary and tertiary structure [8–10]. In some cases, this denaturation is largely reversible upon melting of the ice, and in other cases, substantial loss of activity is observed. This variation is believed to be due to its dependence on the residual volume of liquid water in equilibrium with ice and on the morphology of the ice.

Strategies to Minimize Ice-Liquid Interface

1. Avoid extensive undercooling which leads to flash nucleation and smaller ice crystals.
2. Avoid non-scalable fast-freezing methods such as dry ice/alcohol or liquid nitrogen submersion.
3. Optimize freezing rate to achieve low ice surface area.
4. Investigate the use of formulation components to avoid surface interaction. The addition of cryoprotectants such as polyols and disaccharides (e.g., sorbitol, glycerol, sucrose) and surfactants profoundly attenuates or even eliminates the perturbation.

4.3 Cold Denaturation

While some proteins survive freezing with little or no measurable loss in activity, the freezing process irreversibly inactivates others. Just as proteins undergo thermal denaturation at elevated temperatures, proteins also undergo spontaneous unfolding at very low temperatures, denoted “cold denaturation” [3]. This is partly because of the unsuitable environment created during freezing. As discussed above, as solute species are concentrated, the ionic strength increases, the pH may shift, and most importantly, the “hydrophobic interactions” that stabilize the native conformation of

the protein in water are reduced or eliminated as bulk water is removed from the protein phase. The transition between the denatured and native state is described by changes in enthalpy (ΔH), entropy (ΔS), and Gibbs free energy (ΔG) through the following equation:

$$\Delta G = \Delta H - T\Delta S$$

Gibbs free energy relates to the amount of work required to disrupt the structure of a protein molecule and is used to describe the protein stability. The Gibbs free energy equation has a parabolic shape (Fig. 8), which suggests that both high and cold denaturation is thermodynamically possible.

The maximum stability of the protein at its native state temperature (T_s) occurs when the entropy difference between the native and denatured state is zero. This means that the stability depends mainly on the enthalpy differences between the native and denatured states. The enthalpy of transition can be determined as a function of temperature using either microcalorimetry or modulated DSC. Cold denaturation is not easy to determine experimentally since the declining part of the Gibbs free energy curve below T_s may be below 0°C. Although cold denaturation is not widely reported for protein drugs, it remains a possibility.

The denaturation of protein resulting from cryoconcentration effects and ice-liquid interface adsorption can be eliminated or attenuated through the optimization of critical freezing parameters. If the cause for the protein denaturation during freezing is due to cold denaturation, then addition of small amounts of one of the "excluded solutes," termed cryoprotectants (amino acids, polyols, sugars, and

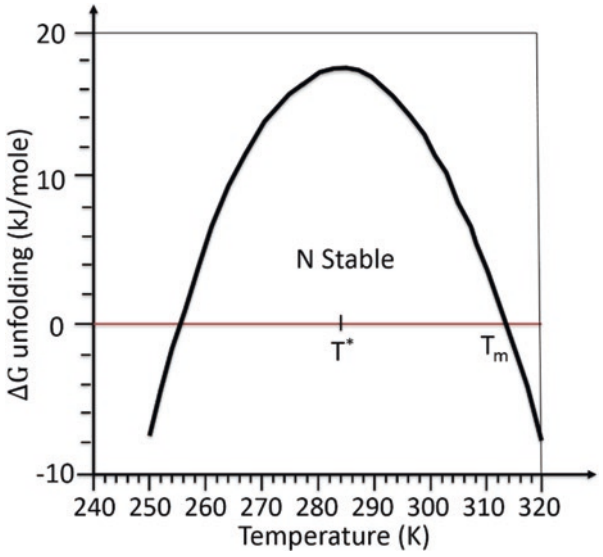

Fig. 8 Schematic representation of the protein stability curve illustrating the temperature dependence of the free energy of unfolding, ΔG. (Replotted from [20])

poly(ethylene) glycols), in molar concentrations will increase the free energy of denaturation. Therefore, the protein is protected against cold denaturation through the preferential exclusion of solutes from the surface of the protein [21–30].

Strategies to Minimize Cold Denaturation

1. Formulation additives to increase freeze-thaw stability
   (a) Thermodynamic stabilizers
   (b) Cryoprotectants
   (c) Glass forming substances
2. Rapid liquid-to-solid phase transition

5 Freeze-Thaw Technologies

Currently, there are two freeze-thaw technologies that are commonly utilized in the cryopreservation of protein solutions: uncontrolled freezing rate (e.g., polycarbonate carboys, stainless steel containers, Teflon, PETG bottles, etc.) and a controlled freezing rate (e.g., stainless steel-based platforms, CryoVessel®, disposable-based platform Celsius bags). In uncontrolled technology, the freezing rate, or rather the ice front velocity, is not constant, while in the controlled rate technology, the ice front velocity is maintained constant along the entire freezing and thawing process throughout the container. The following sections will describe the process development and characterization, robustness, and design space studies of each technology along with some mitigation strategies to address the challenges associated with each.

5.1 Uncontrolled Rate Freeze-Thaw Technology

Process Development

There are three parameters that need to be defined at the end of process development:

1. *Freezing time*: Minimum time required to reach the freezing temperature set point and complete solidification of ice (i.e., minimum hold time at that temperature to ensure complete solidification of ice for a given freezing method). Determined using heat transfer fluid (HTF) flowing through the container or by placing the container in a walk-in freezer (−40 °C or − 80 °C) freezer or on dry ice. This should be determined at the last point to freeze (LPF) location.
2. *Thaw time*: Minimum time required to reach the thawing temperature set point (i.e., 5 °C) and complete melting of ice into water (i.e., minimum hold time at

that temperature to ensure complete conversion of ice into water for a given thawing method (static or dynamic). This should be determined at the last point to thaw (LPT) location.
3. *Mixing time*: Time to reach homogenous solution. It depends on the mixing methodology utilized: mixing during thawing, recirculation of thawed solution, or post thaw mixing such as inversions or gentle shaking/rolling by number or by time following static thaw.

All the above parameters are dependent upon scale, design/geometry of the container, and formulation composition. Hence, they need to be determined at manufacturing scale. The freeze-thaw process cannot be designed using scale-down models, and scale-down models created by matching the aspect ratio of two sized containers are not representative of large scale unless the ice front velocity and the freezing path length are matched between small scale and large scale.

Performing these process development experiments requires large quantities of drug substance, which is not available at the early stage of product/process development. A separate study can be performed as a one-time investment to screen and identify the best surrogate/mimic solution (e.g., PEG, dextran, BSA solution, or any rejected mAb or placebo with same composition as that of drug substance) that matches the viscosity and solid content of the drug substance formulation. Once identified, it can be used as a surrogate to active drug substance material for purpose of equipment testing and qualification/validation and to develop the process at scale. Once developed, this freeze-thaw process or freeze-thaw cycle will serve as a platform freeze-thaw cycle, which can be tested or verified at scale for its suitability and implementation for new products. The at-scale freeze-thaw process development using surrogate solution should use the worst-case parameter conditions as suggested below.

For Freezing Process

1. Use container's maximum working fill volume.
2. Set freezing temperature − 10 °C to −20 °C below the target freezing temperature, or use heat transfer fluid temperature (HTF) + 5 °C above the target.

Note: For storage stability of the active drug substance, it must be stored below the $T_{g'}$ of the formulation. Therefore, while designing the freezing temperature set point, consider the $T_{g'}$ of the formulation and select a freezing temperature 5–10 °C below the $T_{g'}$ value. This combines the highest thermal load with the lowest thermal driving force.

For Thawing Process

1. Use container's maximum working fill volume.
2. Select thawing temperature 5 °C above the target thawing temperature, or use HTF temperature at 5 °C below the target.

Mixing Time

For dynamic thawing through recirculation of thawed solution using a pump, use 80–90% recirculation mixing pump flow rate at 90% of target for the standard recirculation time and the standard recirculation start time. This combines the highest thermal load with the lowest thermal driving force and lowest mixing rate. Completeness of thawing and mixing can be verified by taking samples from LPT and bottom of the vessel to check homogeneity and analyze for conductivity, osmolality, and pH.

Process Characterization, Robustness Studies, and Design Space Considerations for Uncontrolled Rate Freeze-Thaw

As mentioned above, it is difficult to develop a large-scale process using scale-down models. But, it is still useful to evaluate the effect of freeze-thaw stresses such as temperature excursions and multiple freeze-thaw cycles on the product quality by using scale-down models that are designed to be representative of large-scale/commercial manufacturing scale. For example, a representative environment including conditions and/or stressors that the protein sees in a 10 L carboy can be created in a small size bottle (100 mL) by simulating the large-scale freeze-thaw product temperature profile obtained from the above process development studies. This can be accomplished using a controlled rate freezer (CRF), which can be programed such that the product temperature step profile obtained superimposes upon the large-scale product temperature profile. A successful scaling model is achieved once the small-scale product temperature profile overlaps with the large-scale data as schematically illustrated in (Fig. 9).

However, this scale-down model mimics only two types of denaturation or stresses: ice-liquid surface denaturation and cold denaturation. The cryoconcentration mode of denaturation is not considered in this type of model. In order to ensure the

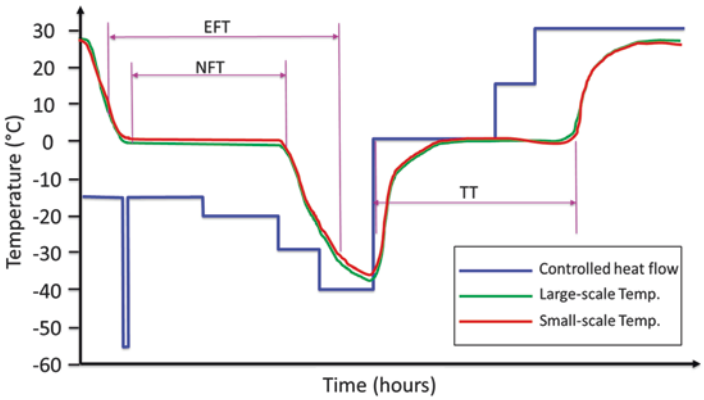

Fig. 9 Schematic representation of a scale-down model to mimic large-scale freezing. EFT, NFT and TT stands for effective freezing, nominal freezing and thawing time, respectively

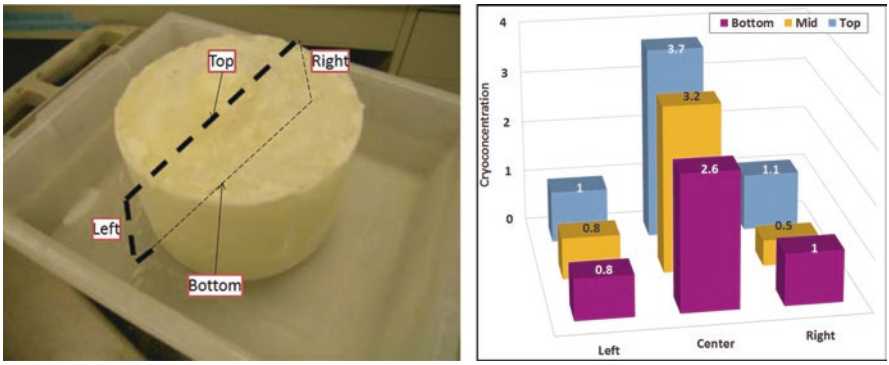

Fig. 10 Cryoconcentration in a 10 L carboy after freezing to −40 °C. (Replotted from Ref. [12])

evaluation covers all of the potential types of denaturation or stresses, the sample should be "pre-concentrated" to three or four times higher concentration than necessary followed by a similar assessment as above. The rational for three or four times higher concentration is supported by the coring studies shown in Fig. 10.

In addition to evaluating the effect of multiple freeze-thaw cycles on product quality, the process robustness studies should include situations mimicking commercial shipping and storage target conditions (time and temperature) to define the proven acceptable ranges (PAR) to cover excursions during manufacturing. In addition to testing the stressed samples at the initial timepoint, the drug substance should also be made into drug product and placed on long-term stability to ensure that these shipping stressors do not impact drug product quality.

5.2 Controlled Rate Freeze-Thaw Technology

Process Development

Like uncontrolled rate technologies, the controlled rate technologies also lack linear or direct scalability, i.e., the process developed at small scale is not linearly transferable to large scale. First, the process needs to be developed at large scale using a surrogate. Then, the resulting product temperature profiles are used to develop freeze-thaw cycles at small scale to characterize the process and understand the impact of process variables on the stability of the drug substance. The set point temperature profile of the heat transfer fluid for the scale-down unit is programmed and adjusted in such a way that the product temperature profiles observed at the last point to freeze in the small-scale system superimpose the ones observed at large scale. Supercooling is rarely observed with large-scale systems but is frequently seen in small-scale systems. If supercooling effects are observed in small-scale units, it can be minimized by transiently dropping the set point temperature to a very low value (e.g., −70 °C) to induce ice nucleation. The process performance equivalency

between the two scales and equipment performance consistency between large- and small-scale equipment can be assessed by comparing nominal freeze times (NFT) and effective freeze times (EFT), respectively. Nominal freeze time is defined as the time it takes the last point to freeze to −5 °C from an initial temperature of 3 °C. It characterizes the ice crystal growth rate which influences both cryoconcentration and morphology of the ice to determine the quality of the product. On the other hand, effective freeze time is defined as the total time required to reduce the temperature at the thermal center of the sample to a sufficiently low temperature (e.g., −30 °C) from a higher initial temperature (e.g., 10 °C).

Since the controlled rate technologies are designed and programmed to minimize the cryoconcentration effects through a controlled freezing rate (e.g., an ice front velocity of 20–25 mm/hour, which creates linear dendrites as opposed to branched dendrites), the cooling rates do not need to be monitored and assessed. However, the other critical parameters of the freezing process need to be evaluated to assess whether they need to be optimized to meet the freezing requirements of the product. To minimize mobility-related interactions, it is suggested that the product be frozen to temperatures below T_g, and sufficient time be allowed for the product to equilibrate to that temperature to ensure complete conversion of all of the free water to ice. The parameters that need to be evaluated include the following: (1) the lowest temperature of solidification and (2) the time to reach complete solidification. These two parameters need to be determined by monitoring the thermocouple placed at the last point to freeze in the container, which is usually at the top center of the container.

The thawing process is normally characterized by the following critical process parameters: time, temperature, and mixing speed. Their design is product-specific and dictated by the stability of the protein. It is recommended to identify and optimize the parameters such that thawing is completed as rapidly as possible with no presence of ice and minimal foam, while maintaining the product temperature below the temperature limit imposed by the product stability. It can be done in two steps. First, a series of experiments can be performed where the temperature and hold times are varied while keeping the mixing speed constant to identify the optimum conditions that allow the thawing process to occur close to the maximum allowed product temperature. Next, various mixing speeds, typically ranging from 50 to 150 RPM, can be studied to identify an optimum mixing speed that results in a clear and homogenous solution with minimal foam generation. If the formulation contains a crystallizable excipient such as mannitol, one may observe settling of fine mannitol crystals at the bottom of the container that formed during the freezing step. Increasing mixing speed will also increase crystal dissolution rates; however, an increase in foaming tendency may also result.

Process Characterization, Robustness Studies, and Design Space: Controlled Rate Freeze-Thaw

The operating parameters in the controlled rate freezing and thawing method need to be characterized to understand the effect of various process variables on the performance of the process and product quality attributes. The effects of fill volume

should also be evaluated as they alter the process performance and product temperature profiles which in turn, affect the product quality. As it is not feasible to examine all possible fill volumes, a bracketed approach covering the extremes can be taken. The extremes are comprised of a maximum load (16 L for Celsius-Pak® and 200 or 300 L for CryoVessel®) and a minimum load (4.2 L for Celsius-Pak® and 30 L for a 200 or 300 L CryoVessel®), thus requiring the development of only two cycles at lab scale to study the effect of fill volumes. For commercial production, it will be desirable to have one freeze-thaw cycle covering all volumes and denominations, as it will be easy to validate and use. In addition to fill volume, the effect of multiple freeze-thaw cycles, freezing rates (minimum and maximum), protein concentration, excipient weight ratios, formulation (liquid and lyophilized), and storage conditions (both warmer temperatures such as −20 °C or −30 °C and colder temperatures corresponding to dry ice after freezing to −50 °C) should also be evaluated.

CryoWedge®

To accompany the large-scale CryoVessel®, Sartorius has designed a scaled-down system called the CryoWedge® that allows for investigation into the impact of freezing and thawing conditions on protein stability. The scaled-down system requires a minimum working volume of 350 mL, but volumes as large as 4 L may be tested. Its wedge shape design mimics one compartment of the symmetrical compartments of the CryoVessel® with identical configuration within the heat exchange surface angles, length, and material of construction [31].

Although the design of the CryoWedge® models the compartments of the vessel, it still requires programming in order to generate product temperature and time profiles similar to those observed in the CryoVessel®. A stepwise freezing and thawing program for the heat transfer fluid needs to be developed using the CryoTrol® software associated with the CryoWedge® unit so that it creates freezing and thawing conditions comparable to what the product would experience in the CryoVessel®. The optimized stepwise freeze-thaw conditions that resulted in superimposable product temperature profiles between the CryoWedge® and CryoVessel® can then be used to study the effect of process variables on the stability of the product. Still, the CryoWedge® cannot be used to develop a large-scale freeze-thaw process as the large-scale CryoVessel® is required for the development of a process of this size.

Celsius Bag: S3 System

The Celsius technology S3 system can be used with bags as small as 30 mL to evaluate the impact of multiple freeze-thaw cycles and other product and process variables and create a design space encompassing the operative space and PAR. The S3 system is designed to represent large-scale freeze-thaw stresses, including cryoconcentration, by keeping the freezing path length and ice front velocity the same.

6 Case Study

In this section we discuss case studies that were previously published by Padala et al. [12] where a systematic study was undertaken by them to understand the differences between controlled and uncontrolled freeze-thaw rates on product quality attributes. Two different types of biomolecules – a fusion protein and a peptibody – were involved in the study. Conventional carboys were used for the uncontrolled freeze-thaw rate study, while CryoVessel® and Celsius-Pak® technologies were explored for the controlled rate study. The integrity of both kinds of molecules against multiple freeze-thaw cycles was evaluated using stability-indicating assays. The rationale behind studying multiple freeze-thaw cycles as opposed to a single freeze-thaw cycle was twofold; first, the impact is not clearly apparent from a single cycle, and second, in practice, the products are frozen and thawed multiple times for various reasons. The results of the case studies demonstrate that while some proteins may not be sensitive to freezing rate-dependent denaturation phenomenon, other biomolecules that tend to lose their integrity upon exposure to uncontrolled rate freeze-thaw processes can be effectively protected using controlled rate freeze-thaw technologies such as Celsius-Pak® or Cryofin® as discussed below in Sects. 6.1 and 6.2.

6.1 Case Study 1 (Fusion Protein)

To mimic uncontrolled freezing and thawing rates, the bulk drug substance of the fusion protein solution was filled into carboys and frozen by placing it inside a walk-in freezer. Thawing was carried out by placing the frozen carboy in a cold room maintained at 2–8 °C. A total of five freezing and thawing cycles were carried out, and samples were collected after the end of each cycle. Size exclusion chromatography was employed to resolve the higher molecular weight species (aggregates) from the main component. The test results indicate a slight increase in the percentage of aggregates after three freeze-thaw cycles within a carboy, as compared to the pre-freeze-thaw control sample (Fig. 11) [12]. However, no further increase in the percentage of aggregates was observed beyond three consecutive freeze-thaw cycles. It was not clear whether the slight increase in the percentage of aggregates observed with SE-HPLC was due to the impact of consecutive freeze-thaw cycles or an artifact from the assay. Regardless, all of the results from the other analytical testing indicated no significant alterations in the integrity of the protein molecule. When this material was further processed through filling and lyophilization, no change in the overall drug product quality attributes was observed, suggesting no adverse impact of five uncontrolled freeze-thaw cycles on this drug product.

The CryoVessel® technology was used as the controlled rate freezing and thawing process, and the impact of the process conditions on the integrity of the fused protein was studied in a CryoWedge®, as it only requires a few liters of material.

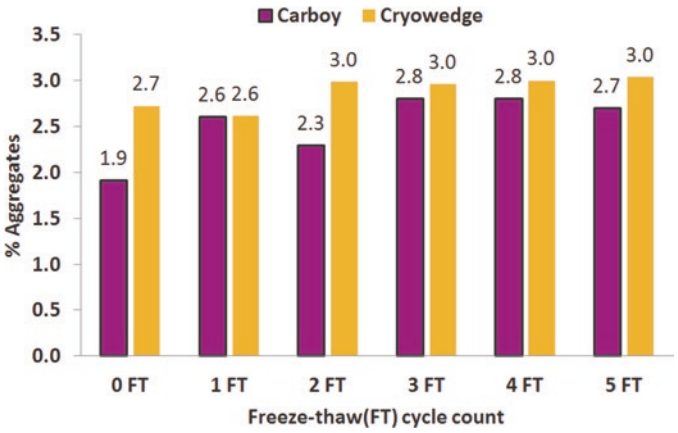

Fig. 11 Results of stability-indicating assays of fusion protein as a function of the number of freeze-thaw cycles in (**a**) carboy (uncontrolled rate freeze-thaw) and (**b**) CryoWedge® (controlled rate freeze-thaw). (Replotted from Ref. [12])

When the same fusion protein solution was then subjected to five consecutive and optimized freeze-thaw cycles in the CryoWedge®, SE-HPLC results indicated no change in the integrity of the protein structure and the quality of fusion protein product compared to the control (Fig. 11) [12]. The side-by-side stability results of the two technologies clearly demonstrate no impact of freezing methodology on the product quality of this fusion protein, suggesting the robustness of the molecule to freezing-induced denaturation processes.

6.2 Case Study 2 (Peptibody)

The bulk drug substance of a peptibody was subjected to multiple uncontrolled rate freeze-thaw cycles in 10 L carboys. The drug product quality attributes were evaluated, and as the number of freeze-thaw cycles increased, differences were observed in the freeze-thaw stressed samples when compared to the control sample (Fig. 12) [12].

Analysis of post-freeze-thaw samples using SE-HPLC indicates an increasing trend in the percentage of higher-order aggregates as the number of freeze-thaw cycles increases. Differences in the percentage of main peak compared to the control were also observed with scale from 2 L to 10 L carboys (Fig. 13) [13]. This data confirms that freezing-induced denaturation is a scale-dependent phenomena in the case of uncontrolled rate freeze-thaw technology.

On the other hand, samples collected and analyzed from Celsius-Pak®, a controlled rate freeze-thaw technology, did not show any significant change in the percentage of aggregates after 5 freeze-thaw cycles. These results suggest that the controlled rate freeze-thaw technology mitigates the denaturation of proteins arising

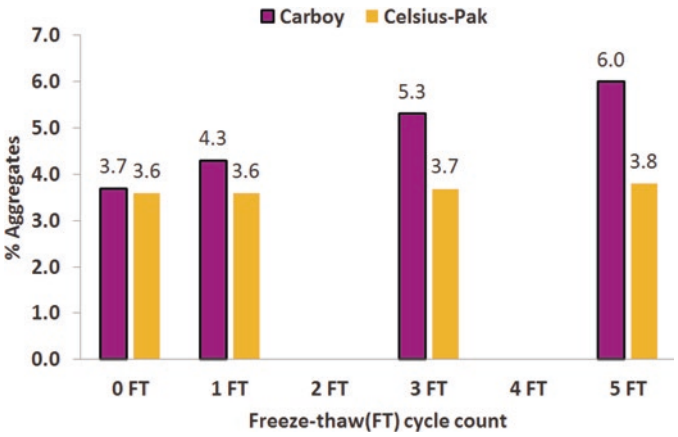

Fig. 12 Results of stability-indicating assays of a peptibody as a function of the number of freeze-thaw cycles in (**a**) carboy (uncontrolled rate freeze-thaw) and (**b**) Celsius-Pak® (controlled rate freeze-thaw). (Replotted from Ref. [12])

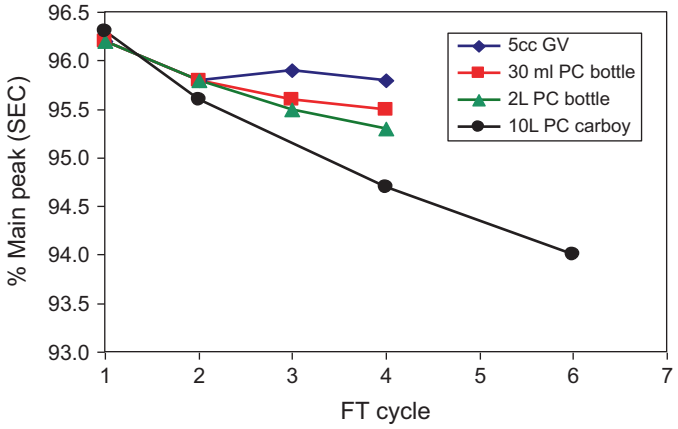

Fig. 13 Effect of container size on freeze-thaw-induced protein aggregation [13]

from uncontrolled rate freeze-thaw technology and assists in preserving products that are sensitive to freeze-thaw stressors.

The results from the described case studies demonstrate that not all biomolecules are sensitive to freezing-induced denaturation phenomena. Some molecules are robust against freeze-thaw effects and can be stored under uncontrolled rate freeze-thaw conditions using plastic or stainless steel containers within a walk-in freezer. However, for other biomolecules that are sensitive to freezing-induced denaturation phenomena, the use of controlled rate technologies such as the Cryofin® or Celsius-Pak® may be required to protect against freezing-induced denaturation.

7 Practical Considerations

7.1 Formulation Considerations

It is quite rare that protein solutions are stored long term in the liquid state as they are often stored frozen. Suitable environments for freezing protein solutions can be identified using pH indicators. Buffers such as citrate, tris, and histidine are less prone to pH shifts during freezing and should be considered instead of phosphate buffers, which undergo significant pH shifts during freezing [32, 33]. During the early stage of product and process design, the sensitivity of the protein to freeze-thaw needs to be evaluated using the appropriate scale-down model that represents the commercial freeze-thaw process. If it is determined that the protein is sensitive to freezing and thawing due to cold or interfacial denaturation, then screening of cryoprotectants and surfactants should be considered. Cryoprotectants protect the protein against cold denaturation in a similar manner as excluded solutes by increasing the free energy of unfolding [21]. Excluded solutes such as mannitol, sorbitol, disaccharides (e.g., sucrose and trehalose), polyethylene glycols, PVP, certain amino acids, methyl amines, and salting-out salts (e.g., ammonium sulfate) are typically used as cryoprotectants and are effective in molar concentrations in the range of 300–500 mM. For proteins that undergo degradation through ice-liquid interfacial denaturation, slow cooling combined with the use of a surfactant may alleviate the observed denaturation [5]. Inclusion of nonionic surfactants such as polysorbate 20 or 80, poloxamer 188, and pluronic F68 in the range of 0.01% to 0.1% in the formulation protects the protein by competing with the protein molecules for ice-liquid interfaces [34, 35]. Because dilute solutions are more prone to inactivation and material loss due to low-level binding to the storage vessel, increasing the initial protein concentration or adding a carrier or filler protein such as purified HSA to 1–5 mg/ml (0.1–0.5%) will help mitigate the adsorption-related protein denaturation.

Conformational perturbations leading to protein instability can occur simply by exposure of the molecule to low temperature without cryoconcentration effects [4], which means that formulation excipients and their concentrations should be selected such that they do not depress the freezing point substantially and demand deep freezing for complete solidification of ice. Formulation excipients should also be chosen to provide eutectic freezing and protein stability at warmer temperatures.

Frozen storage of protein solutions in metal containers (e.g., stainless steel) can result in leaching of metals, especially in the presence of corrosive salts such as NaCl. If the protein contains exposed methionine, cysteine, or sulfhydryl groups, these groups may then undergo metal-catalyzed oxidation. In such cases, a metal chelator such as polyaminocarboxylate (PAC), citrate, or EDTA should be added to a final concentration of 1–5 mM [36]. Finally, addition of protease inhibitors may also be useful to prevent the proteolytic cleavage of proteins, and the addition of preservatives such as sodium azide (NaN_3) and thimerosal will help to limit any microbial growth during liquid storage post thawing.

7.2 Physical Properties and Storage Temperature Considerations

Biological solutions are typically stored frozen at temperatures between −20 °C and − 80 °C. However, it is important that the thermal events such as eutectic melting temperatures and glass transition temperatures of the maximal freeze concentrate ($T_{g'}$) are considered. In addition to the properties of the protein and formulation, the physical properties of plastic containers with respect to their ability to withstand both freezing and autoclaving conditions and rigorous shipping and handling must also be assessed (Table 1) [37]. When using plastic containers for freezing applications, the primary concern is the brittleness temperatures of the plastic, which should be lower than the desired storage temperature.

In addition to protein stability and container integrity, identification of storage temperatures away from the phase transition temperature (10 °C below the thermal event) is also an important step when assessing freeze-thaw liabilities. Depending on the nature of the protein formulation, different storage temperatures may be warranted, but in general, storage at lower temperatures (e.g., −40 °C or −80 °C vs. −20 °C) is preferred for the following reasons:

1. −20 °C is close to the phase transition temperature (eutectic temperature) of many salts that are used in protein formulations. Natural cycling of the temperature above and below −20 °C by the freezers can cause dissolution and recrystallization of salts which causes stress to the protein, in addition to the cryoconcentration effects as mentioned above.
2. The temperature of the freezer will not consistently be −20 °C at all locations within the freezer. It could differ by 5–10 °C which could potentially leave the product in a partially unfrozen state.
3. When stored at lower temperatures (e.g., −40 °C or −80 °C), the rates of adverse reactions arising from cryoconcentration, cold denaturation, or interfacial dena-

Table 1 Physical properties of plastic materials (Ref. [37])

Plastic type	LDPE	HDPE	PP	PMP	PC	PVC	PA
Maximum use temperature	80 °C	120 °C	135 °C	175 °C	135 °C	70 °C	121 °C
Brittleness temperature	100 °C	100 °C	0 °C	20 °C	135 °C	30 °C	40 °C
Transparency	Translucent	Translucent	Translucent	Clear	Clear	Clear	Translucent
Autoclavable	No	No	Yes	Yes	Yes	No	Yes
Sterilization gas	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Dry heat	No	No	No	Yes	No	No	No
Disinfectants	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Specific gravity	0.92	0.95	0.90	0.83	1.20	1.34	0.90
Gravity flexibility	Excellent	Rigid	Rigid	Rigid	Rigid	Rigid	Moderate

Resin codes: *LDPE* low-density polyethylene, *PP* polypropylene, *PMP* polymethylpentene, *PC* polycarbonate, *PVC* polyvinyl chloride, *PA* polyallomer

turation can be slowed compared to those at −20 °C. However, there is a trade-off as lower storage temperatures require higher energy costs and equipment and operational logistics for product storage. To avoid these issues, it may be possible to store some products at −20 °C after freezing them at lower temperatures if there are no phase transitions, and the subsequent annealing that results in secondary crystallization will not impact product stability.

In order to circumvent the adverse effects of repeated freeze-thaw, it is helpful to aliquot material appropriately to align with fill and finish batch requirements. Alternatively, if the protein is sensitive to repeated freeze-thaws, then addition of glycerol or ethylene glycol will prevent solutions from freezing at −20 °C, enabling repeated use from a single stock without warming (i.e., thawing). Ethylene glycol is a better choice than glycerol as it will also prevent microbial growth. Finally, one simple and practical approach to address and minimize cryoconcentration effects is to use small containers with reduced freezing path lengths.

7.3 Heat Flow

The morphology of ice formed during the freezing process varies with the method of freezing and the composition of the formulation. The structural unit of ice formed falls into four categories: vitreous, cubic, dendrites (linear or branched), and spherulites (coarse or evanescent) [38]. The moving solid boundary during freezing could be either flat or dendritic in nature. A moving dendritic ice front allows solutes to become entrapped in the interdendritic space, which promotes a more uniform macroscopic distribution of solutes in the frozen mass and minimizes freeze concentration. If the moving solid-liquid interface were flat, solutes could be more easily excluded from the frozen mass and become increasingly concentrated. One important strategy for controlling dendritic growth for freezing large volumes is to assure directional heat flow. Convection or agitation in the liquid phase of the solution during freezing may cause exclusion of solute molecules from the solution's solidifying mass and gradual cryoconcentration of solutes in the liquid phase. This is caused by the sweeping effect of liquid motion at the solid surface and the suppression of dendritic ice growth. For this reason, it is important to avoid any mechanical agitation during the freezing process.

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Chapter 20
Freeze-Thaw Process Data Analysis and Mechanistic Modeling: Simplified Lumped Capacitance Analysis for Small Fill Volumes

Alina A. Alexeenko, Laura Mozdzen, Sherwin Shang, Michelle A. Long, Grace Kim, and Margaret Musser

1 Background and Motivation

During production of both biologic and small-molecule pharmaceuticals, freezing is commonly used as a processing step to maintain stability and quality of a drug substance or drug product during manufacturing, shipment, and/or storage. Freezing is especially important to limit drug substance degradation pathways. However, the freeze-thaw process involves challenges ranging from destabilizing interfacial stresses on biomolecules at the liquid-ice interface [1], to container-closure integrity challenges upon freeze expansion (especially in the case of products packaged in glass containers [2, 3]), to the lengthy time required to reach the frozen state and thus halt sensitive pharmaceutical degradation.

Whereas many factors related to drug substance stability need to be considered when designing a freeze-thaw process, the main goal of this study is to develop a simplified model to quantify time and conditions for freezing and thawing of a small amount of solution (10s of mL) in a primary package such as a glass cartridge with a stopper. In contrast, freeze-thaw of biologics in large containers such as drug substance bottles (with a fill volume of a few liters), cryo-Celsius bags (10s of liters), and cryo-vessels (100 s of liters) has been addressed by Kantor et al. [5, 6]. The long-term goal is to develop better process understanding and mechanistic modeling capability for designing freeze-thaw conditions for a combination drug-device pharmaceutical product. This would allow a development teams to assess the design options of primary, secondary, and tertiary packaging up to pallet configurations to

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define the best system design for practice. Such process understanding based on mechanistic modeling, data analysis, and experiments forms an integral part of a Quality by Design (QbD) approach to manufacture pharmaceutical drug products [9].

2 Problem Statement

We consider a small-volume container such as a glass cartridge filled initially with a given volume of liquid solution and a stopper seal. The system is exchanging heat with an environment such as in a freezer at an ambient temperature T_a. The goal is to find the dependence of time for freezing or thawing a given amount of solution in a certain primary package when subjected to an environment at a given ambient temperature T_a (Fig. 1).

3 Applicability of Lumped Capacitance Model for Heat Transfer

Freeze-thaw of small volumes of solution is often characterized by relatively uniform temperature due to small Biot numbers. The Biot number is a nondimensional quantity which indicates whether there is spatial variation of temperature inside a body, while it heats or cools due to heat transfer through its surface. Typically, if Bi<0.1, the temperature of the body can be approximated as uniform at any given moment of time, and a simplified "lumped capacitance" model for heat transfer analysis can be applied [4]. The Biot number is defined as

$$\mathrm{Bi} = \frac{L\,h}{k}$$

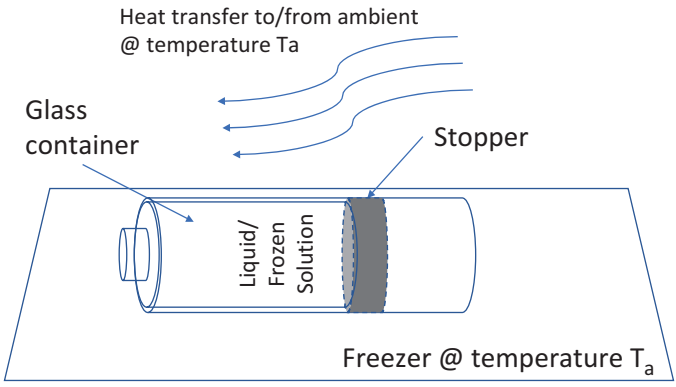

Fig. 1 Schematic of solution in a primary package at ambient temperature T_a

where L is the characteristic length of the body which could be taken as the ratio of volume to surface area: $L = V/A$. The heat transfer coefficient h is dependent on both the packaging and freezing/thawing method. The thermal conductivity of the body is k and is a property of the material.

To assess the applicability of the lumped capacitance model, first we estimate the Biot number for typical conditions described in Sect. 2. For a sample volume of 15 mL filled into a 20 mL cartridge with ID = 23.85 mm, the $L = V/A = 4.4$ mm. The thermal conductivity of pure liquid water k is 0.5917 W/m/K at 290 K = 16.85 °C [7]. A typical heat transfer coefficient value h for such a container at $T_a = -23.5$ °C based on experimental measurements (described in Sect. 5 below) is h~10 W/m^2/K. The resulting Biot number is Bi = 0.074. This is within the range of Biot numbers Bi<0.1 where the error of applying the lumped capacitance method is expected to be small.

Note that for larger container volumes such as drug substance in vessels and cryo-Celsius bags which have fill volumes from a few to tens of liters and more [5], the assumption of small Biot number would be invalidated. Similarly, for much higher heat transfer coefficients such as those expected in forced convection freezers, e.g., a blast freezer, the Biot number would be higher, and spatial variation of temperature should be considered by a higher-fidelity modeling method. On the other hand, the presence of secondary packaging is expected to decrease the heat transfer coefficient h and thus the Biot number. The Biot number, therefore, needs to be characterized as the first step of analysis of a specific container/fill/freezer combination.

Note also that the low Biot number conditions are typically satisfied for lyophilization of pharmaceuticals in typical single dosage container-closure systems such as glass vials and syringes. Lyophilization of pharmaceuticals typically includes a freeze-drying cycle that consists of three main stages: freezing (ice formation), primary drying (ice sublimation), and secondary drying (removal of absorbed moisture). Among these steps, the freeze is often the most critical stage during which most of the destabilizing stresses occur, especially for therapeutic proteins [8]. Additionally, the ice crystal during the freezing stage is determined from the cooling-freezing rate, homogeneous vs. inhomogeneous nucleation, and induced or controlled supercooling-freezing process. In other words, the size of ice crystals is impacted by the nucleation kinetics which in turn depends on the duration and set point temperature during the freezing process. A high degree of supercooling often results in fast nucleation and fine ice crystals and thus increased resistance to vapor flow. While nucleation occurs randomly, controlled ice nucleation techniques have received significant attention because they can significantly reduce the variability in the nucleation event, improve primary drying control, increase the ice crystal size, and reduce primary drying time. Thus the design of the freezing stage of pharmaceutical lyophilization can exploit the lumped capacitance analysis similar to that described and applied below for the freeze-thaw process.

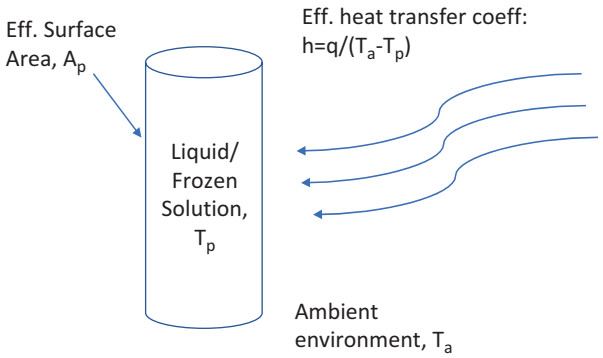

Fig. 2 Notations used in the lumped capacitance model of freezing and thawing of small fill volumes of pharmaceutical product in primary packaging

4 Lumped Capacitance Model for Freezing and Thawing Time of Solution in a Primary Package

A simplified lumped capacitance model is shown schematically in Fig. 2. A typical freeze-thaw cycle will involve multiple heat transfer and phase change stages. For example, during the freezing stage, there is initially a liquid cooling process until the material reaches its freezing temperature, followed by a solidification process during which the phase change from liquid to ice is taking place. After solidification, further cooling of ice may proceed until the temperature between ambient and material equilibrates. During the thaw stage, the ice is warming until it reaches a melting temperature, followed by a solid-liquid phase change process. Finally, the liquid temperature will increase until it reaches equilibrium with the ambient temperature.

The lumped capacitance model assumes the material properties are constant with time allowing an analytical solution for the time evolution of product temperature and the total phase change time as described below.

Cooling/Warming Heat Transfer Process: A volume of material at a uniform temperature T_p is exchanging heat with an ambient environment at a temperature T_a. The surface area available for heat transfer is A_p.

From the conservation of energy for a given mass m of the product

$$\frac{dT_p}{dt} = \frac{A_p h}{m c_p}\left(T_a - T_p\right) \tag{1}$$

where C_p is the specific heat of the product in the given phase, for example, liquid water or ice.

Assuming only T_p varies with time, we can integrate Eq. (1) as

$$T_p(t) = T_a + (T_0 - T_a)\exp\left(-\frac{A_p h}{m c_p} t\right) \tag{2}$$

Here T_0 is the initial temperature of the product at $t = 0$. Note that the simple analytical expression above assumes that the specific heat c_p of liquid or frozen product does not change significantly when the product temperature is changing between T_0 and T_a.

4.1 Solidification/Melting Phase Change Process

The conservation of energy can be used to calculate the duration of the phase change process. Assuming the temperature of the product remains constant during melting or freezing, we get

$$\Delta H \frac{dm}{dt} = -\frac{A_p h}{1 - f_s}(T_f - T_a) \tag{3}$$

where ΔH is the enthalpy change, f_s is the solid fraction, and T_f is the freezing or melting point. Assuming the phase change is a steady process, we get the following expression for the solidification/melting time t_{pc}:

$$t_{pc} = \frac{m\Delta H(1 - f_s)}{A_p h(T_f - T_a)} \tag{4}$$

Note that the approximation above neglects the effect of stopper movement due to expansion of ice after freezing which leads to (a) change in the product surface area available for heat transfer and (b) the work of gliding force. As is seen from the analysis of experimental data in Sect. 5 below, the change in the product surface area is an important factor and can be easily incorporated. The gliding force for the typical stopper/cartridge used in the experiment has negligible effect on solidification or melting time.

5 Experimental Data Analysis and Comparison with the Model

The lumped capacitance heat transfer model described in Sect. 4 was used to analyze the experimental data. Freeze-thaw experiments were performed using a 20 mL cartridge with a stopper filled with 15 mL of water for injection. A sample cartridge with a stopper before and after freezing of 15 mL liquid fill volume of water is

Fig. 3 Sample 20 mL cartridge with a stopper and 15 mL water, before and after freezing

shown in Fig. 3. The freeze-thaw tests were conducted using temperature probes (GRAPHTEC Corporation, model GL220) inserted into the cartridge. The samples were placed in a thermally controlled chamber (Thermotron Environmental Chamber Model SM-16-8200) shown in Fig. 4.

Figure 5 presents a measured temperature history for an experiment performed at chamber temperatures ranging from −23.5 °C in the freezing stage to +4.7 °C for the thawing stage and + 60 °C for the thermal aging stage. The various heat transfer and phase change stages are clearly visible in the temperature history as marked in Fig. 5.

The temperature data in Fig. 5 can be used to estimate the heat transfer coefficient h of the specific solution/package/freezer combination. The heat transfer coefficient can be extracted from one stage (e.g., initial liquid cooling) and can be used to verify by calculated comparison with the data for another heat transfer stage such as liquid heating. Additional formulation-specific parameters such as freezing point depression can be determined from analysis of the solidification stage data.

In combination with the material properties of the product (Table 1), the experimental data in Fig. 5 for the initial cooling of the product were used to extract the effective heat transfer coefficient. Eq. (2) was used to obtain heat transfer coefficient h based on the fit of the temperature data with the following input parameters: m = 1.5E-2 kg, A_p = 3.40923, and C_p = 4.182 kJ/kg/K.

Figure 6 shows the comparison of mechanistic modeling and experimental data for the freeze-thaw cycle at conditions listed in Table 2. The heat transfer coefficient extracted from the data for the initial liquid cooling (green dotted line) is

Fig. 4 Environmental chamber used for freeze-thaw measurements

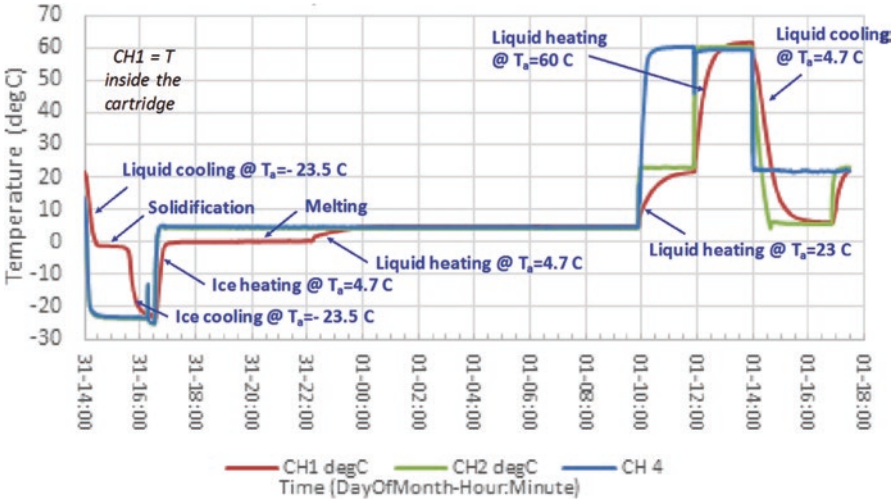

Fig. 5 Sample temperature data for a freeze-thaw study of 15 mL water filled into a 20 mL cartridge with heat transfer and phase change stages at different ambient temperatures

Table 1 Material properties of water and ice

C_p, liquid (20 °C), J/kg/K	4182
C_p, ice (0 °C), J/kg/K	2093
Fusion enthalpy ΔH, J/kg	333,550

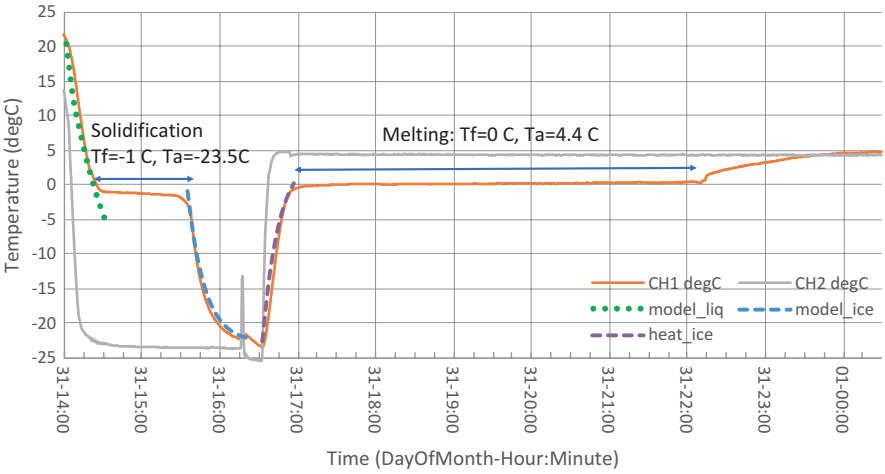

Fig. 6 Comparison of modeling (dashed and dotted lines) and experimental data for freeze-thaw thermal history. Data for 15 mL fill in 20 mL cartridge at conditions in Table 2

Table 2 Freeze-thaw conditions for experimental data in Fig. 6

Container/fill		Thermal conditions	
Inner diameter, mm	23.85	Liquid and ice cooling T_a, °C	−23.5
Fill volume, mL	15	Freezing temperature, T_f°C	−1.0
Fill height, mm	33.58	Ice warming T_a, °C	4.4
Surface area A_p, m^2	3.409E-3	Melting temperature, °C	0.0
Fill mass, kg	0.015		

11.62 W/m^2/K using a two-point fit for product temperatures at 14:07 and 14:14. If a 6-minute interval from 14:05 to 14:11 is used, the extracted heat transfer coefficient is 10.42 W/m^2/K, whereas for a 3-minute interval from 14:11 to 14:14, the effective heat transfer coefficient is 12.08 W/m^2/K. A heat transfer coefficient of h = 11.62 was selected and used in Eq. (2) to predict the profile for ice cooling and ice warming temperature histories in Fig. 6.

The agreement with measured temperatures for ice cooling and ice warming is good, within about 5 minutes of the measured time. Note that the heat transfer calibration error is about 20% for this case and leads to these differences in model results for cooling and warming times.

The freezing duration time in Eq. (4) has been calculated using the extracted effective heat transfer coefficient h = 11.62 W/m^2/K. Because a significant (~3.5 mm) stopper movement due to ice expansion was measured in the experiment, the product surface area was increased. Note that the observed stopper movement is consistent

with an expected increase of ice volume compared to that of liquid by 9.05% for the dominant *I*(*h*) polymorph of ice. Table 3 shows the freezing time with consideration of ice expansion for two values of h derived from the initial cooling.

Figure 7 compares the measured product temperature during the freezing stage with that predicted by the models using the two values of heat transfer coefficient. It is seen that in the physical experiment, the transition between cooling and solidification is gradual, rather than strictly separated as assumed in the model. This is due to the fact that the outer region of the liquid is at a lower temperature and starts to freeze before the core of the cartridge. This temperature nonuniformity is neglected in the lumped capacitance model and assumed negligible when the Biot number is less than 0.1. The overall time to reach −10 °C is within 15% of measured for h = 11.62 W/m^2/K and within 11% for h = 12.08 W/m^2/K. Note that since the model strictly separates the cooling and phase change processes, the predicted times to reach a specified temperature below the freezing point are longer than that in the experiment, giving a conservative estimate.

The work due to stopper gliding was also considered and found to have negligible effect compared to the effect of the surface area change due to ice expansion.

Table 3 Comparison of model and experimental data

Process time, hr	Model		Measurement
	h = 11.62 W/m^2/K	h = 12.08 W/m^2/K	
Freezing at T_a = −23.5 °C			
Solidification at −1 °C	1.48	1.42	1.13
Ice cooling from −2 °C to −20 °C	0.371	0.357	0.45
Thawing at T_a = +4.4 °C			
Ice warming from −20 °C to −2 °C	0.273	0.263	0.25
Melting at 0 °C	7.41	7.13	7.16

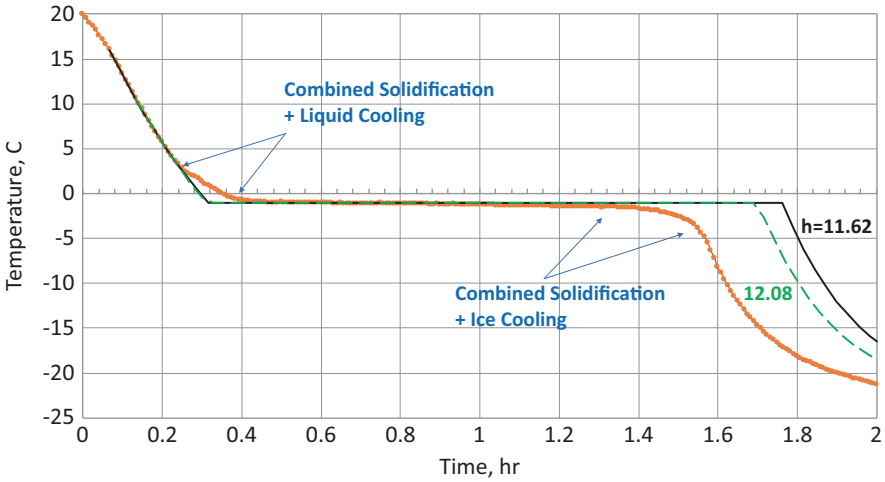

Fig. 7 Comparison of measurements and modeling for cooling and freezing assuming h = 11.62 and h = 12.08 W/m^2/K

The stopper gliding force was measured to be 1–2 N. Given the stopper gliding distance of 3.5 mm, this gives a total gliding work of 3.5–7.0 mJ which is ~0.0001% of the total of about 5 kJ of heat removed during freezing for a 15 mL sample. Thus, the corresponding proportional increase in freezing time is negligible.

6 Application of the Model for Freeze-Thaw Process Design

The heat transfer analysis, based on calibration of the heat transfer coefficient from the experimental data, can be applied to consider the effects of varying ambient temperature on the freezing and thaw times.

From Eq. (2) we can find the time for the given liquid fill volume initially at a temperature T_0 to reach the freezing point T_f due to heat transfer with the ambient environment at a temperature T_a characterized by the heat transfer coefficient h and the thermal capacitance per unit area ($m \cdot c_p/A_p$):

$$t_{T_0 \to T_f, T_a} = \ln\left(\frac{T_0 - T_a}{T_f - T_a}\right)\frac{m \cdot c_{p,liq}}{A_p}\frac{1}{h} \tag{5}$$

We can compare, for example, the effect of changing the ambient temperature from −23.5 °C to a lower or higher value. Using the same cartridge and fill parameters as in the data set in Sect. 5, we can consider how long will it take to freeze the sample and cool it down to −10 °C for the three different ambient temperatures, T_a = −15 °C, −23.5 °C, and −35 °C, using the same cartridge and fill conditions as in Sect. 5 with the heat transfer coefficient h = 11.62 W/m²/K. The results are shown in Fig. 8 and Table 4.

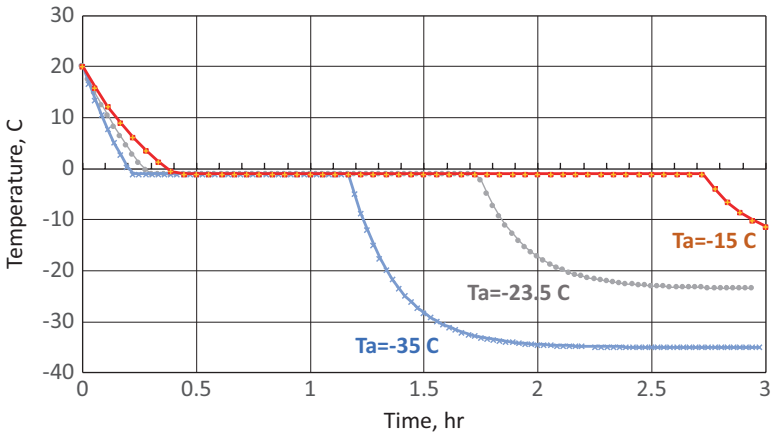

Fig. 8 Calculated product temperature as a function of time for different ambient temperature conditions T_a = −15 °C, −23.5 °C and −35 °C. Same container and fill as in Fig. 6, h = 11.62 W/m²/K

Table 4 Effect of ambient temperature T_a on cooling and freezing time

Process time, hr	$T_a = -15$ °C	$T_a = -23.5$ °C	$T_a = -35$ °C
Liquid cooling +20 °C to −1 °C	0.403	0.290	0.212
Freezing at −1 °C	2.327	1.448	0.958
Ice cooling −1 °C to −10 °C	0.211	0.104	0.063
Total time +20 °C to −10 °C	**2.941**	**1.842**	**1.233**

7 Future Directions for High-Fidelity Mechanistic Modeling of Freeze-Thaw Process

There are two general directions for further development of the modeling of a freeze-thaw process for pharmaceutical products: first, consideration of secondary and tertiary packaging and, second, higher-fidelity modeling that includes spatial nonuniformities due to heat conduction at Bi>0.1.

7.1 Secondary and Tertiary Packaging

Product in cartridges is typically placed in a secondary package such as a carton (typically cardboard box), several of which are in turn packaged on pallets. The heat transfer coefficient would be reduced by this additional packaging and can be similarly characterized by measurements in a controlled freezer. Heat transfer coefficients corresponding to secondary and tertiary packaging can be determined by laboratory measurements and used in modeling Eqs. (1)–(4) accounting for variation of T_a (x,y,z) that corresponds to different locations (x,y,z) within the pallet and variations of the temperature within the large-scale freezers as shown schematically in Fig. 9. Note that the Biot number should be checked for such conditions to satisfy lumped capacitance assumptions.

7.2 Higher-Fidelity Modeling for Bi>0.1

A more detailed heat transfer model using a computational solver which accounts for thermal non-uniformities should be applied for large fill volumes or other conditions leading to large Biot numbers when the lumped capacitance method is not applicable.

Summary: A simplified lumped capacitance model for analysis of the freeze-thaw process for a small fill volume in a primary packaging has been developed. The model requires the heat transfer coefficient for a given package to be calibrated

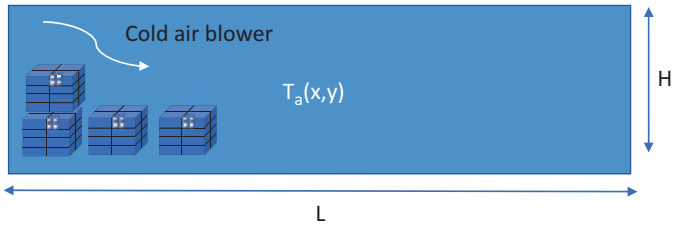

Fig. 9 Schematic of ambient temperature variation within the large-scale freezer

from experimental data for the initial liquid cooling. It can then be applied to predict the freezing and thawing. The predicted time to reach a specified temperature below the freezing point agrees with experimental measurements within 11–15% depending on the quality of fit of the heat transfer coefficient. The model can be improved by including the effect of thermal nonuniformities through more detailed computational simulations of heat conduction within the container.

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Chapter 21
Rational Design of a Freeze-Drying Process for Protein Products

Feroz Jameel, Tong Zhu, Ehab M. Moussa, and Brittney J. Mills

Nomenclature

P_{ch}	Chamber pressure
P_{sub}	Sublimation front pressure
P_{ref}	Reference pressure
T_{sh}	Shelf temperature
T_{surf}	Average shelf surface temperature
T_{inlet}	Shelf heating fluid inlet temperature
T_{bot} (or T_b, T_{pr})	Product bottom temperature
T_e	Eutectic melting temperature
T_c	Collapse temperature
T_{ref}	Reference temperature
T_{crit}	Critical product temperature
$T_{sh,\,crit}$	Critical shelf temperature
T_g	Glass transition temperature of the dry powder
T_g'	Glass transition temperature of the maximum freeze-concentrated solution
W_g'	Residual unfrozen water content at the T_g' temperature
$\dot{Q}_{cond}$	Heat conduction rate in the frozen product
$\dot{Q}_{tot}$	Total heat transfer rate to the vial
$\dot{Q}_{sub}$	Heat transfer rate at the sublimation front
K_v	Vial heat transfer coefficient in general
$K_{v,\,surf}$	Vial heat transfer coefficient between the shelf surface and the vial bottom
$K_{v,\,inlet}$	Total/apparent heat transfer coefficient between the shelf fluid inlet and the vial bottom

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K_{rad}	Radiative heat transfer coefficient to the vial from the surroundings
K_{gas}	Gas conductive heat transfer coefficient between the vial bottom and the shelf/tray
K_C	Pressure independent part of heat transfer coefficient between the vial and the shelf/tray
K_P	Constant parameter expressing the pressure dependence of heat transfer coefficient
K_D	Pressure dependence of heat transfer coefficient specific to a vial geometry
R_p	Area-normalized product resistance
$R_{p,max}$	Maximum area-normalized product resistance for a given product
$c_{solid}\%$	Solid concentration of the formulation (w/v)
R	Universal gas constant
ΔH_s	Latent heat of ice sublimation
A_p	Bottom area of the frozen product
A_v	Outer area of the vial cross section
l_{bot}	Effective gap distance between the vial bottom and the shelf/tray
$l_{pr,\,0}$	Initial frozen product thickness
l_{ck}	Dried cake thickness
V_{vill}	Fill volume
k_{ice}	Heat conductivity of the frozen product
σ	Stefan-Boltzmann constant
λ_0	Heat conductivity of the water vapor at a given pressure
α	Energy accommodation coefficient for water vapor
t_{PriDry}	Primary drying time
ϵ	Porosity of the dried cake
τ	Tortuosity of the dried cake
r	Average radius of the pores in the dried cake
fast-DS	Fast design space

1 Introduction

Many biotherapeutic modalities including several protein-based formats, vaccines, and RNA therapeutics have only limited stability in the liquid state that can't support sufficient long-term shelf-life. Since almost all physical and chemical degradation pathways are typically significantly faster in liquid compared to the solid state, drying technologies such as freeze-drying, spray-drying, and supercritical fluid precipitation are often employed to prepare dried drug products. In the solid state, the stability of molecules typically increases in the order of solution, glassy solid, and crystalline solid mainly due to the increasing restrictions on the mobility of the reacting species in these matrices, respectively [1–3]. Historically, lyophilization has been the method of choice for drying biologics as it does not involve exposure

to high temperatures as is the case for spray-drying and minimizes the waste in drug substance [4].

The freeze-drying process consists of three phases: freezing, primary drying, and secondary drying. In the freezing phase, the water component of the solution is converted into ice leaving behind a matrix of either glassy or crystalline solutes. The primary drying phase involves the sublimation of the frozen ice into vapor under deep vacuum. Finally, in the secondary drying phase, higher temperatures are applied to remove the remaining unfrozen water dispersed throughout the matrix by desorption.

Despite the advantages of using freeze-drying with regard to long-term stability, the freezing and drying processes per se may pose stresses on the protein leading to stability challenges. In addition, the lyophilized product should be pharmaceutically elegant with no or minimal signs of collapse or melt-back, have low residual moisture content and rapid reconstitution time, and be easy to administer and compatible with the reconstitution kit. Moreover, the process should be efficient, be low-cost, and allow manufacturing with minimal equipment capability and process parameter restrictions and hence can be implemented on most typical production freeze-dryer at several manufacturing sites.

In lyophilization, the formulation composition, the primary package configuration (dimensions and fill volume), and certain process parameters are interdependent and should all be considered before designing the drug product. For instance, formulations with certain solid content and/or physical properties can be practically impossible to freeze-dry. Similarly, a well-designed formulation dried using a poorly designed process may take exceedingly long time. So, for the successful scale-up and technology transfer of the lyophilization process to the manufacturing site, it is important to understand the scale-up challenges specifically as it relates to equipment capabilities and limitations, the manufacturing environment differences, and the relevant formulation parameters [5, 6].

In this chapter, we first review the basic principles of the thermal properties of the formulation and the freezing phase of the process. We then focus on the primary drying phase as it represents the most critical step of the process with regard to scale-up and describe the theoretical development of a new method to calculate the design space of the primary drying phase in order to obtain initial guesses of the process parameters. Next, we briefly review the commonly used methods to determine the end of the primary phase. We then discuss the critical considerations for designing the secondary drying phase and for the scale-up and technology transfer of the entire process. Finally, we describe a case study that demonstrates the systematic development of the freeze-drying process for a formulation with challenging thermal properties.

2 Thermal Properties of the Formulation

The design of the formulation and the freeze-drying process should go hand-in-hand. The design of the freeze-drying process depends on the freeze-drying properties of the excipients in the formulation such as the eutectic melting temperature

(T_e), the glass transition temperature of the maximally freeze-concentrated solution (T_g'), crystallization time, and the glass transition temperature of the dry powder (T_g). For example, T_g' and/or T_e values set the limits of the primary drying conditions (i.e., shelf temperature, chamber pressure) and guide the selection of the secondary drying conditions (ramp rate, temperature), and T_g of the cake determines the recommended transportation and storage conditions of the product [4]. The values of the above freeze-drying properties are affected by the nature of the excipient and their weight ratios in the formulation. Accordingly, simple formulation compositions and the rational inclusion of additional ingredients in the formulation are recommended. However, to address and mitigate certain liabilities of the drug molecule (such as low concentration, low solubility, and/or limited stability), to enable a robust and fast process, and to improve patient convenience, a multicomponent system containing low T_g' stabilizers, salts, and/or crystallizing excipients may be used. Most of the excipients commonly used in protein formulations such as buffers, stabilizers, or bulking agents behave differently depending on their relative concentration in solution and the presence or absence of other excipients [7]. Hence, it is critical to characterize the behavior of these components and determine the values of the key thermal properties before locking in the formulation and selecting the process conditions.

The freeze-drying process should be designed to be efficient and amenable to commercial manufacturing, and both are influenced by the critical temperature of the formulation (T_{crit}). Depending on the physical form of the components of the formulation matrix (i.e., amorphous, crystalline, or both), the critical temperature of the formulation could be the collapse temperature (T_c) or T_e. T_c is defined as the maximum product temperature that allows drying to occur without the loss of porous "cake-like" structure with the dimensions equivalent to those of the frozen solid [8]. At this temperature, the mobility in the glass matrix increases significantly, and viscous flow occurs wherein the glassy component transitions into a rubber state, loses structure, and results in the collapse of the cake, which can also result in high residual moisture content and long reconstitution time. Therefore, the product temperature at this interface should be maintained below T_c throughout the primary drying phase. This temperature is related to T_g', which is the temperature at which the maximally freeze-concentrated solution falls out of equilibrium during freezing and forms a glass. T_c can be measured directly using freeze-drying microscopy (FDM) or indirectly estimated from the T_g' measured using modulated differential scanning calorimetry (MDSC). The T_g' of a monophasic multicomponent system can also be estimated from the T_g' values of the individual components using the Fox equation [9]:

$$1/T_g = \left(W_1/T_{g1}\right) + \left(W_2/T_{g2}\right) \tag{1}$$

where W_i is the weight fraction of component "i" and T_{gi} is the glass transition temperature of pure component "i". Equation (1) can be applied to determine the T_g' of systems containing two or more amorphous components wherein T_{gi} is the T_g' of aqueous component "i" and W_i is the weight fractions of the solute relative to the

total mass of solutes. The value of T_g' determined by DSC is approximately 2–3 degrees lower than the actual T_c measured using FDM. Lists of the values of T_c/T_g' and T_g of excipients that are commonly used in freeze-dried pharmaceuticals are provided Tables 1 and 2, respectively.

Table 1 List of the collapse temperature (T_c) and the glass transition temperature (T_g') for selected excipients

Material	T_g' (°C)	Reference	T_c (°C)	Reference
BSA	−11	[10]		
Dextran	−10	[10, 11]	−9	[12–14]
Ficoll	−19	[11]	−19.5	[12–14]
Gelatin	−9	[11]	−8	[15]
PVP (40 k)	−20	[11]	−23	[15]
Dextrose	−44	[11]		
Hydroxypropyl-β-cyclodextrin			−18	(Pikal MJ, Shah S. Unpublished data. Eli Lilly & Co.)
Lactose	−28	[10, 11]	−31	[12–14]
Mannitol	−35	[10, 11]	−35	[12–14]
Raffinose	−27	[16]	−26	[15]
Sorbitol	−46	[11]	−45	[15]
Sucrose	−32	[10], (Pikal MJ, Chang LQ. Unpublished data. University of Connecticut.)	−32	[12–14]
Trehalose	−29	[10, 11]	−34	[12–14]
β-Alanine	−65	[10]		
Glycine	(−62)	[17]	(−62)	[12–14]
Histidine	−33	[10]		
Acetate, potassium	−76	[10]		
Acetate, sodium	−64	[10]		
$CaCl_2$	−95	[10]		
Citric acid	−54	[10]		
Citrate, potassium	−62	[10]		
Citrate, sodium	−41	[10]		
HEPES	−63	[10]		
$NaHCO_3$	−52	[10]		
Phosphate, KH_2PO_4	−55	[10]		
Phosphate, K_2HPO_4	−65	[10]		
Phosphate, NaH_2PO_4	−45	[10]		
Tris base	−51	[10]		
Tris HCl	−65	[10]		
Tris acetate	−54	[10]		
$ZnCl_2$	−88	[10]		

Collapse temperature data were obtained with freeze-drying microscopy, and T_g' data were obtained using DSC at roughly 10 °C/min heating rates and represent mid-points of the glass transition region. The values in parenthesis are estimated by extrapolation from non-crystallizing mixtures to the pure compound

Table 2 List of the glass transition temperatures (T_g) of selected excipients measured by DSC

Compound	T_g (°C)	Reference
Citric acid	11	[18]
Lactose	114	[19]
Maltose	100	[19]
Mannitol	13	[20]
Raffinose	114	[21]
Sorbitol	−3	[9]
Sucrose	75	[21]
Trehalose	118	[21]
Maltodextrin DE 20	141	[19]
Maltodextrin DE 25	121	[19]
Maltodextrin DE 36	100	[19]
PVP k90	176	[21]
PEG 400	41	[22]

Consult the references for specific details of the techniques used

A eutectic system is a mixture of two or more crystalline compounds that melt together at the lowest freezing temperature. In a mixed formulation system where crystalline phase constitutes the major weight fraction of the matrix, the T_e will be the critical temperature of the formulation. Carrying out the primary drying with the product temperature above the T_g' of the amorphous phase of the formulation but below T_e of the crystalline component will result in the collapse of the amorphous component, while the crystalline phase will provide the necessary mechanical support to the cake structure. This is an effective strategy to enable fast and robust freeze-drying cycles, but the impact on the product stability needs to be evaluated [7].

It is critical from the quality perspective that the product is dried with the retention of structure to maintain pharmaceutical elegance, low residual moisture content and good stability, and short reconstitution time. As the critical temperature of a formulation depends on the nature of excipients and their weight ratios, the rational selection of the excipients that have high collapse or eutectic temperature is recommended whenever possible.

3 The Freezing Phase

Once the formulation is completely characterized, the next step is to identify optimal process conditions. Identification of the optimal process parameters requires good understanding of the various phases of the freeze-drying process, the objectives of each phase, and the interdependence of the formulation and the process parameters. In this section, we review the basics of the freezing phase and its impact on the subsequent drying phase.

3.1 Freezing

The main objective of the freezing phase is the complete conversion and solidification of water into ice. For complete solidification, the formulation solution should be cooled to at least 5–10 °C below the T_g' and held for at least 1–2 h depending on the fill volume to ensure the complete crystallization of water. Failure to achieve this will potentially result in the upliftment of the cake when vacuum is applied during primary drying as indicated in Fig. 1.

The morphology and size of the ice crystals formed during the freezing phase influence the performance of the subsequent phases of freeze-drying. Both features are dependent on the freezing protocol such as the cooling rates and the annealing time and temperature. Faster cooling rates typically result in higher degrees of supercooling and hence smaller ice crystals and larger specific surface area (SSA), which provides a large interface. So, while faster cooling minimizes the cryoconcentration effect, it also creates a large interfacial area and hence high potential for denaturation of the protein at the ice-solution interface. Also, from a process point of view, smaller-size ice crystals result in small pore size of the dried layer which results in high resistance to water vapor transport during primary drying. In such case, primary drying times will be longer, but secondary drying times could be shorter due to the high SSA in the dried product [20, 23, 24].

Additionally, the freezing protocol influences the physical state of the excipients and their intended role in the formulation. Certain excipients remain amorphous or crystallize depending upon several factors like molecular structure, solubility,

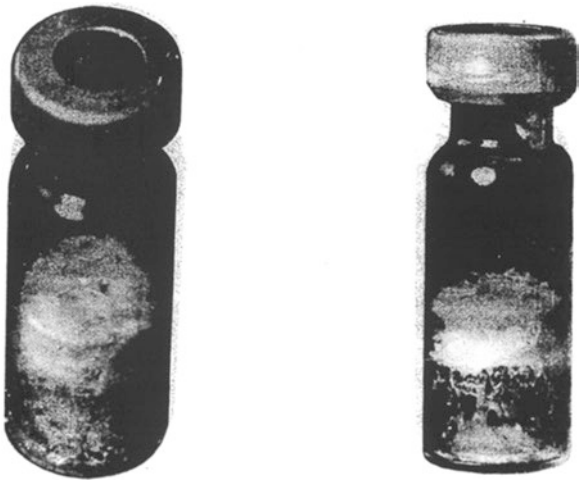


Fig. 1 Example of failure in complete solidification of the formulation during freezing. When material is not fully pre-frozen, bubbles form, and when vacuum is applied, it lifts the rest of the frozen mass. Freezing is completed by evaporative cooling, but the plug is supported in the raised position by a web of solid residue beneath it. Poor thermal contact with the base and shrinkage of the frozen core within the dry cake reduce heat transfer and prolong freeze-drying

concentration, and the presence of other formulation components [25]. For example, sorbitol can remain amorphous or crystallize depending upon the freezing protocol. If the intended role of sorbitol is to stabilize the protein during freeze-drying and upon storage, a certain freezing protocol needs to be followed to ensure that sorbitol remain amorphous and forms a phase within which the protein is molecularly dispersed [4, 26]. Similarly, mannitol may remain amorphous or crystallize. If the intended role of mannitol in the formulation is to serve as a crystalline bulking agent, a specific freezing protocol is employed to enable a near-complete crystallization. (See chapter "Development of Robust Lyophilization Process for Therapeutic Proteins: A Case Study" and Sect. 7 of this chapter.) Partially crystallized mannitol will continue to crystallize during primary drying which may result in vial breakages [27] and/or upon storage which may compromise stability [4]. Furthermore, if the intended role of an excipient is to act as a buffering agent, it is critical that none of its components crystallize upon freezing [28]. In his vein, before designing the freezing regimen to achieve the intended role of each excipient, it is important to characterize the frozen solution of the excipient individually and in the presence of other excipients to understand its freezing properties (see Fig. 2).

3.2 Annealing

As discussed above, specific freezing protocol should be used depending upon the intended physical form of an excipient. One common approach used to ensure the complete crystallization of excipients is annealing. An annealing step involves the thermal treatment of the formulation solution above the T_g' to facilitate near-complete crystallization of the excipients that did not crystalize or crystalized only partially during the first cooling ramp. The temperature and time of maximum crystallization of a particular excipient can be determined in the frozen solutions using real-time X-ray diffraction techniques [30] or by DSC and FDM [25]. Complete crystallization can then be confirmed using several techniques including (1) T_g' annealing temperature curves, (2) the area under the eutectic melting endotherm in a frozen system, (3) the area under the bulking agent melting endotherm in dry powder system, and/or (4) the absence of an exotherm upon heating the dry powder on DSC.

In addition to the complete crystallization of excipients like mannitol, annealing also results in larger ice crystals by Ostwald ripening where large crystals grow at the expense of the smaller crystals when the formulation is annealed at or more than 10 °C above the T_g'. From a process performance prospective, the annealing steps in amorphous systems serve two purposes. First, it eliminates heterogeneity in the product quality arising from the stochasticity of the ice nucleation temperature in the different vials which results in heterogeneity in ice crystal size. Second, the formation of larger-size ice crystals facilitates the mass transfer of water vapor from the sublimation interface through the dried layer of the cake with less product resistance, which renders primary drying faster and decreases the product temperature

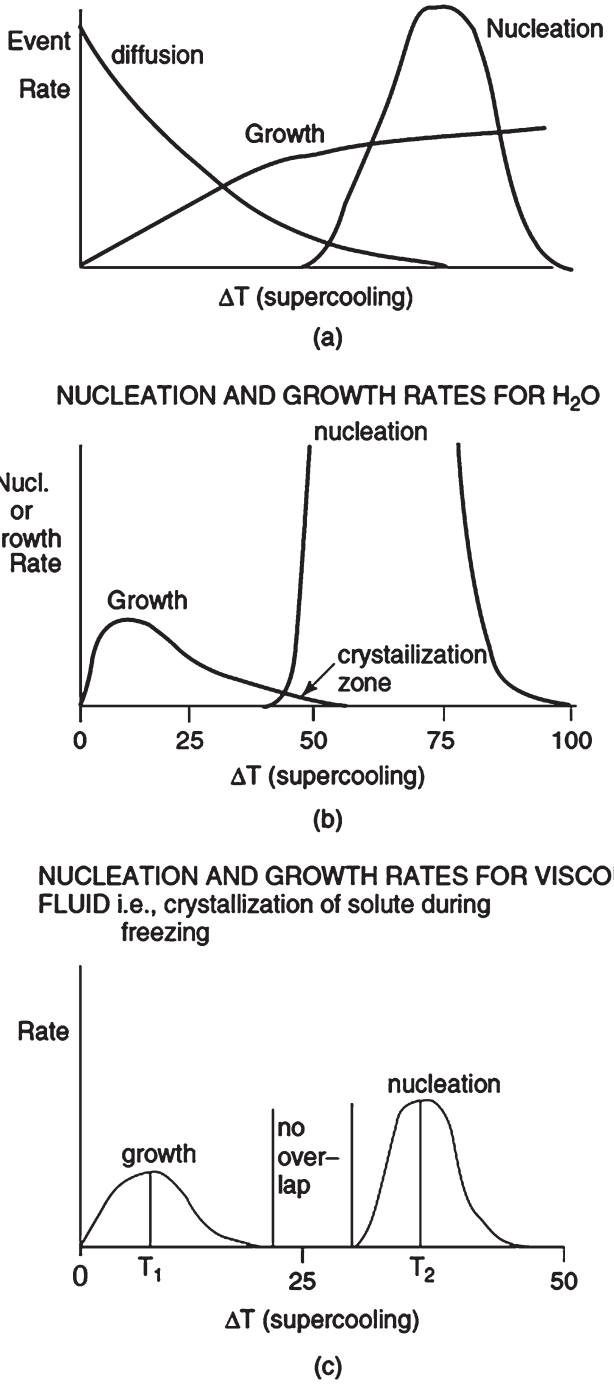


Fig. 2 (**a**) Effect of temperature on nucleation and growth rate. (**b**) Nucleation and growth rate for pure water. (**c**) Nucleation and growth rate for viscous fluid. (Reprinted with permission from Ref. [29])

especially in systems with high solid content [31]. While the annealing step benefits both product quality and process performance, the annealing time should be optimized to minimize the residence time the protein spends in the freeze concentrate where all deleterious effects may occur. The pH can shift due to the selective crystallization of one of the buffer components, and protein-protein interactions and the ionic strength will increase several-folds. For example, if a protein is formulated with 0.15 M NaCl, the concentration of the salt would go up to 6 M in the freeze concentrate (calculated data), which can destabilize the protein [32–34].

The rate of ice crystal growth is a major factor in determining the solidification of ice and the residence time of the product in a freeze-concentrated state. In general, rapid ice growth rate minimizes the residence time of solutes and protein in such state and minimizes the exposure to the adverse conditions of the freeze concentrate such as adsorption to interfaces and high protein and salt concentration. In commercial freeze-drying, heat transfer limits the rate of ice growth. Therefore, in vials and pans where heat removal is through the container bottom, rapid ice growth is facilitated by small fill volume-to-container area ratio (i.e., small fill depth) and good contact between the container bottom and the freeze-dryer shelf. A low shelf temperature also promotes rapid ice growth.

4 The Primary Drying Phase

Following the completion of the freezing phase, the primary drying phase starts by ramping the shelf temperature (T_{sh}) up and the chamber pressure (P_{ch}) down to predetermined values based on the critical product temperature, T_{crit}. As discussed earlier in the text, the main objective of this phase is the complete removal of ice through sublimation while maintaining the temperature of the product below T_{crit}. The choice of T_{crit} for the product usually takes into consideration a safety margin of approximately 2 °C below T_c or T_e. To avoid adjustments in process parameters during the process and to keep the process simple, it is customary to run primary drying at a constant shelf temperature and chamber pressure combination rather than using a variable profile.

4.1 Primary Drying Phase Design

Traditionally, the design of primary drying has been carried out following one of two major practices: empirical (uncoupled) or systemic (coupled) design. The major difference is that the former approach recommends the design inputs independently in an uncoupled way, whereas the latter use a graphical approach to simultaneously determine the design inputs. With the recent emphasis on the application of Quality by Design (QbD) elements to formulation and process development, lyophilization design space has increasingly been used as an efficient process

development tool [6, 35–37]. Deep knowledge of the freeze-drying cycle, however, is usually limited at the early development stage and increases gradually as the project progresses. In this vein, Table 3 summarizes three stages of primary drying phase design in development projects with regard to the gained knowledge of the product and the equipment used for drying.

In Stage 1, only T_{crit}, the solid content of the formulation (c_{solid}%), and the fill volume are known. Therefore, for non-platform formulations, the initial guess approach has been typically employed [38] assuming standard pharmaceutical equipment capabilities. In Stage 2, the process parameters obtained by initial guesses are then adjusted based on the results of Stage 1 experiments. Additionally, estimation of the freeze-drying equipment properties (i.e., the heat transfer coefficient (K_v) and the equipment capability curve) and the product resistance of the formulation (R_p) are done as well. Finally, in Stage 3, the final design is locked in based on knowledge obtained in Stage 2 as well as knowledge of the manufacturing freeze-dryer.

In both Stages 2 and 3, the design space can be computed using the quasi-steady-state 1D model for heat and mass transfer in freeze-drying, which can be represented as:

$$\left[T_{pr,max}, \left(\frac{dm}{dt} \right)_{max}, t_{PriDry} \right] = f\left(T_{sh}, P_{ch}, K_v\left(P_{ch}\right), A_v, l_{pr,0}, R_p, c_{solid}\% \right) \quad (2)$$

where $l_{pr,0} = \frac{V_{fill}}{A_p}$. $T_{pr,max}$ and $\left(\frac{dm}{dt} \right)_{max}$ are the maximum product temperature and sublimation rate, respectively. t_{PriDry} is the primary drying time, A_v is the outer area of the vial cross section, and $l_{pr,0}$ is the initial frozen product thickness.

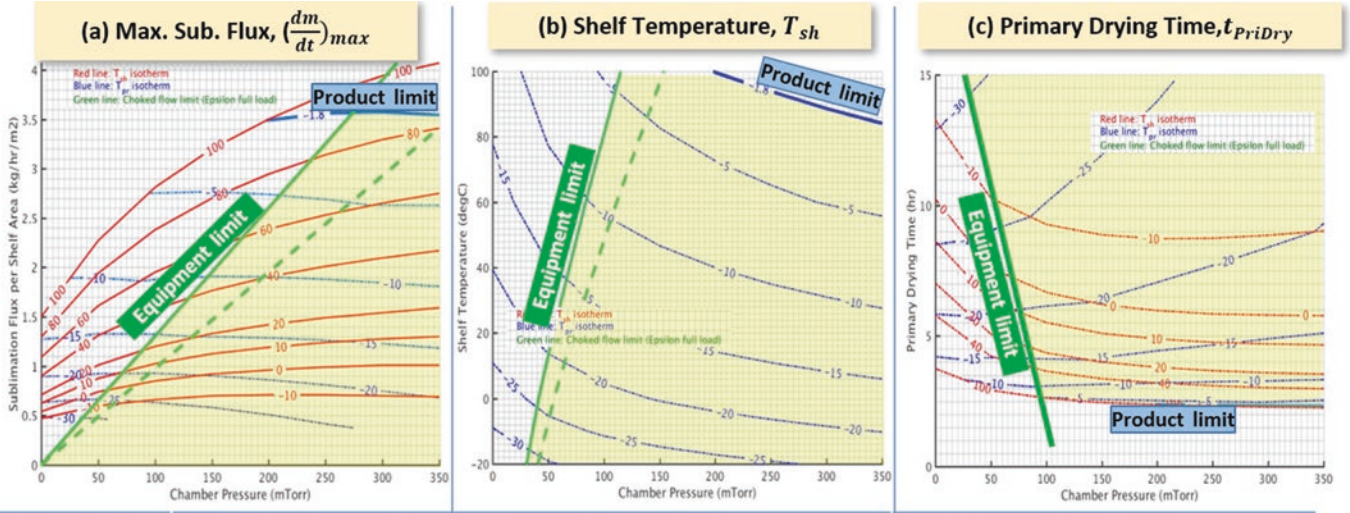
Based on Eq. (2), the design space has multiple input design variables and thus can be constructed in favor of the two key input parameters as the X- and Y-axes in the Cartesian coordinates. Figure 3 illustrates three types of the design space that

Table 3 Summary of the three stages of primary drying phase design

	Stage 1: Initial Guess	Stage 2: Initial Design	Stage 3: Final Design
Inputs needed	T_{crit}, $c_{solid\%}$	T_{crit}, $c_{solid\%}$, K_v (at one P_{ch}), $R_{p,max}$	T_{crit}, $c_{solid\%}$, $K_v = f(P_{ch})$, $R_p = f(l_{ck})$
Tool used	Heat and mass transfer equations for a "target" set-point	Pre-computed[a] matrices of approximate design spaces	Exact design spaces[a]
Accuracy	Low	Moderate	High
Easy to use	Moderate	High	Moderate

Knowledge of the process typically increases significantly moving from Stages 1 through 3. The inputs needed include T_{crit} the critical product temperature, c_{solid}% the solid content of the formulation, $R_{p,max}$ the maximum area-normalized product resistance for a given product, K_v vial heat transfer coefficient, and R_p area-normalized product resistance

[a]Using a Lyo-Calculator [23]



Type of design spaces	Type 1: Sublimation Flux	Type 2: Shelf Temperature	Type 3: Primary Drying Time
X and Y axes choices	X: P_{ch} Y: $\frac{dm}{dtdA}$	X: P_{ch} Y: T_{sh}	X: P_{ch} Y: t_{PriDry}
Contour plot isolines	T_{sh}, T_{pr}	T_{pr}	T_{sh}, T_{pr}
Comments on how to use	Useful for including the equipment capability and avoid choked flow	Useful for the design of an operating condition -- P_{ch}, T_{sh}	Helpful for the estimation of primary drying time, t_{PriDry}

Fig. 3 Illustration of various types of design space created with the following input parameters as the X- and Y-axes (**a**) P_{ch} and *dm/dt/dA* (**b**) T_{sh} and P_{ch} (**c**) P_{ch} and t_{PriDry}. P_{ch} is the chamber pressure, T_{sh} is the shelf temperature, *dm/dt/dA* is the sublimation flux, and t_{PriDry} is the primary drying time

can be created for a formulation with T_{crit} = 1.8 °C in a development freeze-dryer as an example. The X-axis represents the chamber pressure in all the three types of design space diagrams, whereas the Y-axis represents the sublimation flux (Type 1, Fig. 3a), the shelf temperature (Type 2, Fig. 3b), and the primary drying time (Type 3, Fig. 3c), respectively. The Type 1 design space is the most commonly reported in literature for conveniently including the equipment limit (solid green line) as well as the product limit (solid blue line) to form a safe zone of operation (shaded yellow region). On the contrary, the Type 2 design space is the most useful for the design of an operating condition since P_{ch} and T_{sh} are both represented in linear scales. Furthermore, in the initial design stage, small-scale batches with conservative cycle design are typically tested with a minimum risk of reaching the equipment choked flow limit. As such, in the subsequent discussions, we use Type 2 design space for illustration and focus on the product limit.

Figure 4 outlines the typical workflow of the three stages of primary drying phase design. The Initial Guess Design Stage (Stage 1, top flow chart) is based on the work of [38], which has been one of the few practical and widely used guides to both the product and process design of freeze-dried products. However, with regard to the primary drying stage, the approaches described therein have several limitations in terms of condition specificity and operational convenience. Starting from a single design limit posed by the product (i.e., T_{crit}), the choice of the operating conditions (P_{ch} and T_{sh}) is independently recommended using empirical correla-

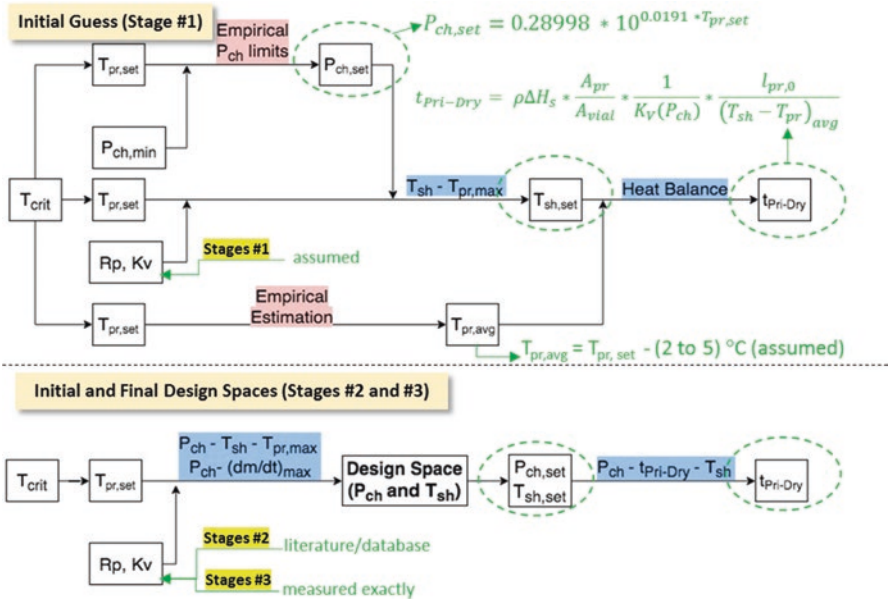


Fig. 4 Comparison of the workflow in the three stages of the primary drying phase design. $T_{pr,set}$, $P_{ch,set}$, and $T_{sh,set}$ are the targeted product temperature, selected chamber pressure, and selected shelf temperature, respectively. $T_{pr,avg}$ is the actual measured average product temperature, and $P_{ch,min}$ is the minimum controllable chamber pressure

tions. For K_v and R_p, empirically selected "average tubing vial K_v" and "solid concentration-based R_p" can be used for initial guesses, which may lead to longer operation time. On the contrary, the Initial and Final Design Stages (Stages 2 and 3, bottom flow chart) are different in that a design space is created using the best estimation of K_v and R_p based on a finite database in Stage 2 (e.g., from literature or prior data) or the exact measurement in Stage 3 (from experimental data). The design space is then used to guide the simultaneous selection of the operating conditions, P_{ch} and T_{sh}, with all design limits considered.

*4.2 A Novel Fast Analytical Design Space (***fast-DS***)*

In this section, we propose a fast "Initial Design" approach for use in Stage 2, in which we set up a matrix of fast design spaces (termed hereinafter as *fast-DS*) using analytical expressions that cover a wide range of K_v and R_p. This approach gives better initial design using moderate information, i.e., only three design inputs: T_{crit}, K_v (at one P_{ch}), and $R_{p,max.}$

Notably, for the purpose of the initial design, we focus on the selection of T_{sh} and P_{ch} rather than the estimation of the primary drying time, which can be efficiently optimized in follow-up runs. In contrast, the choice of T_{sh} and P_{ch} is more critical and is traditionally less optimized after an initial guess is made as was pointed out earlier [38] due to the lack of a non-intrusive measurement of the product temperature that is representative of the entire batch.

In the quasi-steady-state 1D model for heat and mass transfer in the freeze-drying process in vials [23], the total heat transfer rate ($\dot{Q}_{tot}$) to the vial and product is defined as:

$$\dot{Q}_{tot} = A_v K_v \left(T_{sh} - T_b \right) \tag{3}$$

where A_v is the bottom area of the vial, T_{sh} is the shelf temperature, and T_b is the temperature at the bottom of the vial.

The heat conduction rate ($\dot{Q}_{cond}$) in the frozen product is computed as:

$$\dot{Q}_{cond} = -k_{ice} \frac{T_{sub} - T_b}{l_{pr,0} - l_{ck}} A_p \tag{4}$$

where k_{ice} is the heat conductivity of the frozen product, $l_{pr,0}$ and l_{ck} are the initial product and instantaneous cake lengths, and A_p is the bottom area of the frozen product.

The sublimation heat flow rate at the sublimation front of the product ($\dot{Q}_{sub}$) is defined as:

$$\dot{Q}_{sub} = \frac{A_p \left(P_{sub} - P_{ch} \right)}{R_p} \Delta H_s \tag{5}$$

where ΔH_s = 2803 J/g is the sublimation heat of water and

$$P_{sub} = P_{ref} * \exp\left[\frac{\Delta H_s}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T_{sub}}\right)\right] \tag{6}$$

is the Clausius-Clapeyron equation that relates the sublimation pressure, P_{sub}, with the sublimation front temperature, T_{sub}. R = 8.314 J/(mol·K) is the universal gas constant and $P_{ref}(@T_{ref}$ = 233.15 K) = 12.848 Pa is the reference pressure over the ice at the reference temperature, T_{ref}.

This quasi-steady model assumes that most of the heat received goes from bottom to top, and all the incoming heat flow is balanced by the sublimation heat as given by:

$$\dot{Q}_{tot} = \dot{Q}_{sub} \tag{7}$$

If we consider that at the end of primary drying,

$$l_{ck} = l_{pr,0}, R_p = R_{p,max}, T_{sub} = T_{bot} \approx T_{b,max} \tag{8}$$

Then using Eq. (8), an approximate analytical expression for the critical product temperature isotherm of $T_{bot} = T_{crit}$ (in this chapter, it will be called "T_{crit}-isotherm" for short) can be derived where the "critical shelf temperature," $T_{sh,\,crit}$, is a function of the chamber pressure:

$$T_{sh,crit}\left(P_{ch}\right) = \Delta H_s \frac{A_p}{A_v} * \frac{1}{K_v\left(P_{ch}\right) * R_{p,max}} * \left[P_{sub}\left(T_{crit}\right) - P_{ch}\right] + T_{crit} \tag{9}$$

Equation (9) is the key equation for the *fast*-DS which gives $T_{sh,\,crit}$ at a given chamber pressure as the upper limit of shelf temperature that one can operate at without exceeding a product temperature of T_{crit}.

As will be verified next, there is only a very small penalty/error when using the approximate Eqs. (8) and (9), suggesting that this approximation is usually suitable for most popular formulation and filling conditions (i.e., leading to <0.3 °C product temperature prediction). As a result, it has been widely used in several reports in literature [35, 36] in the creation of design spaces without a remark on the presence of the small error.

Verification of the Analytical Product Temperature Isotherm (T_{crit}-isotherm)

Here, we first examine the intrinsic error of using Eq. (9) in the prediction of $T_{b,max}$. There is minimum error if the maximum product temperature, $T_{b,\,max}$, occurs at the end of the primary drying phase. This is the typical case for most purely crystalline-

based formulations where R_p increases with l_{ck} without reaching an apparent plateau. For a typical amorphous-based formulation where R_p increases with l_{ck} and reaches a plateau, however, there is a small error of typically less than 0.5 °C. This is the case mainly for amorphous-based formulations with a high fill height. For example, for the primary drying of a 3.6 ml fill of a sucrose-based formulation in a 20R vial (Fig. 5a), there is a 0.3 °C under-prediction of $T_{b,\,max}$ using Eq. (9).

Figure 5b shows that the analytical isotherm solution (red line) is very close to the standard design space isotherm (thick blue line), which justifies the use of this good enough approximation especially in the early design stages. Furthermore, there is another benefit for using the analytical isotherm solution, which is a much faster computation in creating a complete design space in order to generate a smooth enough isotherm.

Creating the "Fast Design Spaces" (*fast-DS*) Using the Analytical Product Temperature Isotherm (T_{crit}-isotherm)

It is helpful to identify the representative points on this isotherm for various inputs (termed anchor points hereinafter) and then effectively show in one graph the equivalence of multiple design space contours for various K_v and R_p combinations.

Figure 6 shows a schematic of the *fast-DS* and the associated key points. The T_{crit}-isotherm given by Eq. (9) forms one boundary curve for the *fast-DS*, and then two anchor points for this T_{crit}-isotherm can be identified at:

1. Point A: Mass transfer limit at saturation – (Lowest T_{sh}, highest P_{ch}) The limit below which P_{ch} is lower than the T_{crit} such that drying can occur.

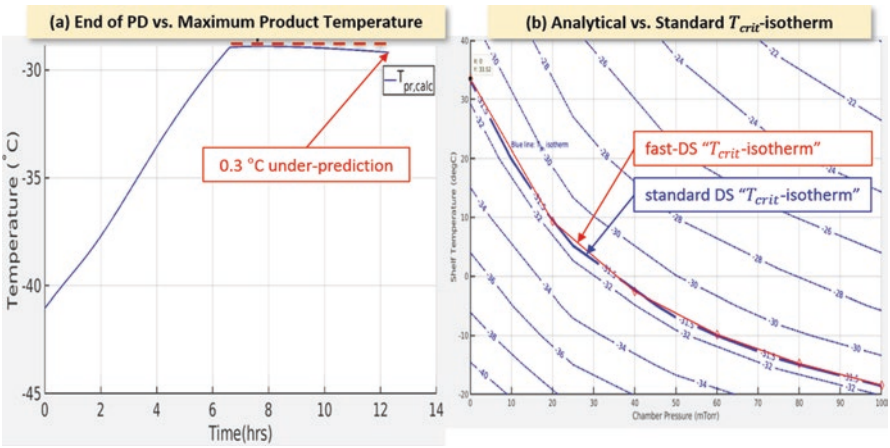


Fig. 5 Verification of the analytical product temperature isotherm by checking as follows: (**a**) product temperature at the end of primary drying and its maximum during primary drying; (**b**) an analytical vs. the standard T_{crit}-isotherm at −31.5 °C

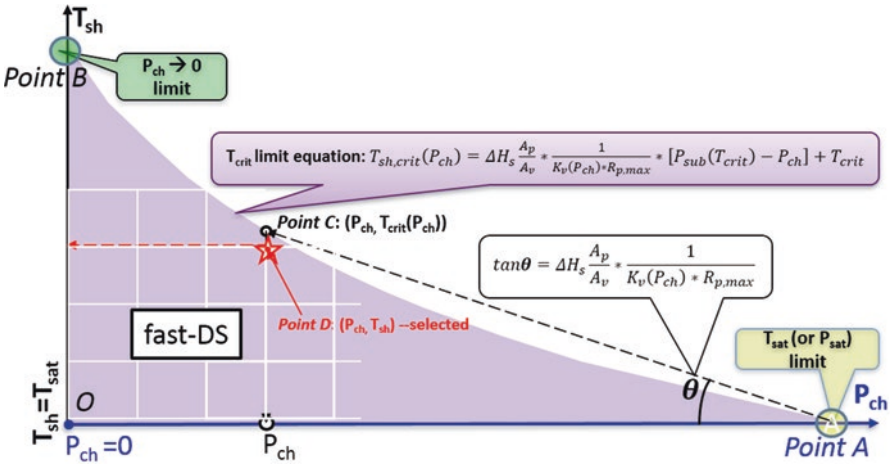


Fig. 6 A *schematic* outline of creating the "fast design space" (*fast-DS*) and identification of associated key points

2. Point B: Heat transfer limit at vacuum (P→0⁺) – The limit below which T_{sh} is lower than the "vacuum critical shelf temperature" $T_{sh,\,crit}(P_{ch} = 0)$ such that T_{crit} is not exceeded.

The corresponding equations for the anchor points are summarized in Table 4.

With these key points identified, the purple-shaded area, $\widehat{OACB}$, is the created *fast-DS* corresponding to T_{crit}. In this work, we assume that the choice of T_{crit} has already taken into consideration a typical safety margin of 2–5 °C below T_c such that it is effectively the boundary of an operational design space. Theoretically, with the safety margin considered in the choice of T_{crit}, we can select the operating conditions right on the T_{crit}-isotherm. In practice, however, it is more convenient to select T_{sh} and P_{ch} values at increments of a grid (shown by the white grid in Fig. 6) of 1 °C and 5 mTorr (or even larger). For example, a chamber pressure P_{ch} is found and the corresponding optimal $T_{sh,\,crit}$ is at "Point C" and then usually "Point D" is selected for practical use.

The Effect of T_{crit}, K_v, and R_p on the Fast Design Space (*fast-DS*)

In this section we consider a design task for a mock-up formulation filled in a 20R tubing vial and freeze-dried in a laboratory freeze-dryer with a three-shelf setting as an example. We call it a "typical condition," which is representative of a commonly used "vial-lyo-product" combination. The related product and process inputs for this condition are summarized in Table 5. It is important to note that only $R_{p,max}$ is needed instead of the entire $R_p = f(l_{ck})$ function. We will create the *fast-DS* for this typical condition and then show the effect of T_{crit}, K_v, and R_p on the *fast-DS* by varying each of them, respectively, from the typical condition and keep all other inputs unchanged.

Table 4 Anchor point limits and the corresponding equations

Anchor point limits	On T_{sh}	On P_{ch}	Equation for the anchor point
Mass transfer limit at saturation	Lowest	Highest	$T_{sh} = T_{crit}$ $P_{ch} = P_{sub}(T_{crit})$
Heat transfer limit at vacuum (P→0⁺)	Highest	Lowest	$T_{sh} = \Delta H_s \frac{A_p}{A_v} * \frac{P_{sub}(T_{crit})}{K_c * R_{p,max}} * + T_{crit}$ $P_{ch} = 0$

Table 5 Product and process inputs for a typical condition

Inputs		Value	Unit
V_{fill}		*1.8*	ml
A_p		*5.98*	cm^2
A_v		*7.07*	cm^2
c_{solid}		*5%*	(% w/v)
T_{crit}		*−31.5*	°C
K_v	K_c	*1.25*	10^{-4} cal/s/K/cm^2
	K_p	*33.20*	10^{-4} cal/s/K/cm^2/Torr
	K_D	*2.52*	/Torr
R_p	$R_{p,max}$	*4.0*	cm^2-Torr-h/g

Part 1: Effect of T_{crit}

As mentioned earlier, T_{crit} for a given formulation is determined based on the collapse/eutectic melting temperature as well as the choice of a safety margin. Figure 7 shows the effect of T_{crit} on the *fast-DS* with both $K_v = f(P_{ch})$ and $R_{p,max}$ fixed at the typical condition. As the X-axis shows the P_{ch} log scale, it is found that the T_{crit}-isotherms for various T_{crit} values appear to be almost straight lines. The T_{crit}-isotherm for the typical condition in Table 5 is highlighted using a dashed gray line. In addition, the T_{sat} limit and a P_{ch} limit are included to form the fast design space of the typical condition. The choice of the P_{ch} limit is specific to the lyophilizer and reflects the equipment capability in terms of vapor transport and pumping down. Since our focus is on the product limit (T_{crit} limit), we assumed a general 50 mTorr in the current work following [38]. As an example, when T_{crit} is increased from −31.5 to −20 °C, the size of the *fast-DS* increased significantly, and $T_{sh,\ crit}$ for the typical condition increased from −10 to 65 °C if P_{ch} = 70 mTorr is selected. It is also seen in both Fig. 7 and Table 4 that T_{crit} alone determines the location of "anchor point A."

Part 2: Effect of K_v

For a given vial and freeze-dryer combination, the pressure-dependent heat transfer coefficient $K_v = f(P_{ch})$ is fixed. With this in mind, we first study the effect of K_v on the T_{crit}-isotherm and in turn the design space.

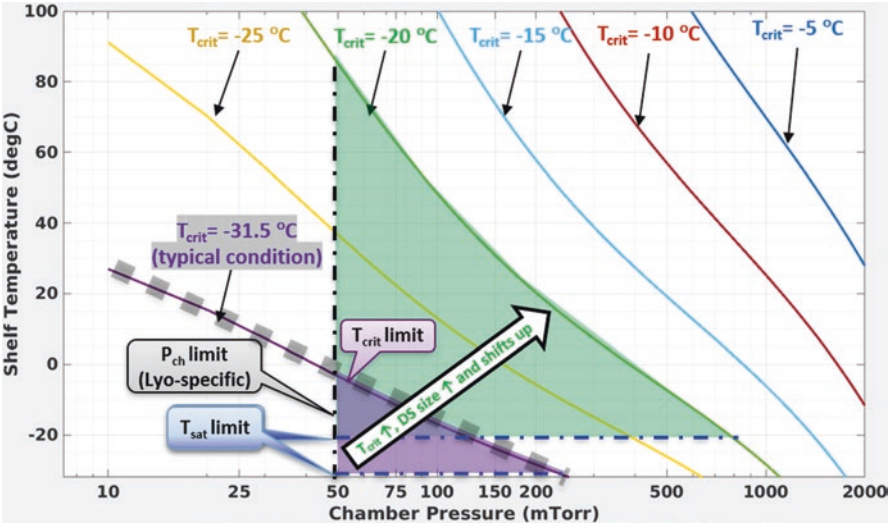


Fig. 7 Effect of T_{crit} on the fast design space. The T_{crit}-isotherm for the typical condition is highlighted using a dashed gray line

The values of K_v depend on the vial, the freeze-dryer, and the process conditions. In the present work, K_v is defined with respect to $T_{sh} = T_{inlet}$ in Eq. (3) based on the fluid inlet temperature (instead of the shelf surface temperature) and is dependent on P_{ch} as given by [23, 39]:

$$K_v = K_c + \frac{K_p \bullet P_{ch}}{1 + K_D \bullet P_{ch}} \tag{10}$$

where K_C is the pressure independent contribution from radiation and the contact between the vial and the shelf/tray and K_p is the parameter expressing the pressure dependence of heat transfer coefficient for water vapor. $K_D = 77.4 l_{bot}$ (cm) is another parameter expressing the pressure dependence of heat transfer coefficient specific to a vial geometry, specifically l_{bot}, which is the effective gap distance between the vial bottom and the shelf/tray. The effects of K_C, K_P, and K_D on K_v components and the major factors that influence them are summarized in Table 6.

Figure 8 parts (a) to (c) show the effects of K_C, K_P, and K_D on the *fast-DS*, respectively, with both T_{crit} and $R_{p,max}$ fixed at the typical condition. From Fig. 8 and Table 4, it is found that T_{crit} and K_C together determine the location of the "anchor point B." Figure 8a shows that the increase of K_C leads to the decrease of $T_{sh,\,crit}$ or the "compression" of the design space with the anchor point A unchanged. Figure 8b shows that the increase of K_P also leads to the decrease of $T_{sh,\,crit}$. However, in this case the design space is not compressed but only lessened with a more curved T_{crit}-isotherm with both anchor points A and B unchanged. For example, when K_P changes as a result of switch from purely water vapor to nitrogen, $T_{sh,\,crit}$ for the

Table 6 Effect of the K_C, K_P and K_D components on K_v and the design space

K_v coeff.	Effect on K_v components	Major factor changes that will increase it	Effect on the T_{crit}-isotherm/ design space
K_c	Solid contact, radiation	Larger nominal contact area Higher shelf-vial solid contact heat conductivity Higher shelf emissivity	Determine anchor point B
K_P	Gas conduction	Higher gas heat conductivity Higher gas heat accommodation coefficient	Determine the curvature of the T_{crit}-isotherm ($\widehat{ACB}$)
K_D	Gas conduction	(Besides all that affects K_P) larger vial bottom curvature	

typical condition decreases from −10 to −5 °C if P_{ch} = 70 mTorr is selected. In Fig. 8c, we found that the decrease of K_D has a similar effect as the increase of K_P on $T_{sh,\,crit}$ and the design space. Particularly, we studied l_{bot} ranging from 0.003 cm (effectively flat bottom vial) up to 30 cm (extreme case of a suspended vial) to show the effect of K_D over the entire possible range. For example, when K_D changes as a result of switching from *Schott*® ISO 20R vial (l_{bot} = 0.03 cm) to Wheaton-50 M vial (l_{bot} = 0.09 cm from [39]), $T_{sh,\,crit}$ for the typical condition decreases from −10 to −7 °C if P_{ch} = 70 mTorr is selected.

Part 3: Effect of R_p

R_p is the area-normalized resistance of the dried layer of the cake to the flow of water vapor subliming from the frozen layer beneath, and $l_{pr,\,o}$ is the initial length of the frozen product. R_p has a typical hyperbolic dependence on the cake length as follows [40]:

$$R_p\left(l_{ck}\right) = R_0 + \frac{A_1 * l_{ck}}{1 + A_2 * l_{ck}} \tag{11}$$

where, R_0, A_1 , and A_2 are the three fitting parameters for R_p and $l_{ck} = l_{pr,\,o} - l_{pr}$ is the cake length. R_p can be obtained by methods such as fitting to the product temperature history [6, 41].

Figure 9 shows the effect of $R_{p,max}$ on the *fast-DS* with both T_{crit} and $K_v = f(P_{ch})$ fixed at the typical condition. When the frozen product of a given formulation with various $R_p = f(l_{ck})$ are considered, anchor point A is not changed for this group of product temperature isotherms for all these frozen products since they all correspond to a same T_{crit} for this formulation. Furthermore, from the "illustrative drawing" of the *fast-DS*, we see that the critical shelf temperature at any given pressure $T_{sh,\,crit}(P_{ch})$ is inversely proportional to $R_{p,max}$. Therefore, the effect of R_p can be recognized as a linear compression factor that "compresses" the fast design space. An example use of this knowledge is given in the subsequent section.

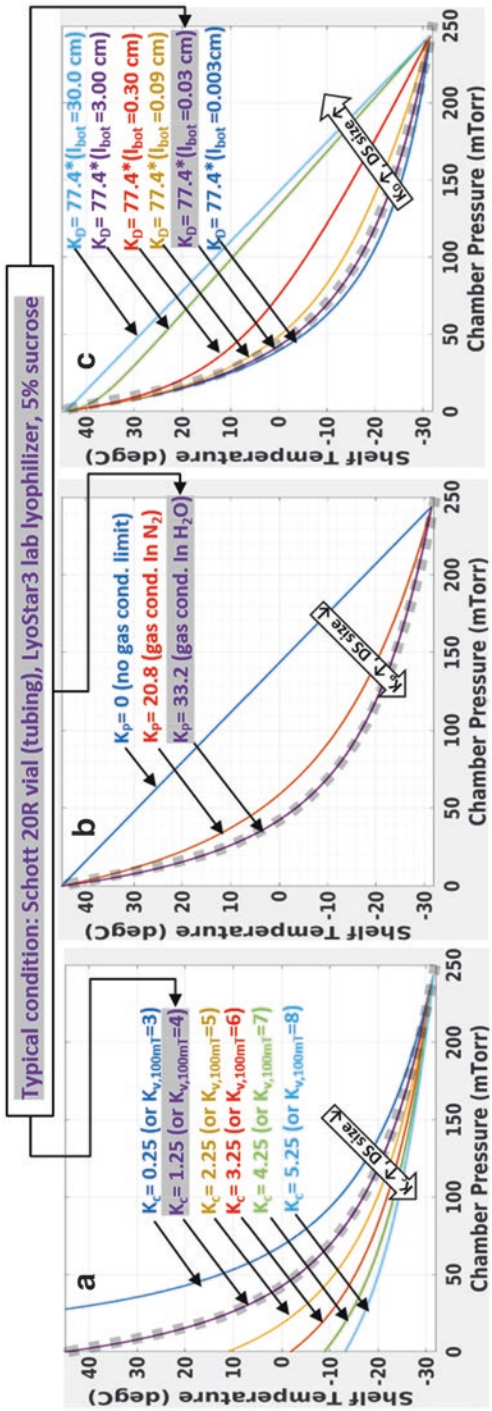


Fig. 8 The effect of various coefficients in the expression of K_v (Eq. 10) on the fast design space: (**a**) K_c in the unit of [10^{-4} cal/s/K/cm^2], (**b**) K_p in the unit of [10^{-4} cal/s/K/cm^2/Torr], and (**c**) K_D in the unit of [/Torr]. The T_{cri}-isotherm for the typical condition is highlighted using a dashed gray line

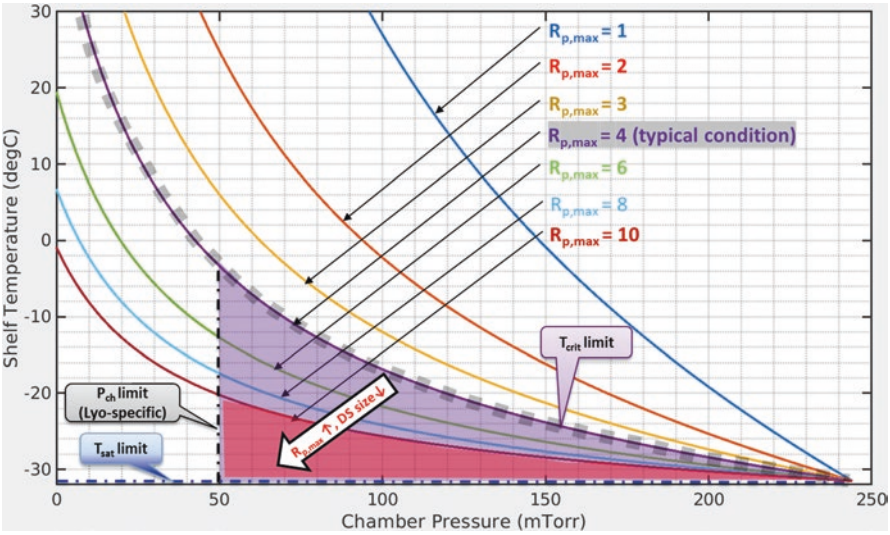


Fig. 9 Effect of $R_{p,max}$ on the fast design space, where $R_{p,max}$ is in the unit of [cm^2-Torr-h/g]. The T_{crit}-isotherm for the typical condition is highlighted using a dashed gray line

Example Use of the *fast-DS*: A Practical Guide

The next task in the *fast-DS* approach is to come up with proper initial estimation of $K_v = f(P_{ch})$ and $R_{p,max}$ in a case-by-case approach. It is important to realize that we are in the initial design stage and only need reasonably approximated $K_v = f(P_{ch})$ and $R_{p,max}$ (instead of accurate measurement, which will only be possible after a cycle is run and optimized). Specifically, K_v and R_p are both functions as were discussed in the preceding sections. We consider the following two cases:

Case 1 If there is a similar product/process condition that was studied before where $K_v = f(P_{ch})$ and R_p (and then $R_{p,max}$) has been obtained in the forms of Eqs. (10) and (11), they can be directly used for this Initial Design stage.

Case 2 Otherwise, K_v and R_p can be approximately estimated using interpolation or extrapolation with available databases for similar formulation and equipment/process conditions.

For Estimating $K_v = f(P_{ch})$

At the minimum, the K_v value at a given operating pressure, P_0, from prior knowledge is available. Figure 10a shows a small database of $K_v = f(P_{ch})$ developed [6] for the popular *Schott*® ISO 6R and 20R vials on various lyophilizers that can be used to help our initial evaluation. It is found from data regression analysis that the varia-

tion of the pressure-dependent K_v across lyophilizers is mostly due to the difference in K_C and that K_D is almost identical for the 6R and 20R vials. Therefore, we assume that K_D is not much different for typical pharmaceutical vial/lyophilizer combinations, and then an initial evaluation of the unknown $K_v = f(P_{ch})$ of interest can be given by:

$$K_v\left(P_{ch}\right) = K_v @ P_0 + \left(\frac{33.2 \bullet P_{ch}}{1 + 2.52 \bullet P_{ch}} - \frac{33.2 \bullet P_0}{1 + 2.52 \bullet P_0}\right) \tag{12}$$

Equation (12) was found to be a good initial guess of K_v for *Schott*® ISO 6R and 20R vials, noting that the possible differences in the contact and radiative component of K_v are both captured in the difference in K_c with the input of K_v @ P_0.

For Estimating $R_{p,max}$

Since the solid concentration and the multiple freezing/drying process conditions that determine the porous structure of the cake can vary a lot, we have to consider only the most critical factor, c_{solid}%, for our study of interest in the initial design. We use a database compiled from a few literature reports that show the effect of solid concentration to guide our initial estimation of $R_{p,max}$. Specifically, the popular amorphous, sucrose-based formulation that covers a typical range of conditions (c_{solid}% between 5% and 15%, uncontrolled nucleation) is considered [42] and is shown in Fig. 10b. Note that there are other factors that affect R_p besides c_{solid}% as evidenced by the variation of R_p for the 5% (w/v) of sucrose in this database. Here, an approximate average of $R_{p,max}$ = 4 [cm^2-Torr-h/g] is adopted for 5% (w/v) of sucrose at a fill height of 0.3 cm. As such, the typical condition in Table 5 is represented by the round symbol "1."

In one example, we study a high solid concentration case with 15% (w/v) sucrose where it is known that the higher solid concentration (c_{solid}%) is the critical factor that with lead to higher R_p. We assume a fill height similar to the typical condition at 0.3 cm, and based on Fig. 10b, we find that $R_{p,max}$ is between 9 and 10 [cm^2-Torr-h/g] (shown by the star symbol "2"). A safe approach is to conservatively round up $R_{p,max}$ to, for example, 10 [cm^2-Torr-h/g] for this 15% (w/v) condition. Compared to the standard 5% (w/v) of sucrose formulation, we find from Fig. 9 that the design space for this 15% (w/v) sucrose formulation is compressed from the purple to the red shaded area. For example, at P_{ch} = 70 mTorr, $T_{sh,crit}$ dropped significantly from −10 to −23 °C and requires set-point adjustment.

In a second example, we study a higher solid concentration case with 10% (w/v) sucrose but lower fill height than the typical condition at 0.1 cm (shown by the square symbol "3"). In this case, since $R_{p,max}$ is 4 [cm^2-Torr-h/g] and is equal to that of the typical condition, we expect the design space for this formulation to be identical to that of the typical condition. This example reinforces the fact that it is $R_{p,max}$ instead of c_{solid}% alone that determines the design space, which should be used to guide the Initial Design stage.

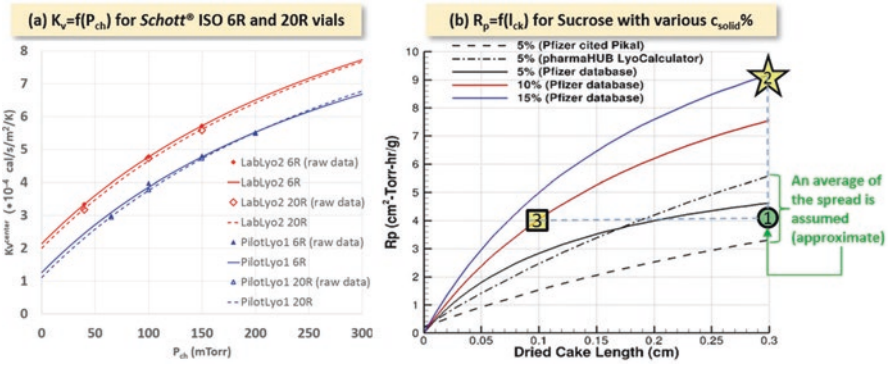


Fig. 10 (**a**) $K_v = f(P_{ch})$ database developed for the popular Schott vial on various lyophilizers [6] and (**b**) $R_p = f(l_{ck})$ database for sucrose with various c_{solid}% [42]

Finally, with these initial estimations of K_v and $R_{p,max}$, we can create the fast easily by plotting the curve from Eq. (9). Since it is an analytical expression, MS Excel® or code-based plotting tools such as MATLAB® or Python can be used.

Comments on the Benefit of the New "*fast-DS*" Tool in the Initial Design

With the pre-computed matrices of *fast-DS*, we can make fit-for-purpose quick design for a new cycle development task. It can also help in the later "final design" stage by visualizing the impact of the scale-up process (where K_v and R_p are changed), as well as the impact of uncertainty in K_v and R_p for a given cycle. Once several successful drying experiments have been conducted using the guidance of the *fast-DS*, the accurate heat and mass transfer coefficient of the process ($K_v = f(P_{ch})$, $R_p = f(L_{ck})$) can then be obtained for the Final Design stage using the Lyo-Calculator [23] as well as Eq. (2).

4.3 Determination of the Endpoint of Primary Drying

After completing the selection of T_{sh} and P_{ch} using the design space proposed in Sect. 4.2, the actual completion time of the primary drying stage should be estimated as the premature advancement to the secondary drying phase poses the risk of melt-back and batch failure. Determination of the endpoint of the primary drying phase is critical for the design and optimization of the process and for large-scale manufacturing. The length of the primary drying phase is determined by the ice sublimation rate, which is dependent on P_{ch}, T_{sh}, K_v, the fill volume, and R_p. Ideally, the primary drying time, t_{PriDry}, can be predicted by Eq. (2) in Sect. 4.2. However, the

preceding input parameters show variability across a batch of product, especially for R_p due to the nucleation heterogeneity during the preceding freezing stage; therefore, t_{PriDry} for the individual vials shows variability, as well. In the following discussion, the primary drying endpoint refers to the completion time for an entire batch of vials to finish the primary drying stage.

There are several commercially available process analytical technologies (PAT) that are used to determine the end of the primary drying phase in single vials or for the entire batch [43]. These methods are based on the measurements of physical quantities related to the heat or mass transfer and the changes in gas composition within the drying chamber. The single vial methods provide useful information on the heterogeneity within the freeze-dryer, whereas the batch methods are more representative and hence useful for process scale-up and technology transfer. In this section, the commonly used PAT to determine the end of primary drying are briefly discussed.

Single Vial Methods

Single vial methods are often heat transfer-based temperature sensors placed inside the product vials, which in most cases are not compatible with production equipment and sterility requirements. Their use is typically limited to the process development phases due to the fact they are not representative of the entire batch.

Wired thermocouples and resistance thermal detectors (RTDs) are commonly used in the development laboratories due to their low cost and the fact that sterility is not required. They are placed so that they measure the temperature at the bottom center in vials at selected locations across the shelves of the freeze-dryer. At the end of primary drying when the cooling effect of ice sublimation decreases, the product temperature increases and converges onto that of the shelf temperature. While indicative of the end of primary drying, the sensors serve as ice nucleation sites per se, which results in higher ice nucleation temperature during the freezing phase compared to the un-probed vials, which leads to the formation of larger ice crystals and lower R_p. As such, this method is not representative of the whole batch. Compared to thermocouples, RTDs are more suitable for use in production freeze-dryers due to their compatibility with the sterilization process, but they are larger in size and measure the temperature over the sensor surface area rather than a single point, which renders the reading error larger.

Temperature remote interrogation system (TEMPRIS) is a wireless battery-free sensor that is used to measure T_p at the bottom of the vial. The temperature values measured by TEMPRIS have been found to be in good agreement with the those measured using standard thermocouples and Manometric Temperature Measurements (MTM) (see section "The Residual Gas Analyzer Mass Spectrometer (LYOPLUS™)") [44]. Compared to wired sensors, TEMPRIS are more suitable for use in the sterile manufacturing environment; however, TEMPRIS and other similar wireless sensors are similarly non-representative of the rest of the batch due to the induction of ice nucleation at higher temperatures.

Batch PAT Methods

Several PAT methods that are used to monitor and control the freeze-drying process can also be used to indicate the completion of primary drying of the whole batch. Examples of these technologies include Pirani vs. capacitance gauge readings convergence, pressure rise test, and partial water vapor pressure measurements.

Capacitance Manometer and Pirani Gauge

Capacitance manometers (e.g., MKS Baratron gauge) are typically used to measure and control the pressure in the chamber and the condenser during drying. Pirani gauge pressure measurements are based on the thermal conductivity of the gas and are dependent on the gas composition. During steady-state primary drying where the chamber is predominantly filled with water vapor, the Pirani gauge reads at higher values because the thermal conductivity is approximately 1.6 times higher than nitrogen. At the end of primary drying, the reading of the Pirani gauge gradually decreases until it converges with that of the capacitance manometer when the gas inside the chamber is predominantly nitrogen. This method is the most commonly used in manufacturing freeze-dryers because it represents the entire batch and because sterilizable Pirani gauges are relatively cheap.

Dew Point Monitor

Dew point monitors are electronic moisture sensors that measure the dew point temperature at which water starts to condense from the gas phase on a colder surface. Like the pressure value measured by the Pirani gauge, the dew point temperature decreases as the composition of the gas becomes predominantly nitrogen; thereby, it can be used to determine the end of primary drying of the batch. Additionally, water desorption during secondary drying can also be measured by a small increase in dew point temperature. Figure 11 demonstrates good agreement in the determination of the primary drying phase endpoint as indicated by Pirani gauge pressure and by dew point sensor measurements. Compared to the convergence of the pressure measurements of the capacitance manometer and Pirani gauge, the dew point measurement has been reported to be more sensitive [45].

The Residual Gas Analyzer Mass Spectrometer (LYOPLUS™)

The residual gas analyzer is a relatively new PAT that uses a quadrupole mass spectrometer that determines the composition of the gas inside the drying chamber [46]. Using this technology, the endpoint of primary drying can be determined by monitoring the concentration of water and nitrogen inside the chamber. Due to its high

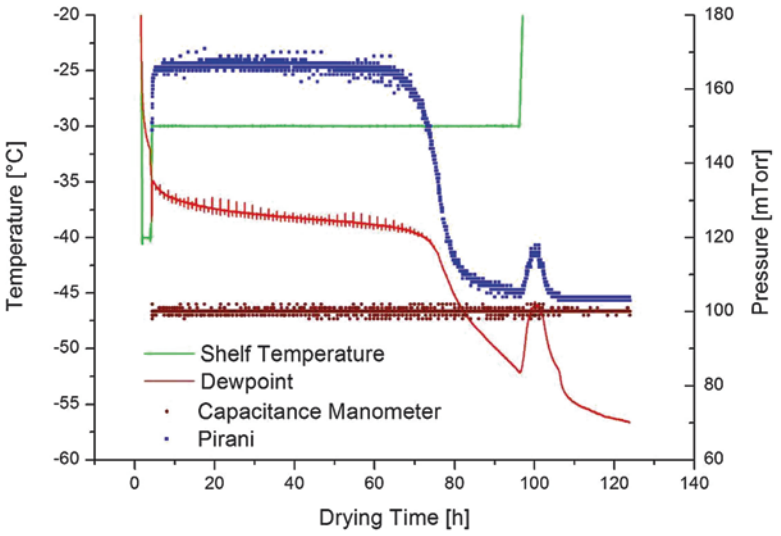


Fig. 11 Comparison of primary drying phase endpoint detection using both Pirani gauge and dew point sensors. (Reprinted with permission from Ref. [43])

cost, the use of the residual gas analyzer to determine the end of primary drying does not add more value to the use of the conventional pressure rise or the comparative pressure tests (see next section). Nevertheless, the residual gas analyzer is a very useful technique to detect leaks and ingress of gases and solvents from the heat transfer fluid, the vacuum pump oils, or the cleaning solvents and is hence use for this purpose.

Pressure Rise Techniques

The basic principle of the pressure rise test (PRT) is that when the valve in the duct connecting the drying chamber and the condenser is closed for approximately 30 s, the pressure rises in the chamber following a specific pattern during the drying. At the end of primary drying, the pressure rise during the closure time of the valve will decrease and will indicate the end of primary drying when the maximum pressure rise value is less than a certain predetermined threshold. Similar to the comparative pressure test, the PRT has been commonly used in manufacturing freeze-dryers because it is straightforward to use and represents the entire batch.

The pressure rise data collected over the period of 30 s can also be used to mathematically compute the batch average temperature and vapor pressure of ice at the sublimation interface. This technique is called Manometric Temperature Measurement (MTM) and is commonly used in development laboratory freeze-dryers to determine T_p and R_p and to optimize the drying cycle. Since the pressure

rise pattern is determined by R_p, ice sublimation area, and chamber volume, the use of the MTM technique is limited in large-scale freeze-dryers due to the large volume of the chamber relative to the sublimation area especially when a partial load is used.

The accuracy of the values calculated using MTM calculations can have some limitations. Generally, the accuracy of these values is limited to approximately two thirds of the drying phase after which the pressure rise is significantly reduced, which renders the calculations more erroneous. In addition, error in the vapor pressure data may occur due to the tendency of the amorphous lyophilizates to re-absorb water vapor during the closure time [44].

Another technique that is based on the pressure rise method is the thermodynamic lyophilization control (TLC). In this technique, the valve between the chamber and the condenser is closed, but only for 3 s, and the pressure rise is used to calculate T_p at the sublimation front. Such short measurement time in TLC eliminates the risks of warming the product up and the reabsorption of water vapor that may occur during MTM. Similar to the MTM-based SMART® technology, the calculated temperature using TLC can be used in a feedback system to automatically adjust the process and optimize the drying time.

Gas Plasma Spectroscopy (Lyotrack)

Gas plasma spectroscopy (Lyotrack) measures the concentration of water vapor in the drying chamber at pressures ranging from 4 to 400 mTorr using a cold plasma source and can determine the endpoint of both primary and secondary drying [47]. The device consists of a plasma generator and an optical spectrometer. The plasma generator ionizes the gas present in the chamber, and the spectrometer analyzes the gas species based on the wavelength-dependent fluorescence emitted by the ionized gas. One advantage of the Lyotrack sensor is that it can be easily calibrated against a reference system and readily implemented into existing freeze-dryers. It is also compatible with sterilization in place (SIP) and cleaning in place (CIP) procedures and has good measurement sensitivity that allows detection of ice in less than 1% of the vials [48]. The broader applicability of this technique, however, is restricted due to it creates free radicals that can negatively impact the stability of the product by inducing oxidation. This effect is especially important when drying oxygen-sensitive products but can be moderated or eliminated by installing the device in the spool that connects the chamber to the condenser instead of placing it in the chamber. Since the gas composition profile of the Lyotrack is the same as the pressure profile measured by a Pirani gauge, it has a small added value.

Tunable Diode Laser Absorption Spectroscopy (TDLAS)

The absorption of electromagnetic waves of specific wavelength by gas molecules can be used to determine the concentration of a specific gas. The tunable diode laser absorption spectroscopy (TDLAS) is a method that applies this principle by using

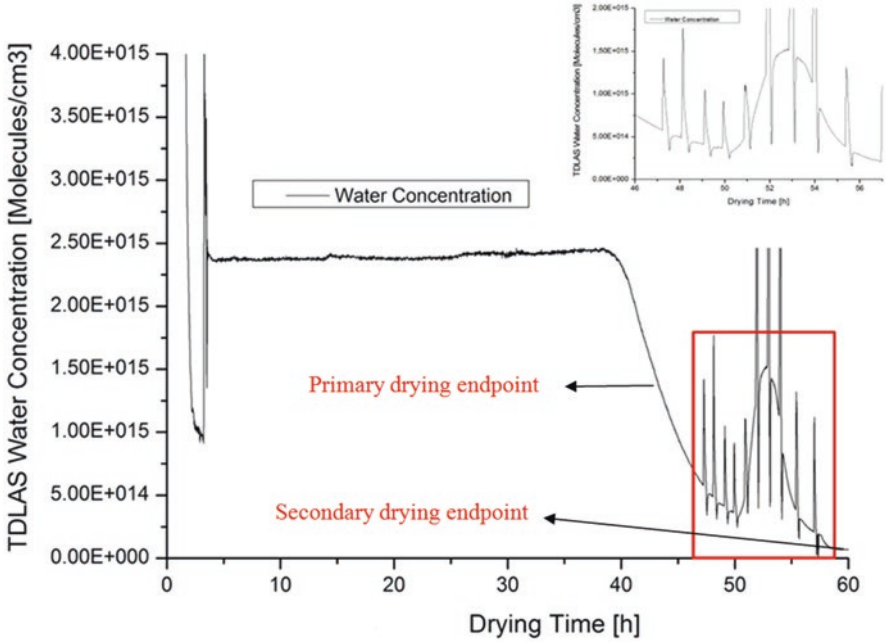


Fig. 12 TDLAS water vapor concentration temporal measurement profile during lyophilization of 5% w/w sucrose in a laboratory scale dryer. (Reprinted with permission from Ref. [43])

near-infrared absorption spectroscopy to directly measure the concentration and velocity of water vapor in the duct connecting the drying chamber to the condenser [49]. These measurements are then used to calculate the instantaneous mass flow rate (dm/dt), which can then be integrated over time to determine the total amount of water removed from the vials as function of time and hence can be used to determine the end of primary drying when the TDLAS water concentration drops. As the sublimation of ice is near completion, the composition of the gas in the drying chamber changes from nearly all water vapor to nearly all nitrogen and a sharp drop in the TDLAS water concentration curve can be observed. The inflection point of the curve can be used as the endpoint of primary drying. An example of TDLAS data is illustrated in Fig. 12. TDLAS is suitable to be used for freeze-dryers at all scales and is useful also in determining batch average value of K_v as well as R_p of the drug product.

5 Secondary Drying

At the end of the primary drying, approximately 5–20% of water remains bound to and dispersed throughout in the dried matrix. The residual moisture content at the end of primary drying would be relatively high or low if the formulation is amor-

phous or crystalline in nature, respectively. Therefore, the main objective of the secondary drying is to remove the bound water by evaporation to bring the moisture content to less than 1%.

The high moisture content present at the end of primary drying renders the T_g low, which may result in cake collapse as the shelf temperature is ramped up to the set-point of the secondary drying phase. A common approach to overcome this issue is to implement a slow temperature ramp rate of 0.2–0.5 degrees per minute, depending on the physical nature of the formulation (i.e., amorphous vs. crystalline).

The shelf temperature for secondary drying depends on the nature of the product. For example, high temperature is usually required for mannitol in order to convert the hemihydrate form into the anhydrous form [50]. Traditionally, the chamber pressure is lowered in the secondary drying phase. Nevertheless, the same pressure of the primary drying phase can also be used for secondary drying [23]. The time required to complete the secondary drying phase can be determined by pulling vials at different time points during the cycle using a sample sieve and test them for moisture content. In order to achieve suitable uniformity of moisture across the cake and between the vials, it is recommended to conduct secondary drying at relatively low temperatures and for longer time than at very high temperatures for short time.

6 Considerations for Scale-Up and Manufacturing Challenges

The main cause for scale-up and technology transfer challenges of a freeze-drying process is the lack of understanding of the differences between the laboratory and the manufacturing freeze-dryers and the failure to consider them during the development of the process. The major differences are in the equipment design, the environment (laboratory vs. class 100/A area), and the load size (fractional vs. full load). For the equipment design, differences in the condenser design and/or volume, the refrigeration system, the geometry design and location of the spool connecting the chamber to the condenser, the valves (solenoid vs. PTD), and booster pumps impact the minimum controllable pressure at the maximum sublimation rate (also termed as the choke flow limit) of the freeze-dryer. In addition, variations in the dryer chamber design, especially the flow pattern of the heat transfer fluid through the shelves, and the walls and shelves polish (emissivity) impact K_v. As for the manufacturing environment, differences in the cleanliness of the environment (i.e., the amount of particles) impact the degree of supercooling and the nucleation temperature which in turn impacts R_p, T_p, and the drying time (i.e., in the manufacturing clean room environment, ice nucleation temperature will be lower, and the product will dry slower and warmer). The product load size (fractional vs. full load) is another factor that needs to be considered as it impacts the fraction of the vials constituting the edge vials and the composition of the gas in the chamber which will impact the drying process differently.

Due to these differences, there should be complete characterization of both freeze-dryers: the development freeze-dryer where the process will be developed and the manufacturing freeze-dryer where the process will be scaled up and transferred for manufacturing. The equipment capability, i.e., ability to control minimum chamber pressure at the maximum sublimation rate, can be determined experimentally through ice slab sublimation experiments [51] or modeled using computational fluid dynamics (CFD) simulations [6, 52–54] if the geometry of the freeze-dryer is known. In addition, the shelf temperature mapping of the hot and cold spots across the shelf and between shelves and the K_v as a function of P_{ch} can be determined using water sublimation tests as reported previously [51, 53].

In addition to equipment characterization, the effect of particle-free environment on the nucleation temperature and in turn on R_p can be characterized following two approaches. The first approach is to determine R_p of the formulation using MTM at different nucleation temperatures using controlled ice nucleation technology to create a correlation curve between R_p and the nucleation temperature and then estimate R_p for the manufacturing environment by extrapolation using an arbitrary nucleation temperature of −20 °C. The second approach is to measure the SSA of the cake that was lyophilized under particle-free environment using the BET method and then relate SSA to R_p [55] following the correlation curve in the first approach. Using this approach, R_p can be determined more accurately instead of assuming a nucleation temperature in the manufacturing environment. Nevertheless, this approach requires having access to vials from previous batches from the same manufacturing freeze-dryer. In addition, several vials have to be tested to cover the heterogeneity in R_p especially for cycles where annealing was not used.

Finally, the information from the equipment characterization and the formulation R_p can then be used as input parameters for first-principle predictive models in order to generate a design space of the formulation and the process for each specific freeze-dryer as discussed in Sect. 4.

7 Case Study

In this section, we discuss a case study that demonstrates the interdependence of formulation composition and the drying process parameters. The case study has been published earlier in [29] and is reprinted here with permission. Another case study demonstrating the rational design of a freeze-drying process is presented in chapter "Development of Robust Lyophilization Process for Therapeutic Proteins: A Case Study" of the book.

The case study described herein illustrates a systematic investigation aimed at developing a freeze-drying process for a protein formulation with challenging thermal properties, which required specific freezing and secondary drying protocols. In the first attempt to freeze-dry this protein using a standard formulation and drying process, the protein demonstrated instability during the freezing and upon storage.

To mitigate this issue, different formulations including various combinations of stabilizers and bulking agents were evaluated. Based on the initial accelerated stability data, the following formulation was selected for further development: 0.1 mg protein, mannitol (bulking agent), trehalose (stabilizer), polysorbate 80, and a small amount of NaCl at pH 7.0.

To determine the optimal annealing temperature for mannitol crystallization, MDSC and FDM were used to characterize the freezing behavior of the formulation. These investigations showed that mannitol can be crystallized at or below −26 °C (see Fig. 13) presumably due to low viscosity and protein concentration. Nevertheless, the T_g' of the formulation was found to be −53 °C. As a result, the first attempt to freeze-dry the formulation resulted in non-elegant product probably due to incomplete crystallization of mannitol as evidenced by the exotherm observed in the non-reversible heat flow signal of the MDSC thermogram of the dried powder as well as the low temperature glass transition observed in the reversing heat flow signal (see Fig. 14), which indicate that the high T_g of trehalose was suppressed by the presence of amorphous mannitol.

The reason for the incomplete crystallization of mannitol and the low T_g' was found to be due to NaCl, which has been found to depress the T_g' of sucrose and interfere with the crystallization of mannitol. In addition, X-ray powder diffraction of the dried powder showed that mannitol hemihydrates are present (see Fig. 15). In order to address these issues, NaCl has to either be removed from the formulation or completely crystallize NaCl before annealing mannitol so that it does not interfere with the crystallization of mannitol. Due to limitations on changing the drug substance process, removing NaCl from the formulation was not considered leaving the crystallization thereof as the only viable option. Accordingly, the crystallization of NaCl was systematically investigated in the presence and absence of the other excipients using MDSC and FDM in order to determine the minimum concentration required for crystallization, the nucleation temperature, and the optimal annealing temperature and time. The results of these investigations showed that a minimum concentration of 150 mM NaCl and freezing below −50 °C for at least 1 h before annealing at −35 °C for 2 h are required to achieve complete crystallization (see Fig. 16).

In order to crystallize NaCl first before crystallizing mannitol, the freezing protocol had to be modified as follows: the freezing temperature was changed from −40 to −50 °C, and annealing at −35 °C for 2 h was performed to enable the crystallization of NaCl prior to the annealing step at −26 °C to crystalize mannitol. In addition to the modified freezing protocol, the secondary drying temperature was changed to 50 °C in order to eliminate the hemihydrate form of mannitol. Using this optimized cycle, elegant lyophilizate (see Fig. 17) with completely crystalized mannitol and a high T_g was obtained as evidenced by the absence of the mannitol crystallization exotherm in the MDSC thermogram and by the increase in the T_g from 64 to 86 °C (Fig. 18). Moreover, characterization of the lyophilizate using X-ray diffraction showed that the degree of crystallinity of both NaCl and mannitol increased and that the hemihydrate form is absent.

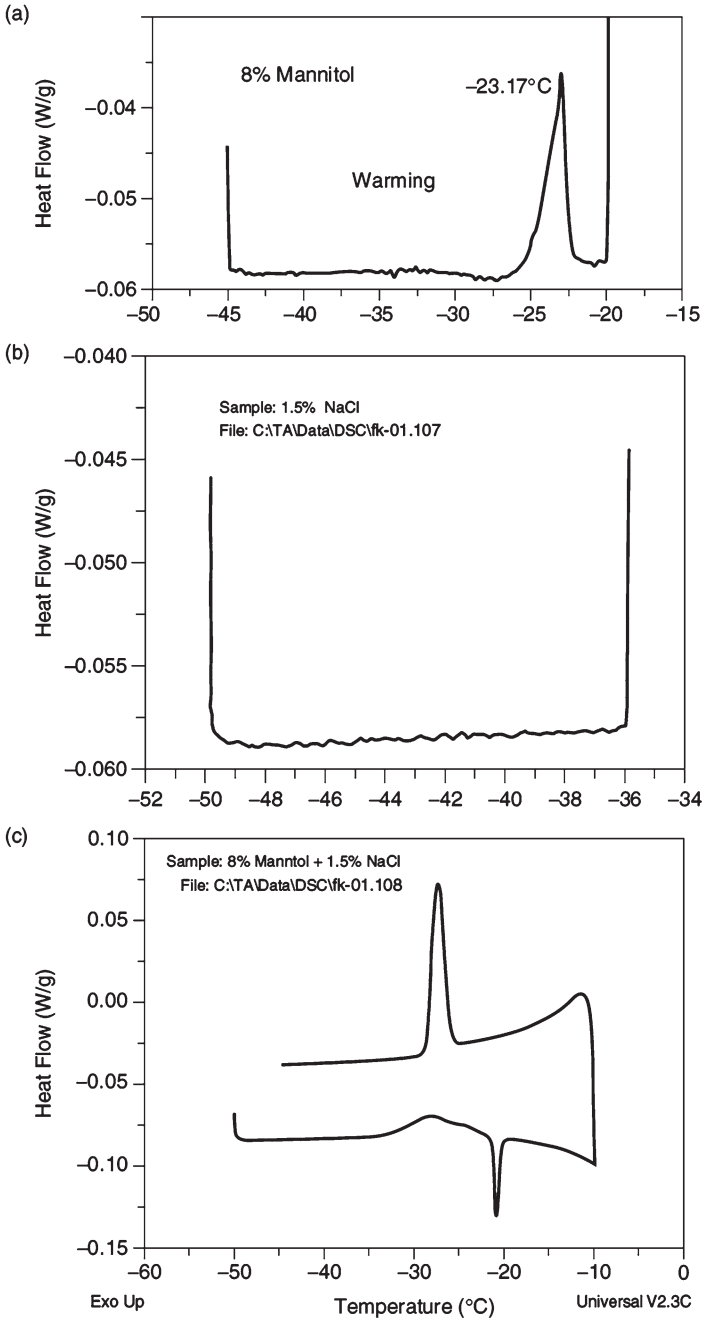


Fig. 13 MDSC thermograms showing the crystallization of mannitol and sodium chloride. (**a**) Crystallization of mannitol on heating the sample. (**b**) Crystallization of mannitol and sodium chloride in a mixture solution followed by a eutectic melt endotherm of sodium chloride and further crystallization of mannitol upon freezing. (**c**) A eutectic melt endotherm of sodium chloride and further crystallization of mannitol on freezing. (Reprinted with permission from Ref. [29])

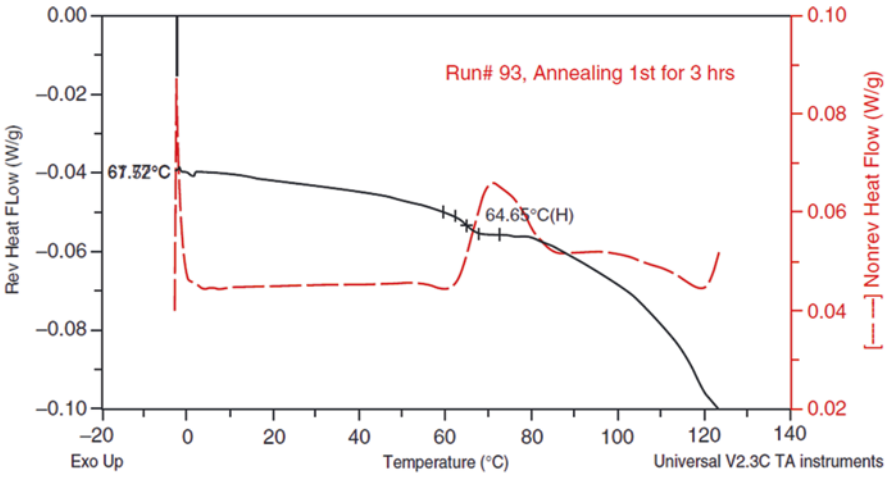


Fig. 14 MDSC thermogram of mannitol and trehalose freeze-dried powder showing glass transition in the reversible heat flow signal and an exotherm of mannitol crystallization in the non-reversible signal. (Reprinted with permission from Ref. [29])

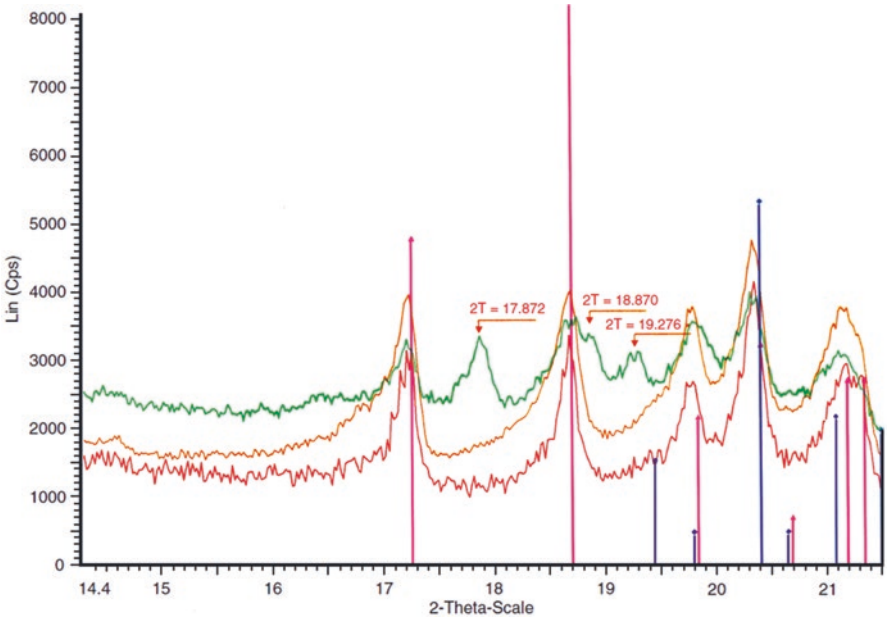


Fig. 15 X-ray powder diffraction of mannitol showing the presence of hydrates and their elimination through secondary drying at elevated temperature. (Reprinted with permission from Ref. [29])

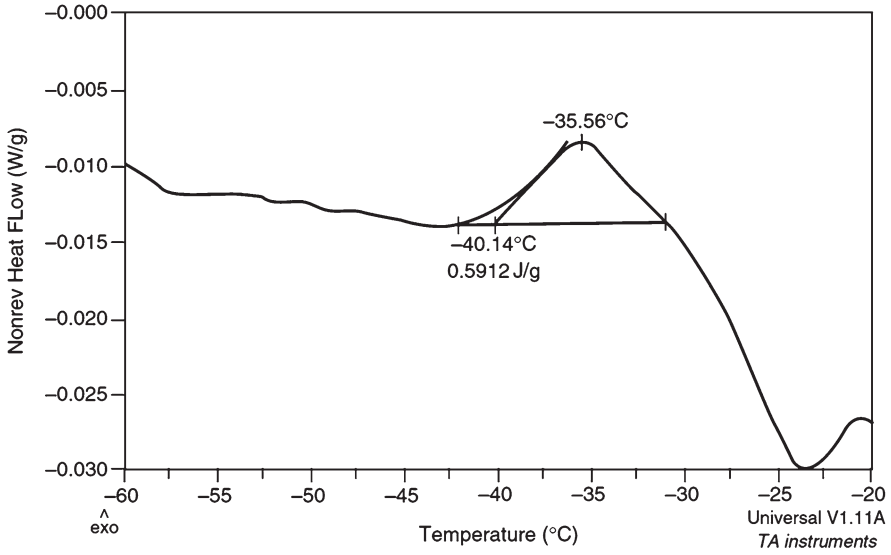


Fig. 16 Thermogram exhibiting crystallization and eutectic temperature of sodium chloride. (Reprinted with permission from Ref. [29])

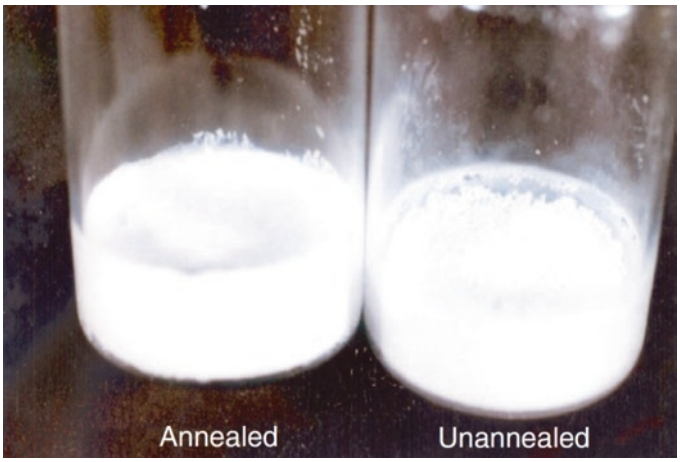


Fig. 17 Appearance of cake before and after annealing of mannitol and sodium chloride. (Reprinted with permission from Ref. [29])

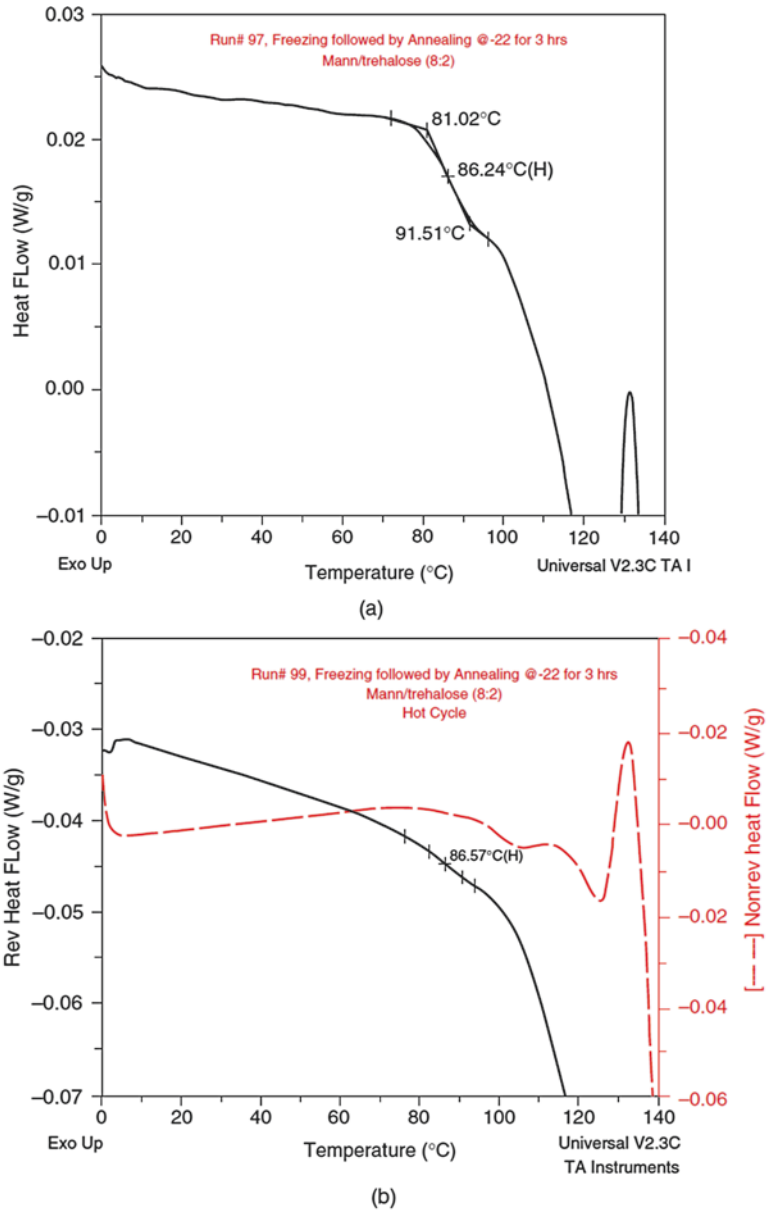


Fig. 18 Thermograms depicting increase in T_g value and no crystallization peak of mannitol and sodium chloride after annealing: (**a**) the total heat flow and (**b**) reversible and non-reversible heat flow. (Reprinted with permission from Ref. [29])

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Chapter 22
Development and Scale-Up of the Mixing Process for Biopharmaceuticals

Feroz Jameel, Ann M. Czyzewski, Tong Zhu, Kushal Sinha, and Nandkishor K. Nere

Nomenclature

a: Specific interfacial area [m^2]
α_{air}: Air volume fraction
C: Impeller clearance [m]
C_L: Gas concentration
C∗: Equilibrium gas concentration
D: Impeller diameter [m]
d_b: Volume-averaged bubble diameter [m]
D_M: Molecular diffusivity [m^2/s]
γ_B: Bulk shear rate [1/s]
γ_I: Impeller tip shear rate [1/s]
H: Height of liquid [m]
J: Baffle width [m]
K_La: Gas-liquid mass transfer coefficient
K_{SL}: Solid-liquid mass transfer coefficient [$1/(m^2 * s)$]
L: Agitator blade length [m]
μ: Liquid viscosity [Pa∗s]
n: Rotational speed [1/s]
N_B: Impeller blend number
N_P: Impeller power number
N_Q: Impeller pumping number
P: Impeller power [W/m^3]
q: Volumetric flow rate [m^3/s]

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Re: Reynolds number
ρ: Liquid density [kg/m^3]
Sc: Schmidt number
T: Tank diameter [m]
t: Torque
Θ: Theoretical mixing time [s]
W: Agitator blade width [m]
ν: Tangential velocity [m/s]
V_B: Bulk velocity [m/s]

1 Introduction

Mixing is defined as the act of combining substances, generally through the application of mechanical stirring, to blend the constituents into one homogeneous mass. Mixing is impacted by all aspects of hydrodynamics that influence transport phenomena (heat and mass transfer) as well as phase dispersion characteristics [1–4]. Many equipment, process, and product considerations influence hydrodynamics including vessel geometry, internal geometry, fluid physicochemical properties, and operating parameters, as summarized in Fig. 1. Robust process design and scale-up are contingent upon understanding and controlling hydrodynamics, which impacts important quality attributes, including homogeneity and physical properties. The reader is referred to Ranade for further discussion of hydrodynamics [5].

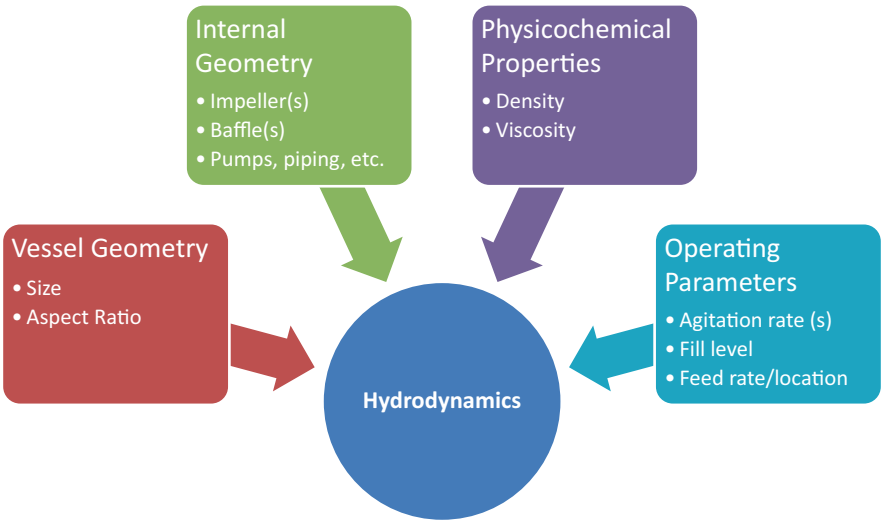

Fig. 1 Equipment, process, and product considerations that impact hydrodynamics

Mixing can be applied to single and multicomponent mixtures of solids, liquids, and gases (as well as combinations of phases). The systems most commonly used for biopharmaceutical processing include single-phase liquid blending as well as liquid-liquid, gas-liquid, and solid-liquid biphasic applications. In this chapter, the discussion is therefore focused on systems comprised of these most relevant single and biphasic mixtures.

The performance of many different unit operations associated with drug product processing of biomolecules is significantly impacted by hydrodynamic considerations. The most basic unit operations, such as pooling of single-phase shear-insensitive materials, are performed simply to achieve homogenization. However, blending of excipient mixtures is an example of a unit operation impacted by hydrodynamics in more complex ways because it involves solid-liquid mixtures and dissolution processes.

The propensity of biomolecules to aggregate or denature in the presence of high shear and gas-liquid interfaces is another important design consideration for bioprocesses. Biological processes are often subjected to sparged gases, such as oxygen, which results in two-phase gas/liquid systems. Additionally, high agitation can lead to gas entrainment from the headspace of the mixing vessel. While there has been some debate in the literature on the relative impact of shear and interfacial effects on protein integrity [6–10], both shear and interfacial effects should be understood in a well-designed process. Many different approaches and tools are available that guide reliable scale-up of these various unit operations, ranging from simple empirical correlations to first-principle modeling coupled with model-guided experiments.

In this chapter, we begin with the basic concepts and considerations for characterizing mixing performance. We discuss general guidelines and available references that can be applied to the simplest mixing applications (e.g., pooling of homogenous solutions), along with the limitations of such approaches. We then delve into more complex mixing systems that are prevalent in the processing of biomolecules, including mixing of two phases (drug substance and drug product solutions), dissolution of solids (excipients), mixing with entrained gas, and shear-sensitive product mixtures (protein solutions). More sophisticated experimental and modeling approaches, capable of capturing more specific detail associated with a mixing system, are then discussed, along with relevant examples to demonstrate their utility.

2 Mixing Fundamentals and Basic Scale-Up Considerations

2.1 Turbulence

Increasing turbulence in fluid flow improves mixing performance, even in the absence of an external mixing device. An average measure of turbulence is captured in the Reynolds number, which is defined as a dimensionless ratio of inertial to

viscous drag forces within a fluid. The Reynolds number can be expressed as a function of the rotational speed (n), the impeller diameter (D), the liquid density (ρ), and the liquid viscosity (μ) as shown in Eq. 1 of Table 2.

The Reynolds number can be used to predict the transition between laminar flow (with no lateral mixing) and turbulent flow, which exhibits cross-current flows and eddies. Laminar flow is present in systems characterized by low Reynolds numbers, when viscous drag forces dominate, whereas turbulent flow is evident in systems with high Reynolds numbers, where inertial forces dictate fluid motion. Laminar flow systems are the most difficult to mix; hence, conservatively low Reynolds numbers are typically chosen to assess poor mixing performance.

For applications that involve the mixing of low viscosity liquids, the inertial forces may result in sufficient turbulence to homogenize the fluids in the absence of an external mixing device. In most cases, however, mixing needs to be aided by the presence of an agitation and/or baffling device. Baffles, which are stationary devices used to restrict and regulate fluid flow, provide an effective means of increasing turbulence. For example, vortexing and swirling rotation of a liquid mass in a stirred tank are disrupted by the presence of baffles, resulting in significantly improved mixing performance (except at high Reynolds numbers [4, 11]). Agitation applied to a system is most efficient when it (1) enables the fluid to sweep the entire vessel in a process-relevant time and (2) directs fluid flow into all areas of the tank, thus minimizing "dead zones." Impeller design, as discussed in the next section, is a very important consideration that determines agitation effectiveness.

2.2 Basic Impeller Design

Impellers can be classified into two main groups:

- Impellers with a small blade area, which rotate at high speeds and are used to mix low to medium viscosity liquids (turbines and marine propellers).
- Impellers with a large blade area, which rotate at low speeds and are effective for high viscosity and shear-thickening liquids (anchors, paddles, and helical screws).

Several general guiding documents have been published that can be useful in selecting an appropriate impeller [12, 13]. Figure 2 is a tool for selecting an impeller type based on liquid viscosity and tank volume; Table 1 illustrates another selection guidance based on liquid viscosity alone. These tools are based on only a small fraction of all the considerations that impact hydrodynamics (see Fig. 1) and therefore are not capable of providing process-specific information. However, they can provide guidance as a good starting point for designing a well-mixed system.

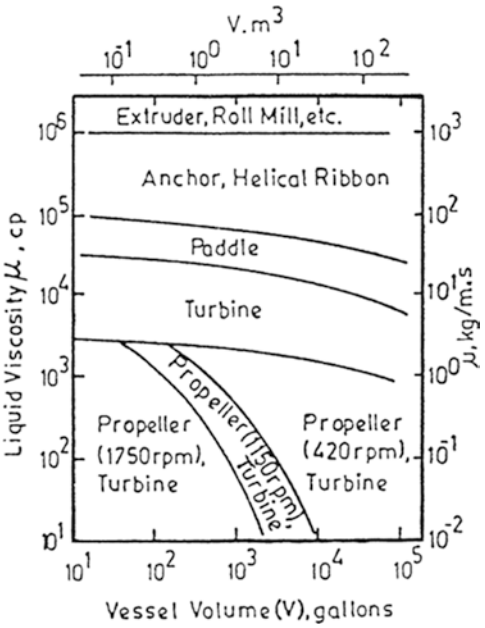

Fig. 2 Impeller selection guide [12]

Table 1 Impeller selection guide based on liquid viscosity [13]

Impeller selection guide		
Type of impeller	Range of liquid, cP	Viscosity, kg/m – sec
Anchor	10^2–2×10^3	10^{-1} – 2
Propeller	10^0–10^4	10^{-3} – 10^1
Flat-blade turbine	10^0–3×10^4	10^{-3} – 3×10^1
Paddle	10^2–3×10^1	10^{-1} – 3×10^1
Gate	10^3–10^5	10^0–10^2
Helical screw	3×10^3–3×10^5	3–3×10^2
Helical ribbon	10^4–2×10^6	10^1–2×10^3
Extruders	$>10^6$	$>10^3$

Source: Holland and Chapman [13]

2.3 *Platform Approaches and Empirical Correlation-Based Scale-Up*

From a manufacturing point of view, it is highly desirable to have a standard mixing process platform that could be applied to multiple products and all batch sizes. While this may be possible for materials that are insensitive to shear, it may not be feasible to consider this approach for shear-sensitive applications. In systems that

are insensitive to shear, agitation can be designed conservatively to achieve homogenization without concern for product degradation, which lends itself to a platform approach. In shear-sensitive systems, however, excessive mixing can be deleterious to product quality, and a platform approach may not be appropriate. In this section, we discuss some simple approaches useful for determining acceptable mixing conditions for shear-insensitive systems. We also touch on scale-up considerations as a starting point for understanding shear-sensitive systems; a comprehensive discussion of more advanced approaches applicable to complex, shear-sensitive materials is found in Sect. 3.

Platform Approach to Determining Agitation Rate and Mixing Time for Shear-Insensitive Systems

A simple empirical approach based on identifying the most forcing conditions may be useful for selecting a target mixing speed and mixing time. The "worst-case" conditions identified based on scientific rationale lead to a conservative estimate of appropriate mixing conditions. This approach is depicted graphically in Fig. 3.

Robust and scalable drug product formulation processes avoid mixing conditions that result in foaming and splashing, as protein denaturation is promoted at air-liquid interfaces [8]. Conditions that exacerbate splashing and foaming include minimum batch size, minimum solution viscosity/density (modulated by PEG content),

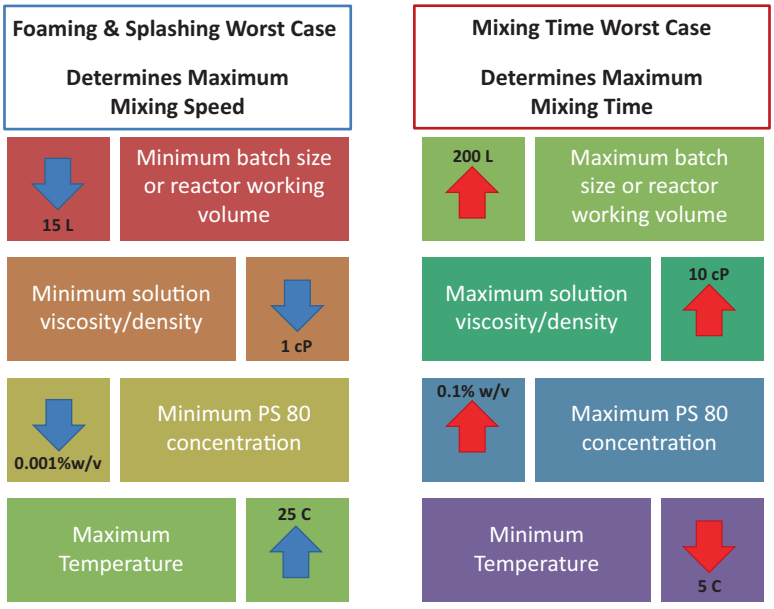

Fig. 3 Platform approach for determining agitation rate and time

minimum polysorbate 80 (PS 80) content, and maximum process temperature, as summarized in Fig. 3. Using this approach, this combination of conditions is applied in a system of interest, and the agitation speed is incrementally increased while observing mixing performance. Based on a visual assessment of the mixing speed at which splashing/foaming occurs under these worst-case conditions, an estimate of an appropriate upper limit of agitation speed is determined.

Using a complimentary approach, the maximum mixing time can be determined by considering the opposite case, which uses maximum batch size/volume, maximum viscosity/density (20% w/v PEG 400 corresponding to 10 cP [14], maximum PS 80 concentration, and minimum temperature surrogate solution. This combination of formulation parameters corresponds to worst case with respect to mixing time. Upon creating the process conditions outlined in Fig. 3, mixing is begun at low speed, and samples are withdrawn periodically at different locations within the mix tank. Samples are analyzed for PEG using refractive index and for PS 80 using mixed mode HPLC column CAD. The maximum mixing time corresponds to the sample point at which concentrations of PEG and PS 80 reach and sustain expected endpoint values based on charged quantities.

Empirical Correlation-Based Scale-Up

Empirical correlations can be useful in determining appropriate mixing conditions to scale-up and scale-down processes in different equipment and to scale mixing as a function of vessel fill volume. Correlations are inherently not always capable capturing important differences in vessel geometry and internal configuration, which can significantly impact hydrodynamics. Hence, this approach is most appropriately applied in cases where there is geometric similarity between the processing equipment being scaled. In simple cases, this approach can be used to establish an acceptable range of agitation rate and mixing time (i.e., design space) to achieve sufficient homogenization as a function of fill volume or upon scale-up/scale-down, as described below.

There are several dimensionless quantities that are commonly used to characterize the hydrodynamics of biological fluids. The definitions and of these quantities, which are functions of fluid properties and/or geometric aspects, are summarized in Table 2. Common correlation-based scale-up rules for geometrically similar vessels are summarized in Table 3. Scaling based on power per unit volume is most commonly used in process design scale-up, as it provides a conservative basis on which to scale-up process performance.

Correlations widely available in the literature can be used to generate an operating range (design space) for the mixing time as a function of agitation rate. Standard agitation systems conform to common geometric proportions summarized in Table 4. The impeller diameter (D), liquid height in tank (H), and baffle width (J) occur in standard ratios to the tank diameter (T). The impeller clearance (C), the agitator blade width (W), and the agitator length (L) occur in standard ratios to the impeller diameter.

Table 2 Definitions of common dimensionless numbers

Quantity	Mathematical definition	Eqn.	Comments
Reynolds number (re)	$\mathrm{Re} = \frac{nD^2\rho}{\mu}$	1	Defined as the ratio of inertial to viscous forces. This relationship can take various forms for non-Newtonian fluids for which viscosity is a function of the shear rate
Impeller blend number (N_B)	$N_B = n\theta\left(\frac{D}{T}\right)^{2.3}$	2	Blend number can be used for blend time prediction
Impeller power number (N_p)	$N_p = \frac{P}{\rho n^3 D^5}$	3	Power number is used to calculate power and torque delivered by the impeller
Impeller pumping number (N_Q)	$N_Q = \frac{q}{nD^3}$	4	Pumping number is used to calculate the impeller pumping capacity

Table 3 Common scale-up bases for geometrically similar vessels

Scale-up basis	Mathematical relationship	Eqn.
Reynolds number	$\mathrm{Re} = \left(\frac{nD^2\rho}{\mu}\right)_{scale1} = \left(\frac{nD^2\rho}{\mu}\right)_{scale2}$	5
Impeller tip speed	$Tip\ Speed = (n\pi D)_{scale\ 1} = (n\pi D)_{scale\ 2}$	6
Power per unit volume	$\frac{P}{V} = \left(N_p\rho n^3 D^5\right)_{scale1} = \left(N_p\rho n^3 D^5\right)_{scale2}$	7

Table 4 Geometric proportions for standard agitated vessels

Standard geometric proportions in ratio to tank diameter (T)	Standard geometric proportions in ratio to impeller diameter (D)
$\frac{\boldsymbol{D}}{\boldsymbol{T}} = \frac{\mathbf{1}}{\mathbf{3}}$	$\frac{C}{D} = 1$
$\frac{\boldsymbol{H}}{\boldsymbol{T}} = \mathbf{1}$	$\frac{W}{D} = \frac{1}{5}$
$\frac{\boldsymbol{J}}{\boldsymbol{T}} = \frac{\mathbf{1}}{\mathbf{12}}$	$\frac{L}{D} = \frac{1}{4}$

The theoretical mixing time (Θ) in the turbulent regime as a function of agitation rate (n), liquid height (H), and geometric characteristics discussed above can be determined using the correlations listed in Table 5 for axial and radial flow impellers. Consider the case with impeller diameter of .19304 m, tank diameter of 0.57912 m, fill volume of 200 L, dynamic viscosity of 1 cP, and density of 1 g/cm^3. Figure 4a, c are contour plots showing the design space for the mixing time as a

Table 5 Mixing time correlations for axial and radial flow impellers

Impeller type	Mixing time correlation	Eqn.	Reference
Axial flow impellers	$n\cdot\Theta = 5.0\left(\frac{2H}{D}+\frac{T}{D}\right)\frac{C}{D}$	8	Raghav Rao and Joshi [15]
Radial flow impellers	$n\cdot\Theta = 9.43\left(\frac{1.33H+T}{T}\right)\left(\frac{T}{D}\right)^{13/6}\frac{W}{D}$	9	Joshi et al. [16]

function of two input parameters (the rotational speed and the height of fluid) for axial and radial flow impellers, respectively. For a rotational speed of 3.33rps (shown by the red cross symbol), the calculated mixing times for the axial and radial flow impellers are 16 and 30 seconds, respectively. Figure 4b, d represent surface plots can be used to show the same design space. These plots can be more helpful for the detection of trends (i.e., inverse proportionality between the mixing time and rotational speed) than for guiding quantitative mixing parameter design.

Average Shear Rate Calculations

Shear stress and shear rate are tensor quantities that are dependent on time, spatial location, and magnitude. However, correlations can be used to calculate the average shear rate, which is an important consideration for proteins.

Viscosity is a measure of resistance of a fluid to deform under shear stress. Fluid layers move at different velocities due to viscosity; fluid "thickness" is a manifestation of the shear stress between layers that oppose any applied force. This phenomenon is captured in Newton's law, which relates shear stress (τ) to viscosity (μ) and the velocity gradient (shear rate) in the y-direction ($\frac{\partial v}{\partial y}$) as shown in Eq. 10 [17].

$$\tau = \mu \cdot \frac{\partial v}{\partial y} \tag{10}$$

Equation 10 in tensor form applies to Newtonian fluids. The true shear stress applied on a fluid element is a tensor quantity and a function of the local environment [18]. The shear stress generated during mixing is due to complex velocity gradients and phase interfaces. This shear, coupled with interfacial effects, may cause damage to biomolecules [6–10].

Rheometers can be used to apply a known shear stress to a biomolecule mixture; the shear rate is then calculated based on the following equations. Protein quality after exposure to shear can then be evaluated using stability-indicating assays.

The following equations provide a simple means to estimating the shear rate during mixing. The bulk shear rate (γ_B) for an unbaffled tank is a function of the bulk velocity (V_B) and tank diameter (T) as shown in Eq. 11.

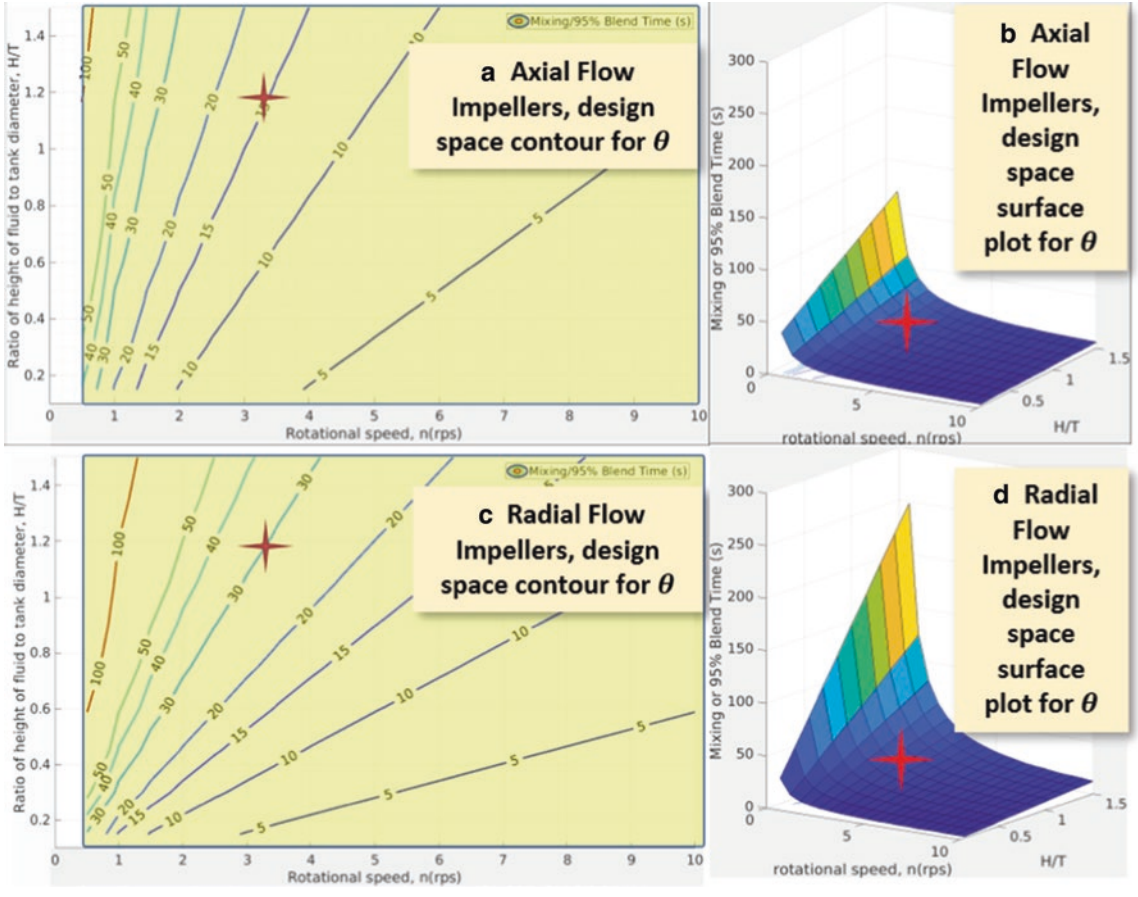

Fig. 4 The design space for the mixing time (or the 95% blend time), θ, as a function of the rotational speed and the height of fluid (normalized by the tank diameter) for (**a**) axial flow impellers, contour plot; (**b**) axial flow impellers, surface plot; (**c**) radial flow impellers, contour plot; (**d**) radial flow impellers turbine, surface plot. Note: Standard agitation system with D/T = 1/3, C/D = 1, W/D = 1/5 and arbitrary tank diameter and liquid fill height. The shaded yellow area corresponds to completely turbulent flow

$$\gamma_B = \frac{32.777 V_B}{\left(\frac{T}{2}\right)} \tag{11}$$

The impeller tip shear rate can be expressed as a function of the impeller diameter (D), impeller rotational speed (n), as shown in Eq. 12.

$$\gamma_I = \left[\frac{\pi Dn}{60\left(\frac{T-D}{2}\right)}\right] \tag{12}$$

Equations (11) and (12) are based on conservation of momentum and simplified assumptions, as discussed in the literature [19].

3 Computational and Experimental Assessment of Mixing and Shear

3.1 Small-Scale Shear and Gas Entrapment Assessment

Correlation-based assessments of mixing and shear as exemplified in Fig. 4 and Eqs. 11 and 12, respectively, can be useful to scale-up mixing time and shear between geometrically similar vessels. However, oftentimes, the characteristics of the vessels, impellers, and internals across laboratory, pilot, and commercial scales are not geometrically similar and/or do not conform to the standard agitation system design. In these cases, correlation-based scale-up criteria are inadequate; computational fluid dynamics (CFD) modeling poses a robust alternative for appropriately scaling processes in nonstandard systems.

Mechanical agitation is an effective means to achieving dissolution and homogeneity, which may be important in various unit operations of drug product processing. This agitation exposes the active molecules to shear and gas-liquid interfaces created due to headspace gas drawn into the solution. Hence, it is advisable to carry out small-scale experimental assessment to ascertain the impact of these conditions on product quality. It is important that these small-scale experiments be appropriately designed to adequately capture the shear and gas entrainment exposure upon scale-up. The following section describes a methodology for designing small-scale experiments that provide a reliable basis for guiding accurate scale-up.

3.2 Methodology for Small-Scale Assessment

The methodology to carry out the small-scale assessment is outlined as follows:

1. Characterization of hydrodynamics in large-scale mixing vessels to be used, potentially using computational fluid dynamics (CFD) to extract.
   (a) The maximum energy dissipation rate.
   (b) Shear strain.
   (c) Number of fluid passes through the high shear impeller region.
   (d) Mass transfer coefficient to predict the mixing time to achieve required dissolution and/or homogeneity.
2. Characterization of small-scale experimental setup to extract energy dissipation rates and shear strains at various agitation rates relevant to nominal and worst-case scenarios at scale.
3. Design of small-scale experiments.
   (a) Sterilize the complete experimental setup, and charge the product solution to the small-scale vessel. Pull a sample from the vessel prior to shear exposure. This sample will be analyzed as an experimental control, providing a baseline comparator for test samples.
   (b) Ensure that the lid is in place to avoid any headspace gas entrainment.
      (i) Subject the solution to three different levels of shear and energy dissipation rates starting with low agitation speed and progressing to the maximum speed.
      (ii) Duration for each mixing condition should sufficiently cover the number of passes through impeller region equal to the number of passes expected in the large-scale vessel at the highest agitation speed.
      (iii) Withdraw appropriate samples from each experiment, and analyze them as well as the control sample for the desired quality attributes.
   (c) Remove the lid to promote headspace gas entrainment into the solution.
      (i) Subject the solution to three different levels of shear and energy dissipation rates starting with low agitation speed and progressing to the maximum speed.
      (ii) Duration for each mixing condition should sufficiently cover the number of passes through impeller region equal to the number of passes expected in the large-scale vessel at the highest agitation speed.

In the absence of rigorous CFD characterization and scale-down setup, the shear assessment can alternatively be carried out using ThermoHaake VT 550, CVO rheometer (see Fig. 5), and/or capillary rheometer, where the drug product solution is aliquoted and dispensed into the cup and bob rotor configuration of the rheometer. The sample is then sheared at a mixing shear rate obtained from Eqs. 11 and 12. For example, exposing a sample to a shear rate of 60 per second at two temperatures

Fig. 5 Picture of ThermoHaake VT 550 CVO rheometer

(2–8 °C and 25 °C) for three different time intervals (20, 40, and 60 minutes) covers a range of the mixing times for different fill volumes. At the end of each time interval, samples are removed and analyzed to assess the impact of mixing shear on product quality attributes using stability-indicating assays, such as SE-HPLC, subvisible particulate, CEX, and potency. This methodology has been illustrated through the case study presented in Sect. 3.3.

3.3 Use of Models and Experiments to Guide Shear-Sensitive Process Scale-Up and Scale-Down

Biomolecules can display different levels of sensitivity to the shear exposure experienced during processing. Monoclonal antibodies (mAbs), for example, are known to be particularly sensitive to mechanical forces [20]. Agitation during fill-finish operations is typically considered low shear; however, locally high shear rates are often observed in the impeller zone around tank baffles [8]. Moreover, shear damage can occur at much lower impeller speeds if the vessel is sparged due to shear effects associated with bubbles [14]. The agitation used to mix biomolecules, therefore, must limit the intensity of shear while still providing adequate blending of constituents.

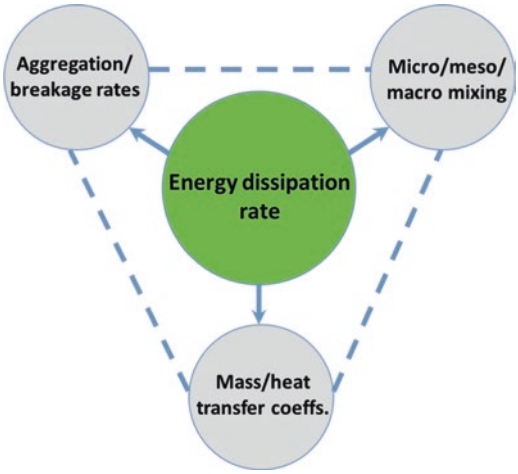

Fig. 6 Scale-up triangle showing the energy dissipation rate controlling several key aspects of process scale-up

In such cases where significant sensitivity to mixing exists, computational fluid dynamics (CFD) is a very useful tool to ensure reliable scale-up and reproducible product attributes. As discussed in Sect. 1, understanding and controlling hydrodynamics is critical to ensure robust process design and scale-up. The fundamental parameter characterizing the hydrodynamics of a system is the turbulent kinetic energy dissipation rate (ε), which is defined as the rate of turbulent kinetic energy loss due to viscous forces in turbulent flow. In any mixed system, the turbulent kinetic energy dissipation rate exhibits spatiotemporal heterogeneity. As depicted in Fig. 6, the turbulent kinetic energy dissipation rate determines the kinetics of various underlying processes (i.e., heat and mass transfer, breakage/agglomeration, phase dispersions, etc.).

While these mixing characteristics associated with the energy dissipation rate can be difficult to measure using experiments, single-phase CFD simulations provide insight into properties that are otherwise inaccessible by experimentation. Moreover, standard impeller power numbers and correlations (discussed in Sect. 2) are not designed to account for differences in reactor/impeller configuration across scales that may be nontrivial. CFD provides a first-principle solution to governing equations describing transfer of momentum, mass, and energy. Thus, CFD is used to simulate spatial-temporal profiles of flow patterns, turbulence, temperature, and species concentrations that determine overall equipment and process performance.

In the case of biomolecule drug product processing, mixing being too low or too high can impact quality attributes in different ways. Insufficient mixing can result in non-homogeneity throughout the batch, ultimately resulting in drug product assay variability and risk to patient safety. Moreover, dissolution (e.g., excipient components), which is driven by good mixing, may also need to be considered to form a fully homogenous mixture in a biomolecule drug product processing. If mixing is too vigorous, on the other hand, the shear imparted can cause the protein to denature

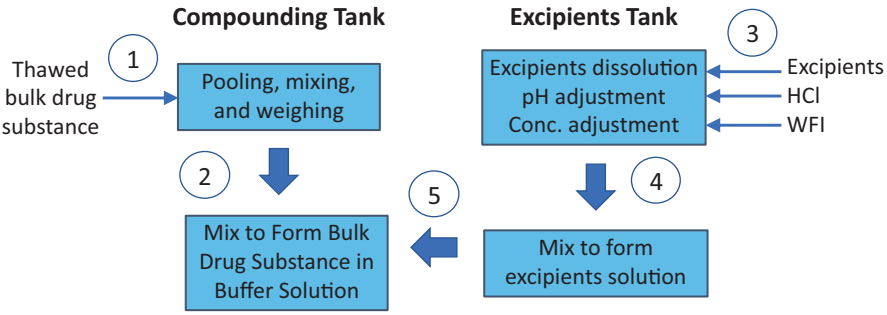

Fig. 7 Case study drug product process flow diagram

and aggregates to form. In summary, the agitation must be sufficient to ensure homogenization while not causing undue damage to shear-sensitive products.

We present a case study demonstrating the utility of CFD to guide appropriate scale-up of biologic drug product mixing processes. The threefold objective of this study included the following: (1) assess mixing parameters and batch size for the compounding process, (2) assess the impact of high shear stress conditions on product stability in a scale-down model, and (3) examine the excipient solution mixing speeds and time to support full dissolution before use. This combined approach of modeling and experiment was designed to provide a complete assessment of the justified operating ranges and impact on critical quality attributes.

The basic design of the drug product process is outlined in Fig. 7. The thawed BDS is pooled, mixed, and weighed in the compounding tank. The excipient solution is prepared by dissolving excipient components in water, adjusting the pH, and then adjusting the weight with the appropriate amount of water for injection (WFI). The excipient solution is then transferred onto the bulk drug substance solution, and the two phases are mixed to form the homogeneous drug product solution.

The geometry of the mixing vessels and impellers for both the compounding tank and excipients tank were modeled in MixIT (version 3.2) software to closely resemble the actual geometry. The technical drawing of the impeller (used for both tanks) in comparison to the modeled geometry is shown in Fig. 8a. The modeled compounding tank geometry with the bottom-mounted impeller is shown in Fig. 8b.

Assess Mixing Parameters and Batch Size for the Compounding Process

The first objective of this study was to explore how agitation rate (110, 110, and 120 rpm) and batch sizes (20 L and 30 L) impact the time needed to homogenize a drug substance/excipient solution mixture in the compounding process. Single-phase simulations were performed for each of six scenarios summarized in Fig. 9 using appropriate fluid property characteristics. All operational conditions exhibit turbulent flow by definition (Reynolds number > 10^4); hence, a realizable k-ε turbulence model was used to simulate the flow pattern in the mixing tank.

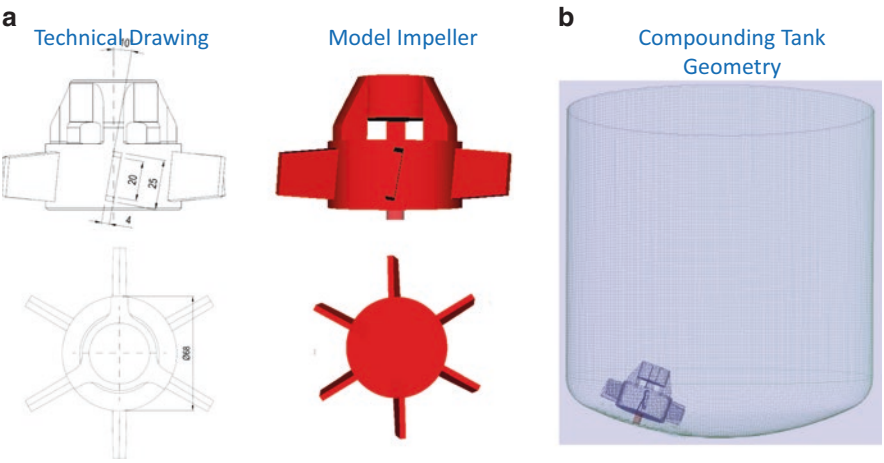

Fig. 8 (**a**) Technical drawing and model of bottom-mounted agitator. (**b**) Simulated geometry of mixing tank equipped with bottom-mounted impeller

Fig. 9 Summary of conditions used to run six different single-phase CFD simulations

	Agitation Rates		
	100	110	120
Batch Size 20L	Sim 1	Sim 2	Sim 3
Batch Size 30L	Sim 4	Sim 5	Sim 6

The velocity vector plot resultants from each simulation are shown in Fig. 10. The arrow direction and color depict the direction of flow and speed, respectively. The strength of flow circulation increases as the agitator speed is increased from 100 rpm to 120 rpm, and the highest fluid velocities are observed around the impeller region.

These simulation results were used to estimate the mixing time needed to homogenize a multiphase system (drug substance and excipient solutions). Figure 11 shows an example velocity vector flow pattern along with the corresponding plot of flow velocity in the z-direction along a line across the diameter of the vessel at a position that approximates the interface of the BDS and excipient mixtures (dashed line in Fig. 11 velocity vector profile). Negative velocities indicate downward flow, while positive velocities indicate upward flow. The downward velocity represents the movement of fluid from the top of the tank (excipient layer) to the bottom layer (BDS), which is the primary mode of mixing. Two reference points are provided in the axial velocity profile in Fig. 11. Point "**a**" is to the left of the impeller and represents a local downward flow due to its proximity to the impeller, which impedes the recirculating flow pattern. Point "**b**" is along the right tank wall and represents the area with a consistent positive velocity (upward flow). The average downward

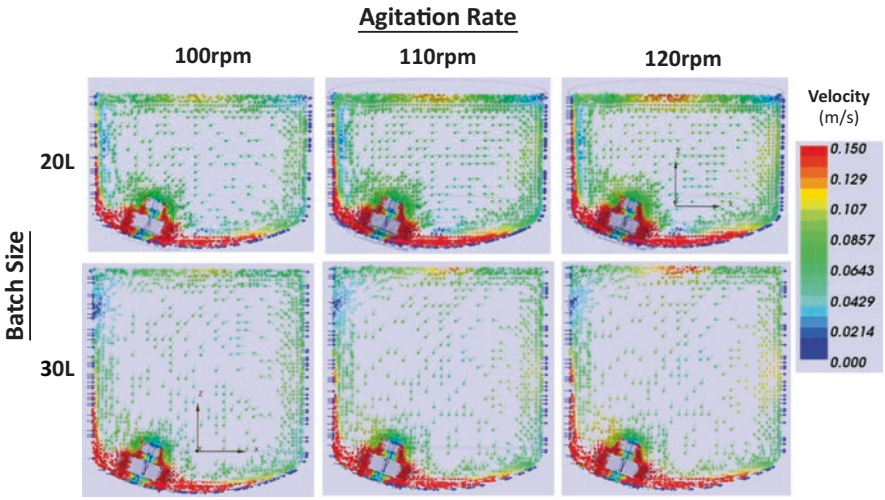

Fig. 10 Velocity vector plots of simulated mixing process conditions

velocity was calculated by integrating the downward velocity across the diameter of the vessel at the interface between the two phases.

The post-processing described above was performed on each of the six simulations to yield an average downward velocity for each scenario of interest. Assuming a conservatively long recirculation path of traversing the full dimensions of liquid volume (2 times liquid height + 2 times tank diameter), the circulation time is estimated based on the average downward velocity. The total mixing time required is taken as five times the circulation time [19]. The average downward velocity, circulation time, and total mixing time for each of the six scenarios is summarized in Table 6. This analysis provides a scientific basis for justifying the lower boundary of the mixing time to be 5 minutes. An assessment of the impact of shear exposure will factor into defining the upper limit, as discussed in the next section.

Assessment of Shear Exposure

Computational fluid dynamic simulations provide an understanding of the shear rate resultant from processing conditions modeled. As an example, Fig. 12 shows the resultant strain rate histogram and contour plot for the 20 L batch size 100 rpm simulation. Much of the bulk fluid volume far from the impeller experiences a relatively low strain rate. Although high shear rates comprise a relatively small volume of the reactor volume, the high shear regions in the impeller region can be damaging to biomolecules. In order to understand the impact of shear on product quality, a scale-down model of the commercial process operating at the highest agitation rate (120 rpm) was created, following the methodology outlined in Sect. 3.2.

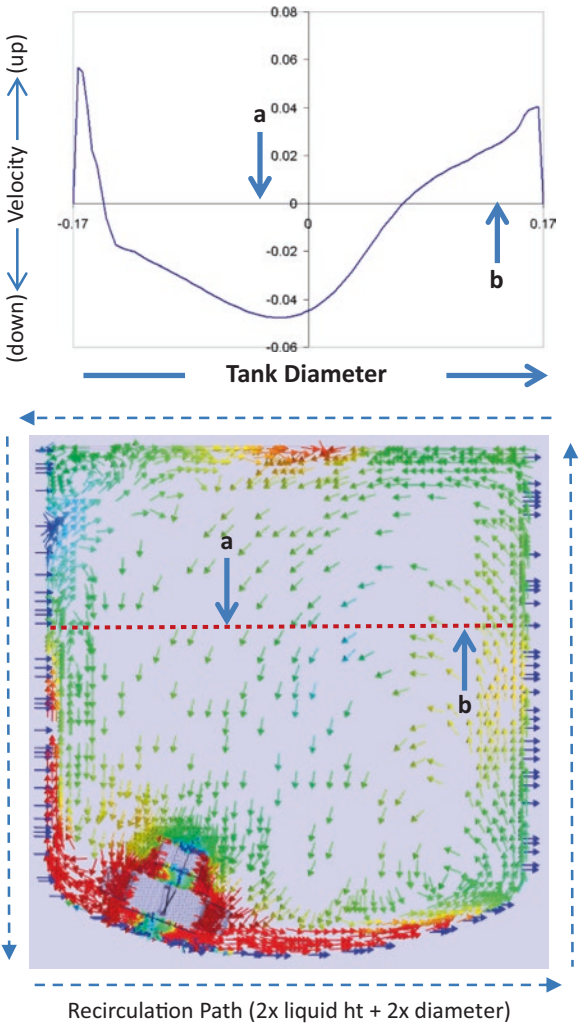

Fig. 11 Velocity vector profile in the z-direction and velocity vector flow pattern

Table 6 Mixing characteristics summary

Sim.	RPM	Average downward velocity, (m/s)	Circulation time, (s)	Mixing time (min)
20 L batch size				
1	100	0.022	52	4.3
2	110	0.025	46	3.8
3	120	0.027	42	3.5
30 L batch size				
4	100	0.026	51	4.2
5	110	0.029	46	3.8
6	120	0.032	42	3.5

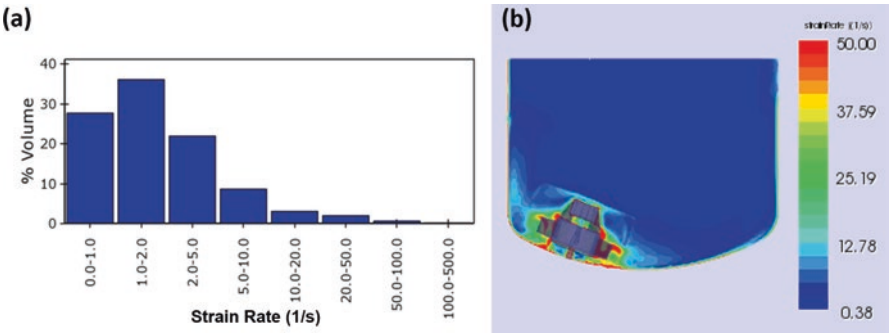

Fig. 12 (**a**) Strain rate histogram and (**b**) contour plot of strain rate for 20 L batch size, stirring at 100 rpm

The average energy dissipation rate (shear) was calculated from a simulation of the commercial process at the highest agitation rate. As a conservative approach, 100 times the average energy dissipation rate in commercial equipment was selected as the basis for scaling a 100 mL lab-scale experiment.

The solution was agitated at 90 rpm for 40 minutes, and samples were pulled after 5, 10, 20, and 40 minutes of mixing (in addition to the control, which was pulled prior to mixing). The samples were then analyzed using multiple techniques to assess potential impact on quality, including:

1. Size exclusion high-performance liquid chromatography (SEC-HPLC) to assess aggregate formation (high molecular weight species) and protein damage (low molecular weight species).
2. Imaged capillary isoelectric focusing (icIEF) to assess charge variant species.
3. Capillary SDS-gel electrophoresis (CE-SDS non-reduced) to assess purity of the product.
4. Analysis of subvisible particles to assess protein integrity.

These analyses showed no impact to the product stability compared to the untreated control sample or typical values.

Dissolution of Excipients

The third part of this study was focused on dissolution and homogenization of excipients prior to introduction into the compounding process. The objective of this study was to evaluate the time required for dissolution of the excipient and subsequent mixing. The timescales of the dissolution and mixing processes were then compared to determine the overall rate-limiting step to the homogenization process.

A single-phase CFD simulation of the excipient mixing process was performed commensurate with the appropriate details of vessel and internal geometry, fluid properties, and operating conditions as described previously. The impeller power

number, N_p, derived from this simulation was then used along with a characteristic particle size of the excipient, d_p, to determine the solid-liquid mass transfer coefficient K_{SL} in accordance with Eq. 13 [21].

$$K_{SL} = \frac{D_M}{\rho}\left[2 + 0.52\left(\frac{\mu}{\rho D_M}\right)\left(\frac{\epsilon d_p^4}{\nu^3}\right)^{0.17}\right] \tag{13}$$

The time required to homogenize the solution was again determined as described previously. In this case, the total mixing time for the process was sufficient to cover the dissolution time (~20 min) as well as the mixing time (~5 min).

In this example, we demonstrate the power of combining CFD with model-guided experiments to provide insight into product- and process-relevant hydrodynamics. CFD enabled us to quantitatively understand the flow pattern in a vessel with a non-conventional bottom-mounted mixer across a range of process conditions of interest. We then used an understanding of the average downward velocity at the BDS/excipient layer interface to calculate the mixing time under various conditions to evaluate operating parameters of interest. These results, taken together with results from a scale-down model used to interrogate the impact of shear on the biomolecule, were used to justify operating ranges that result in an acceptable product quality. Lastly, an understanding of the overall mixing time required to achieve dissolution and homogenization of the excipient mixture was achieved by (1) using the CFD-derived impeller power number and characteristic particle size of the excipient to calculate the mass transfer coefficient (K_{SL}) and (2) determining the mixing time of the solution once dissolved. The level of understanding achieved in this study could not have been achieved by using CFD or experimentation alone, and this example demonstrates the value of these complimentary approaches.

3.4 Use of Models and Experiments to Guide Oxygen-Sensitive Process Scale-Up and Scale-Down

Monoclonal antibodies (mABs) are often prone to oxidative degradation, which can impact product critical quality attribute such as potency, stability, and/or color [22–24]. Careful small-scale laboratory experimentation as discussed in Sect. 3.2 is useful to diagnose the oxygen sensitivity of biomolecules during drug product unit operations such as dissolution, pooling, and filling. The oxygen content of biomolecule solutions, subjected to varying degrees of oxygen exposure, can be quantified using a dissolved oxygen (DO) probe. Measuring the quality attributes with known oxygen sensitivity for each of these solutions provides a quantitative understanding of the impact of oxidative degradation.

As in the case of shear-sensitive biomolecules, understanding hydrodynamics in process-relevant conditions is also necessary to ensure reliable scale-up of

oxygen-sensitive processes. Positive and negative deviations from optimized mixing conditions (both excessive mixing and insufficient mixing) can cause different deleterious effects on product quality. While excessive mixing can result in high entrainment of gas from the vessel headspace (thus favoring oxidative degradation), insufficient mixing can lead to product inhomogeneity and extended processing times. Hence, understanding of hydrodynamics for oxygen-sensitive systems is of paramount importance.

When entrained gas from the headspace of the stirred vessel poses risk to product quality attributes, the following three hydrodynamic characteristics inform process scale-up.

1. Gas distribution in the stirred vessel, which is a measure of the heterogeneity in gas content throughout the processing fluid.
2. Gas holdup, which is a measure of the overall gas volume fraction in the vessel.
3. Gas-liquid mass transfer rate, K_La, and its distribution, which is a measure of the transfer of gas into the liquid phase in local pockets.

CFD Approaches to Understand Oxygen Impact

Measuring these quantities at large scale or predicting them from lab-scale experiments based on correlations may be quite challenging and cost-prohibitive in terms of both time and resource investment. However, CFD modeling is a tool that can provide insight into these quantities that inform process scale-up. Single-phase CFD modeling, which is a well-established platform that is computationally cheap compared to multiphase simulations, can be used to infer a lot about the behavior of two-phase mixtures. For some applications, multiphase simulations may be the best tool to appropriately characterize the hydrodynamics of a given system. In this section, various approaches to understanding mixing behavior, including the use of CFD simulations (single and multiphase) along with model-guided experiments, are discussed.

Oftentimes, there is a need to scale-up oxygen-sensitive drug product processes from small pilot-scale vessels to nonstandard manufacturing-scale vessels of dissimilar geometry under tight project timelines and with limited material availability. For example, the formulation vessel at Pilot Plant A might be a 40 L cylindrical vessel with a hydrofoil impeller mounted centrically (Fig. 13a, c) and the process vessel at Manufacturing Site B, an 80 L cylindrical vessel with a pitch-blade impeller mounted at an angle (Fig. 13b, d). The batch size increase from 5 L in 40 L vessel in Pilot Plant A to 20 L in 80 L vessel at Manufacturing Site B cannot be informed by correlations discussed in Sect. 2.3.2 due to the nonstandard design of these vessels.

To understand the potential to entrain gas from the headspace during processing, single-phase CFD simulations that closely represent vessel and internal geometry as well as fluid properties in both systems are performed. Figure 14 shows the resultant pressure distribution at the liquid surface of these two vessels mixing at steady state.

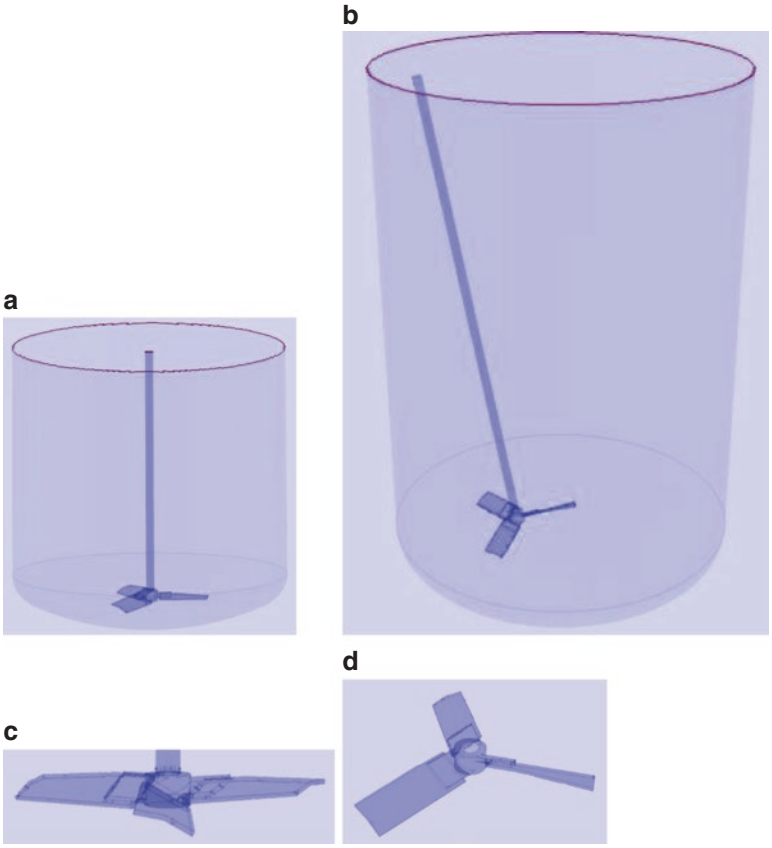

Fig. 13 (**a**) Formulation vessel at pilot scale, (**b**) formulation vessel at manufacturing site, (**c**) close-up view of hydrofoil impeller at pilot scale, and (**d**) close-up view of pitched-blade impeller at manufacturing scale

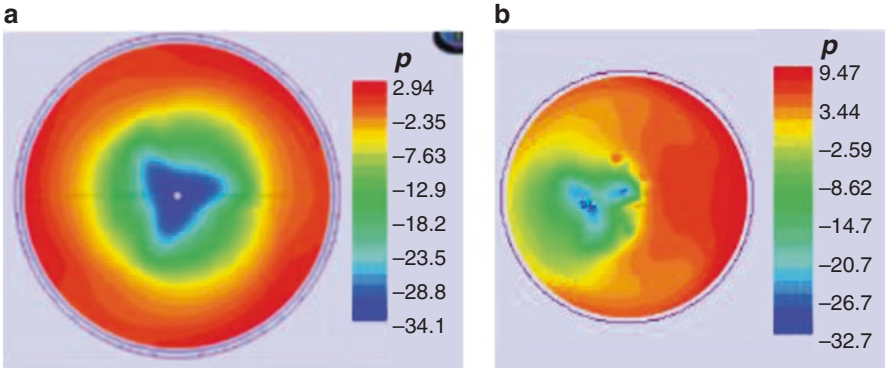

Fig. 14 Contour plot of pressure distribution on the top surface for (**a**) 5 L fill level at 119 RPM in 40 L vessel and (**b**) 20 L fill level at 120 RPM in 80 L vessel

The negative pressure is indicative of regions where headspace gas will be drawn more strongly into the liquid. These data show that the area of low pressure is significantly reduced at manufacturing scale compared to pilot scale at the selected operating condition, ensuring reduced gas entrainment. While lowering the agitation rate would further reduce gas entrainment, agitation must be maintained at an appropriate level to ensure product homogeneity.

Multiphase CFD simulations are useful for interrogating the gas distribution within the liquid phase and its potential impact on product quality. The several multiphase approaches to understand gas distribution include volume of fluid (VOF) method [25], level-set method [26], Lattice-Boltzmann method (LBM) [27], phase-field method [28], immersed boundary methods (IBM) [29], and boundary integral method (BIM) [30]. Each method has benefits and limitations that guide the choice of model for a given application. For a detailed description of these methods, readers are referred to Marchisio et al. [31, 32] and Joshi et al. [33].

VOF approaches have been shown to be relatively simple to implement and exhibit reasonable accuracy for free surface modeling [34, 35]. Figure 15 shows an example from the literature in which VOF was used to understand the onset of gas entrainment and the distribution of gas throughout the liquid. The color scale indicates the volume fraction of gas, which is assigned a value of 1 in the gas phase and 0 in the liquid phase. These data can also be used to calculate gas holdup as a function of impeller agitation rate.

The quality of oxygen-sensitive biomolecules is most strongly influenced by the magnitude of the gas-liquid mass transfer coefficient, $K_{L,}$ and the specific interfacial surface area, a. These quantities impact directly how much oxygen is entering in the liquid phase, leading to a possible oxidative degradation. Following classical Higbie's penetration theory [37, 38], K_L can be expressed as a function of the energy

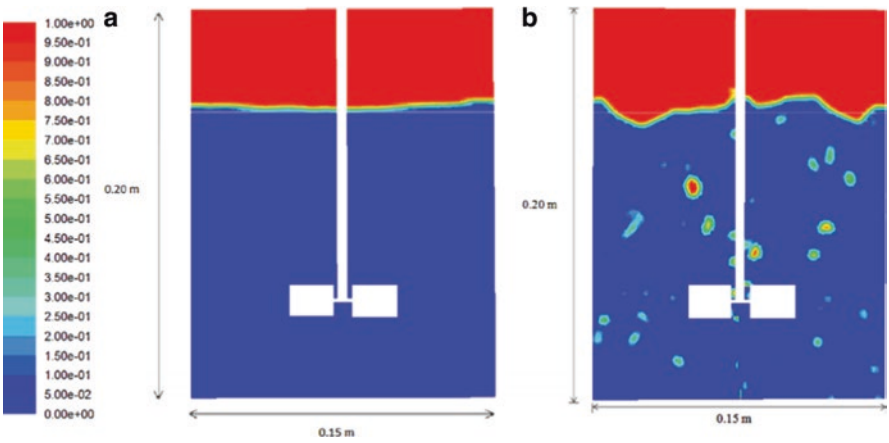

Fig. 15 Contours of volume fraction of air in the mid baffle plane for disk turbine system at the (**a**) below onset (RPM: 400, t = 13.5 s) and (**b**) onset conditions (RPM: 650, t = 6.63 s) of gas entrainment. (Figure adapted from Kulkarni and Patwardhan [36])

dissipation rate (ε, determined from CFD simulations), the kinematic viscosity (v), and the Schmidt number (Sc) as shown in Eq. 14:

$$K_L = \frac{2}{\sqrt{\pi}} (\varepsilon v)^{1/4} Sc^{-1/2}, \tag{14}$$

The specific interfacial surface area, a, for bubbly flows (constant bubble diameter) can be written as a function of the air volume fraction (α_{air},calculated from VOF CFD simulation) and the volume averaged bubble diameter $d_b$as shown in Eq. 15.

$$a = \frac{6\alpha_{air}}{d_b} \tag{15}$$

K_La defines the overall mass transfer coefficient for oxygen transfer from the gas phase to the liquid phase. Hence, it is essential to understand this for products that are strongly impacted by presence of oxygen. In such cases, CFD simulations can be coupled with the population balance modeling (PBM) to accurately account for the evolution of gas bubble distribution and more accurately calculate the average bubble diameter, d_b, as shown in Fig. 16 for a bioreactor application with gas sparging.

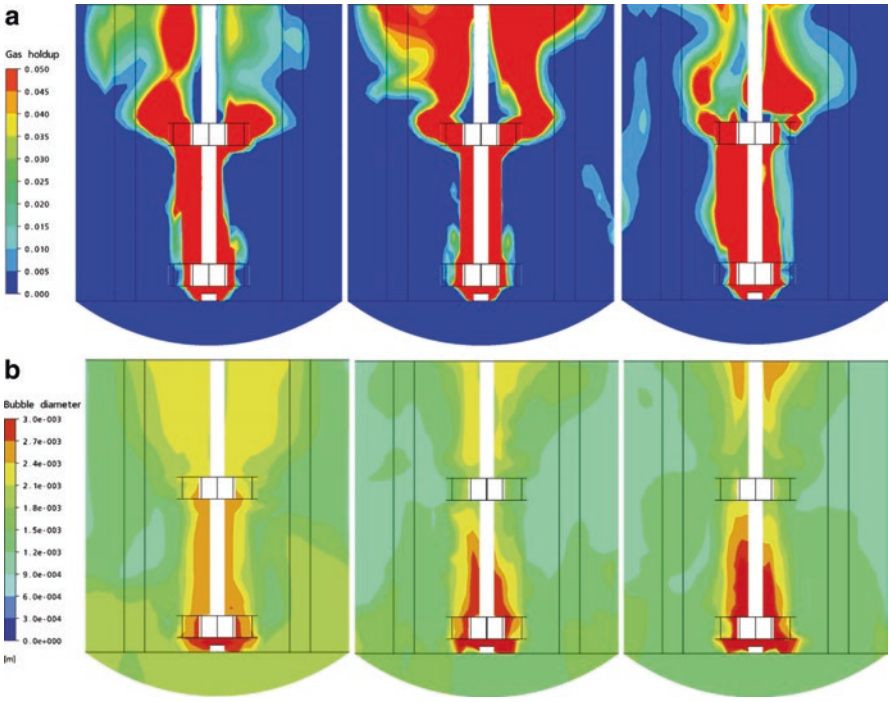

Fig. 16 Contours of (**a**) transient gas holdup distribution and (**b**) transient bubble diameter at t = 10, 40 and 70s in panels from left to right at 400 RPM and sparging air flow rate of 6 L/min. (Adapted from Wang et al. [39])

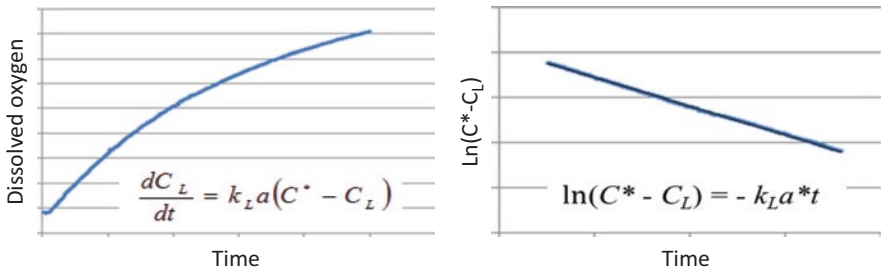

Fig. 17 (**a**) Dissolved oxygen as a function of time as measured using a DO probe in the vessel and (**b**) $\ln(C^*–C_L)$ plotted as a function of time to extract K_La for a given operating condition in a vessel

Experimental Approach to Understand Oxygen Impact

In situations where multiphase CFD is not available, simple experiments can be performed for K_La measurements in processing vessels to enable robust and reliable scale-up. A dissolved oxygen (DO) probe is inserted into the vessel, and the quantity of dissolved oxygen is measured as a function of time as shown in Fig. 17. Single-phase CFD can be used to guide the optimum location of the DO probe to avoid placement in local dead zone. Knowing the equilibrium oxygen concentration, C^*, in the fluid at a given temperature and pressure, the quantity $\ln(C^* – C_L)$ is plotted as function of time and the slope is the K_La. Performing this experiment systematically at different fill volume (V) and impeller agitation rates (n), we can then fit K_La as shown in Eq. 16.

$$77K_La = a_1\left(\frac{\sum_i \rho N_p n^3 D^5}{V}\right)^{a_2} \tag{16}$$

The fitting parameters, a_1 and a_2, are specific to a given vessel/impeller configuration. Performing experimental K_La characterization of a given mixing system is a one-time exercise that can benefit the understanding of all oxygen and/or interface-sensitive biomolecules.

In order to scale-up oxygen-sensitive processes, scaling the process such that K_La remains constant is a best practice. Lab-scale experiments can be used to identify the K_La that does not lead to product degradation at lab scale; the fill level (batch size) and impeller agitation rate can then be selected for scale-up in such a way that K_La remains constant across scale.

4 Concluding Remarks

The performance and product quality resultant from any drug product unit operations is often greatly impacted by the hydrodynamics within a stirred vessel. Standard, empirically derived correlations may in some cases be able to provide a starting

point for understanding appropriate mixing conditions. Most often, however, simple correlations cannot sufficiently capture processing complexities known to impact the quality of biomolecules. Many vessels commonly used in industry do not conform to standard designs, and scale-up is required into a vessel of dissimilar geometry. Correlations do not adequately capture the important hydrodynamic characteristics in this case. As discussed, biomolecules are also often sensitive to shear and interfacial effects; sufficient mixing is required to homogenize the product, but excessive shear and gas entrainment can compromise product quality. For these applications, a combination of first principles modeling and model-guided experiments can provide the best information to guide reliable scale-up.

The several examples discussed demonstrate that computational fluid dynamic modeling is a reliable means of understanding hydrodynamic characteristics that are inaccessible by mere experimentation. Much can be inferred about the hydrodynamics in multiphase, shear-sensitive mixing operations from single-phase CFD simulations. Moreover, software packages are available that make single-phase CFD a tool that can be leveraged by both novices and experts. More advanced multiphase simulations may be the most appropriate means of generating understanding of the gas distribution, gas holdup, and mass transfer coefficients in two-phase systems. Appropriate CFD modeling, coupled with model-guided experiments (when possible), can provide a comprehensive understanding of process-specific hydrodynamics to guide reliable process scale-up.

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Chapter 23
Case Study for the Implementation and Utilization of a Technology Platform for Sterile-Grade Filtration

Christine Rinn and Michael Siedler

1 Introduction

The aim of this chapter is to exemplify how Quality by Design (QbD) principles can be implemented for the sterile filtration process of biologics. The chapter shows the systematic approach to establish a platform technology where the in-depth characterization and the increased level of process understanding allow the definition of a lead and backup filter configuration to be used as a standard for sterile-grade filtration.

The systematic approach starts with the identification of parameters that could potentially affect the filtration unit operation as shown in Fig. 1. The identified parameters are being used to conduct an initial risk assessment that describes the relationship between all identified process parameters and critical quality attributes (CQAs) summarized in Table 4.

In the next step, the available prior knowledge is evaluated and used to define the studies needed to sufficiently explore the required knowledge space, to identify the critical process parameters (CPPs), and to establish a sterile filtration technology platform.

Finally, it is described how the established platform can be applied to justify a reduced product-specific filter test program for future molecules.

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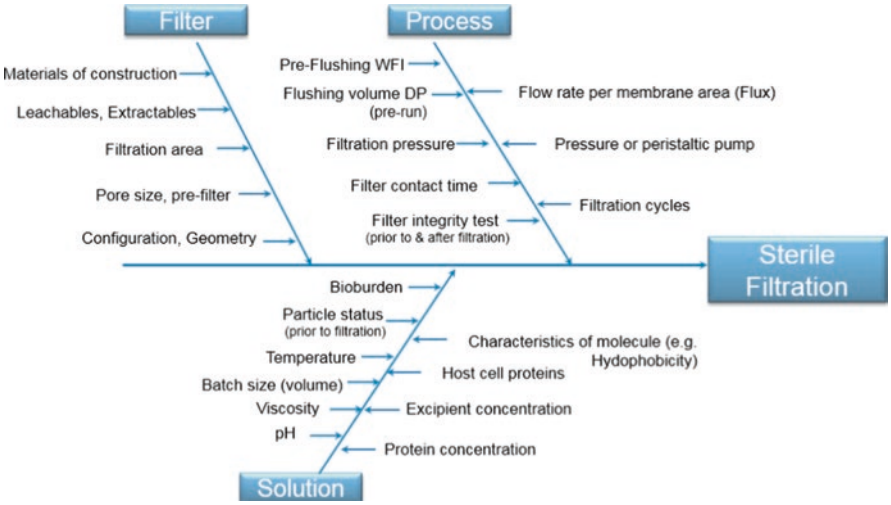


Fig. 1 Identification of parameter potentially affecting the filtration process

2 Identification of Process Parameter

All parameters that may have an effect on the filtration unit operation were systematically identified, categorized, and visualized using a fishbone diagram as shown in Fig. 1. The identified three main parameter categories affecting the filtration unit operation are the characteristics of the respective filter (e.g., materials of construction), the process parameters (e.g., filtration pressure), and the characteristics of the solution to be filtered (e.g., viscosity).

These identified parameters were fed into the initial risk analysis where the respective impact on the drug product critical quality attributes (CQAs) was assessed. A more detailed description of tools and the process of how CQA can be defined are described in the A-Mab Case Study [1] and therefore will not be discussed in detail.

2.1 Initial Risk Analysis

This section describes the application of the initial cause and effect analysis tool that allows assigning a numerical risk score to each process parameter that is applicable for the sterile filtration unit operation based on the potential impact on the respective CQAs. As shown in Table 1, the process parameters were sorted into four different impact categories based on the available prior knowledge and their potential impact on the drug product CQAs.

CQAs are defined typically as physical, chemical, or microbiological properties or characteristics of the drug product [2].

Table 1 Process parameter impact scale

Definition	Numerical impact level
Indicates that there exists a correlation between the process parameter and quality attribute and that additional development is required to better understand and control the risk	10
Indicates that there may be a correlation between the process parameter and the quality attribute and that additional development is required to better characterize and control the risk	7
Indicates that there may be a correlation between the process parameter and quality attribute, but the relationship is well understood and/or controlled	5
Indicates that there is no correlation between the process parameter and critical quality attribute	1

Table 2 Definition of the criticality of a quality attribute on drug product safety and efficacy

Quality attribute criticality	Quality attribute criticality scale
Strong relationship on safety or efficacy known based on data in hand or experience	10
Strong relationship is expected on safety or efficacy	7
Not-so-strong relationship expected or unknown on safety or efficacy	5
Known to not have a relationship on safety or efficacy	1

Attributes that could potentially affect the safety, efficacy profile, or quality of a molecule are rated according to their designated criticality. It is assumed that the criticality is not a digital value (either high or none) but falls into a continuum of criticality.

Following this paradigm, Table 2 was used to classify the CQAs of the drug product according to their impact on overall product quality (safety and efficacy). Only those quality attributes relevant to the filtration process were considered in the initial risk assessment shown in Table 4.

The initial risk assessment allows the identification of those process parameters that have a high risk and have to be examined in more detail.

The initial risk assessment will help to identify gaps in the existing prior knowledge guiding the experiments that need to be performed to explore the knowledge space sufficiently to identify the design space for a specific molecule. The rationale for process parameter designation and the respective color code is defined in Tables 3 and 4.

In order to generate the required product and process understanding for the listed sterile filtration parameters and their respective impact on CQAs, the available prior knowledge as described in the respective guidelines and regulatory requirements was used as a starting point to setup an experimental program for establishing a sterile filtration technology platform.

Table 3 Rationale for process parameter designation

Parameter designation	Rationale
GPP	General Process Parameter No, low or controlled correlation between the process parameter and the critical quality attributes. All risk levels are 1 or 5 (no or low risk)
KPP	Key Process Parameter The process parameter may have impact on at least one quality attribute and additional development activities required. At least one risk level is 7 (medium risk)
CPP	Critical Process Parameter Correlation between a process parameter and the CQAs exists and additional development activities are required At least one risk level is 10 (high risk)

3 Available Prior Knowledge from Guidelines and Regulatory Requirements

Characterization and validation of the sterile filtration process is one of the most critical steps in the aseptic manufacturing process. Various guidelines from regulatory authorities and organizations such as the Parenteral Drug Association (PDA) are available and constitute the regulatory requirements and state of the art in filter testing. A brief overview and the corresponding references are provided in Table 5, but additional guidelines may be applicable and should be considered for filter evaluation and validation.

4 Exploring the Knowledge Space and Establishing a Sterile Filtration Technology Platform

Based on the initial risk assessment for the filtration process as shown in Table 4, an experimental program was designed to evaluate different filter materials in various studies to explore the impact of process parameters on drug product CQAs and select a platform filter configuration and process based on three antibody solutions. In Table 6, the reference to the identified process parameter from the initial risk assessment to the respective sterile filtration platform study is provided.

The experimental program to establish the platform process/filter was subdivided into three consecutive modules as depicted in Fig. 2:

1. *Pre-selection*:
   Technical data provided by the three filter vendors allowed a pre-selection of filters for the experimental program.
2. *Characterization of fluid characteristics* [6, 9, 10]*:*
   Capacity (V_{max}) studies with 0.22 µm filters and pre-filter disk filters including justification of scale-down model and validity of V_{max} calculations
3. *Compatibility assessment of filter capsules* [6–12].
   Adsorption and particle shedding studies, definition of filter flush volumes, and filter contact studies. Influence of multiple filtrations on drug product CQAs

Table 4 Initial risk assessment for sterile filtration unit operation

Category	Quality Attribute Parameter	10 Protein content	10 Potency	10 Size variants (monomer)	7 Size variants (aggregates)	5 Size variants (fragments)	7 Charge variants (main isoforms)	7 Charge variants (acidic species)	7 Charge variants (basic species)	5 Color	5 Clarity and opalescence (turbidity)	10 Visible particles	7 Sub-visible particles	10 pH-value	10 Bacterial endotoxin	10 Sterility	5 Osmolality	7 Viscosity	1 Conductivity	7 Process related impurities	10 Contaminants Leachables/ extractables	7 DP excipient concentration	5 Oxidation-related variants	Rationale/comment	Risk value
Filter	Materials of construction	7	5	5	5	5	5	5	5	1	5	5	5	5	1	1	5	1	5	10	10	10	10	Adsorption of formulation components onto the filter membrane, particles shedding from the filter and release oxidative species	847
	Leachables / extractables	1	5	5	5	5	5	5	5	5	7	7	7	7	1	1	1	1	5	1	10	1	10	Release of e.g. oxidative species	725
	Filter filtration area	7	1	1	1	1	1	1	1	1	1	1	1	1	1	7	1	1	1	7	1	7	5	Filter size has to be adapted to the respective batch size	386
	Pore size/pre-filter	1	1	7	7	7	5	7	5	1	7	5	7	1	10	10	1	5	1	5	1	1	5	Bacterial/particle retention, induction of particle formation, may change due to chemical incompatibility	760
	Configuration/ geometry	5	1	5	5	5	5	5	5	1	1	1	1	1	1	1	1	1	1	5	1	1	1	Shear stress caused by the geometry/materials of the filter (fluid dynamics)	402
Process/ Filter	Flow rate per membrane area (Flux)	1	1	7	7	7	5	5	5	1	7	1	5	1	1	1	1	1	1	1	1	1	1	High flow may induce a shear stress (aggregation)	436
Process	Filter flush volume WFI prior use	1	1	5	5	5	5	5	5	1	5	7	7	1	1	1	5	1	1	1	7	5	1	Flush with WFI to rinse membrane (oxidative species, particles) and wet for filter integrity test prior use	564
	Flushing (pre-run) volume DP bulk solution	10	1	5	5	5	5	5	5	1	5	7	7	1	1	1	7	5	1	5	7	10	7	Adsorption of formulation components onto the filter membrane and particle shedding from the filter	785
	Filtration pressure	1	1	5	7	5	1	1	1	1	5	1	1	1	1	1	1	1	1	1	1	1	1	The pressure will impact flow rate that might lead to shear stress and foaming	270
	Filter contact time	5	1	5	5	5	5	5	5	1	1	1	1	1	1	7	1	1	5	1	7	5	1	Contamination due to microbial growth through the membrane and compatibility of solution with filter materials	526
	Filter integrity test	1	1	1	1	1	1	1	1	1	1	1	1	1	10	10	1	1	1	5	1	1	1	Impact on particulate matter, microbial growth and sterility	502
	Filtration cycles	5	1	7	7	5	5	5	5	1	7	7	10	1	1	5	1	1	1	5	1	7	5	Shear stress may lead to aggregation and visible particle/sub-visible particles formation	715
	Pressure or peristaltic pump	1	1	5	5	5	1	1	1	1	5	1	1	1	1	1	1	1	1	1	1	1	1	The pressure /peristaltic pumping will impact flow rate that might lead to shear stress and foaming	270
Solution	Bioburden prior filtration	1	1	1	1	1	1	1	1	1	1	1	1	1	7	7	1	1	1	1	1	1	1	Bioburden level can affect filtration process efficacy	282
	Particle status (prior to filtration)	1	1	5	5	5	1	1	1	1	5	5	5	1	1	1	1	1	1	1	1	1	1	Impact on filter clogging (filter capacity V_{max}) and flow rate	338
	Filtration temperature	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	No impact on QAs expected, room temperature	162
	Batch size (Filtration vol.)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	No impact on QAs expected. Filter size must be adapted to the respective batch size	162

Table 5 Summary of existing guidelines and regulatory requirements for sterile-grade filtration [2–4]

Category	Parameter	Guidelines/regulatory requirements
Filter	Materials of construction	PDA: Effects of the filter on final product should be evaluated (compatibility with entire device) [3] FDA: Type of filter membrane used for commercial must be evaluated in filter validation studies [4]
Process	Flushing (pre-run) volume DP bulk solution	PDA: Effects of the filter on final product should be evaluated (product adsorption) [3]
Filter	Pore size/pre-filter	Ph.Eur. 5.1.1, Ph.Eur. 2.6.1: <0.2 µm pore size "sterile grade" [5] PDA: Classification of the membrane (manufacturer) and demonstrating product-specific microbial removal [3] FDA: Pore size of 0.2 µm or smaller [4]
Process	Filtration cycles	FDA: Filter should be discarded after a single lot or maximum number of lots specified [4]
Filter	Leachables/ extractables	PDA: Effects of the filter on final product should be evaluated [3]
Process	Filter flush volume WFI prior use	PDA: Particle contamination from the filter should be evaluated; preflushing may be performed to reduce particulates and contaminants prior to integrity testing [3]
Process	Filter contact time	PDA: Effects of the filter on final product should be evaluated (compatibility) and validation of max. filtration time required. Increasing filtration times may increase potential for bacterial penetration [3] FDA: Maximum filter time must be validated [4]
Process/ filter	Flow rate per membrane area (flux)	PDA: Impact on bacterial retention [4]
Process	Filter integrity test (prior to and after filtration)	Ph.Eur. chapter 5.1.1, Ph.Eur. 2.6.1, (FDA): Filter integrity test as in-process control (the primary filter in the filter train should be the last filter in the train) [5] PDA: Pre- and post-filtration integrity test should be used. In case of redundant filtration, the second redundant filter must pass integrity testing; additional filter does not require testing [3] FDA: Post-use can be done prior to processing, forward flow and bubble-point test, test specification consistent with filter validation studies [4]
Filter	Filter filtration area	PDA: Revalidation required, if volume for a given filtration area increases [3]
Filter	Configuration/ geometry	PDA: Two or more filters in a series can be used to ensure sterile effluent, but if both filters are a process requirement and validated to ensure sterility, both filters must pass integrity testing FDA: Redundant filters should be considered [3]
Process	Filtration pressure	PDA: Operating ranges provided by filter manufactures, variables should be evaluated for compatibility with the operating limits and filtration area [3] FDA: Maximum filter pressure must be validated, production conditions [4]

(continued)

Table 5 (continued)

Category	Parameter	Guidelines/regulatory requirements
Process	Pressure or peristaltic pump	Not defined
Solution	Particle status (prior to filtration)	Not defined
Solution	Bioburden prior filtration	FDA: Retains the classic challenge of at least 1×10^7 colony forming units of *B. diminuta* ATCC-19146/cm^2 of effective filtration area at pressures up to 30 psi (2 bar) Acceptance criteria: Expected value excipients and drug substance [4] FDA: <100 cfu/ml [4], PDA Tech. Guide 26: Control by raw materials and process
Solution	Filtration temperature	PDA: Operating ranges provided by filter manufactures, high temperatures could result in increased leachables [3]
Solution	Batch size (filtration vol.)	PDA: Revalidation required, if volume for a given filtration area increases [3] FDA: Production conditions must be validated [4]

Table 6 Summary of identified studies required to establish a sterile filtration technology platform

Category	Process parameter	Data from sterile filtration platform (MAB-A, MAB-B, MAB-C)
Filter	Materials of construction	Filter suitability assessment, description of filter materials
Process	Flushing (pre-run) volume DP bulk solution	Adsorption study with filter disks and capsules
Filter	Pore size/pre-filter	Pre-filter evaluation (Pre-filter V_{max}), multiple filtrations, DoE V_{max} 0.2 µm with various concentrations [6]
Process	Filtration cycles	Multiple filtration study
Filter	Leachables/extractables	Not tested
Process	Filter flush volume WFI prior use	Adsorption study capsule, residual water, particle shedding study [7, 8]
Process	Filter contact time	Filter contact study
Process/ filter	Flow rate per membrane area (flux)	DoE V_{max} 0.2 µm testing [6]
Process	Filter integrity test (prior to and after filtration)	Filter contact study
Filter	Filter filtration area	Filter suitability assessment, DoE V_{max} 0.2 µm testing
Filter	Configuration/geometry	Filter suitability assessment
Process	Filtration pressure	Multiple filtration study, DoE V_{max} 0.2 µm
Process	Pressure or peristaltic pump	Pressure filtration tested
Solution	Particle status (prior to filtration)	Pre-filter DoE V_{max} 0.2 µm, filtration after diafiltration (high particle load)
Solution	Bioburden prior filtration	Not investigated
Solution	Filtration temperature (RT)	No testing, constant
Solution	Batch size (filtration vol.)	DoE V_{max} 0.2 µm

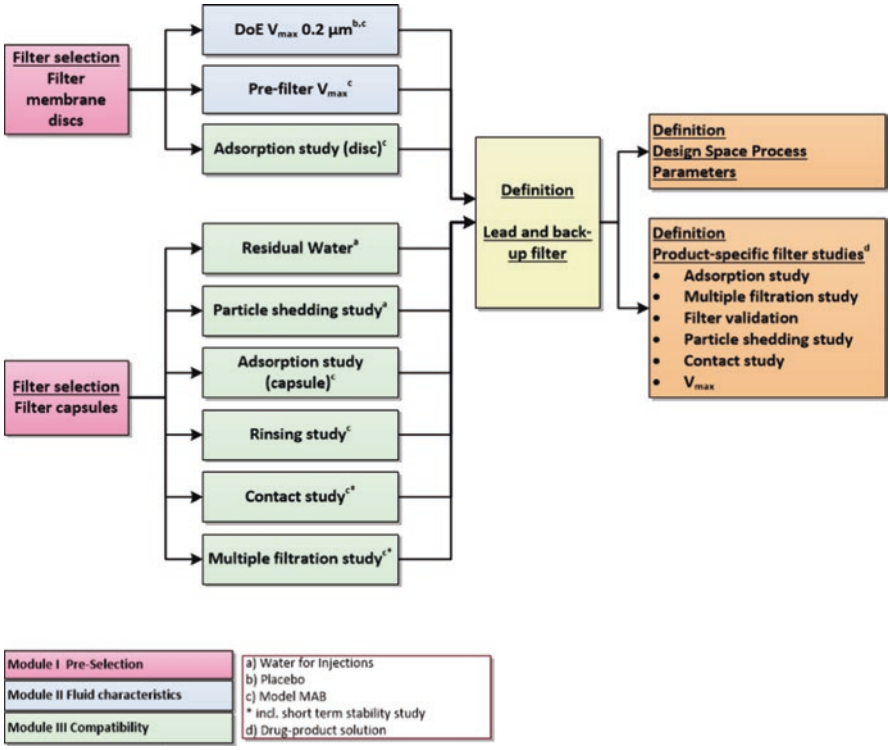


Fig. 2 General filter selection program flowchart of studies to evaluate the performance of sterile-grade filter leading to the identification of a lead and backup filter configuration, the design space for process parameters, and the definition of product-specific filter tests

Pre-selection Module The aim of the first module was to evaluate a broad range of filters regarding their overall suitability for the intended sterile-grade filtration process based on vendor specifications and literature.

A screening of potential filters was performed on filters from three different vendors with regard to their suitability in the laboratory (e.g., to allow scale-down models) and in GMP manufacturing facilities (e.g., manufacturing of clinical supplies and commercial drug product). This includes, e.g., the availability of all required certificates from the filter manufacturers in order to enable the usage in GMP-controlled areas. Since not all filter suitability criteria have the same importance, the weight of each criteria was defined according to Table 7.

Rationale for filter suitability criteria rated with "10" – high weight scale

- *Capsule availability:* filter should be available as a single-use, disposable unit.
- *Scalability range:* filter type should cover filter sizes (membrane areas) in the same configuration for different batch sizes.
- *Sterilization methods:* should be available sterile and re-sterilizable.

Table 7 Filter suitability criteria weight scale

Filter suitability criteria	Weight scale
Important selection criteria and established or expected direct relationship to filter suitability	10
Unlikely to be selection criteria, but influences filtration process	5
No important filter selection criteria	1

- *Leachables/extractables:* should be low, may impact product solution.
- *Operating experience:* handling experience as well as impact on product.

Rationale for filter suitability criteria rated with "5" – medium weight scale

- *Membrane material:* should be compatible with product solution, low adsorption. Data available that materials evaluated are suitable for sterilizing-grade filtrations for biologic liquids.
- *Connections*: should have a broad range for use in laboratory and manufacturing facilities.
- *Double Layer:* to be tested if pre-filter is beneficial.
- *Flow rate*: high flow rates reduce filtration process times, but might impact drug product solution.
- *Flush volume*: low flush volumes reduce product solution loss for filter flush. However this value must be investigated.
- *Hold-up volume*: large hold-up volumes of water after sterilization dilute drug product solution and impact filter pre-run.

Rationale for filter suitability criteria rated with "1" – low weight scale

- *Size, housing, and supporting material*: have only low impact on the filtration process or materials show all good properties

In order to assess to what extent the various filters fulfill the predefined filter suitability criteria, each of the filter was analyzed according to their respective characteristics as stated in Table 8. For data that were not available, a "5" was assigned.

Criteria for the suitability ranking

- *Membrane material:* Filters with pre-filter and 0.2 μm filter with the same membrane material were rated "10" and capsules with different materials "5."
- *Capsule availability*: Filter material available as a single-use, disposable filter capsule, ready-to-use "10" and external filter housing required "5." (Only filters in disposable capsule were evaluated.)
- *Connections:* Filter capsule with a broad range of different connector types and size available for different manufacturing areas. Filters with a broad range of connectors were rated "10," only hose barb and one triclamp "5" hose barb "1."
- *Scalability range:* Filter type availability with a broad range of different filter membrane areas in the same filter configuration. "10" for capsule scalability from laboratory scale to production scale, "5" only for laboratory scale and small batch sizes, and "1" either only laboratory-scale or only production-scale filters.

Table 8 Suitability ranking scale

Suitability ranking	Suitability scale	Rationale
Fulfills all requirements	10	Filter is completely suitable for sterile filtration (e.g., all configurations; connections are available; low hold-up volume; all sterilization methods can be applied)
Partly fulfills requirements	5	Filter is only available in some configurations, few connections medium hold-up volume or only some sterilization methods can be applied
Does not fulfill requirements	1	Filter has only few or none of the required criteria

- *Double layer:* Availability of integrated pre-filters: "10" various pre-filter pore size available, "5" only one pre-filter size, and "1" no integrated pre-filter available.
- *Flow rate:* Manufacturer specification with high flow rate with WFI "10" for short filtration times and "5" for low flow rates or data not provided.
- *Size*: Since large filter housing may not fit in filling/isolator lines, smaller filter are preferred. "10" for filters smaller than 14 cm and "5" data not provided or size larger than 14 cm.
- *Housing/supporting material*: Should be compatible with the solutions, but no discrimination could be made at the initial assessment, so "10" was chosen for all capsules.
- *Sterilization methods*: Filters available sterile, ready-to-use, and autoclavable were rated with "10," filters either available sterile but not re-sterilizable *or* not sterile but autoclavable were rated with "5," and filters only available "sterile" but no sterilization possible were rated with "1."
- *Flush volume*: Required flush volume from the manufacturer low rated "10" and data not provided "5."
- *Hold-up volume:* Volume that remains in the filter capsule after use; low (<6 mL) rated "10" and data not provided "5."
- *Leachables/extractables:* low (meets USP/CFR standards) rated "10" and data not provided "5."
- *Operating experience:* Filters already used in laboratory/manufacturing area "10," filters tested in laboratory environment "5," and filters never used before "1."

The outcome of the filter suitability assessment is a numerical suitability score, reflecting the overall suitability of the filter. Only filters rated with a percentage performance score greater than 70% (≈ performance score >546) were selected for the full fluid characteristics and compatibility module. This means that the tested filter fulfills more than 70% of the maximum possible selection criteria (weighted with criticality).

Based on the filter suitability assessment in Table 9, seven different filter capsules from three vendors were identified, as shown in Table 10.

Table 9 Filter suitability assessment

Weight scale	5	10	5	10	5	5	1	1	1	10	5	5	10	5		
Filter suitability criteria / *Filter type*	Membrane material	Capsule availability	Connections	Scalability range (filtration area (m^2)	Double layer–(pre-filter)	Flow rate (WFI)	Size (length)	Housing material	Supporting material	Sterilization method options	Recommended flush volume	Hold-up volume	Leachables/ extractables	Operating experience	Suitability Score	% Suitability score
Vendor A Filter 1 (PVDF disk)	10	10	10	5	1	5	10	10	10	10	10	5	5	10	585	75.0
Vendor A Filter 2 (PVDF pleated)	10	10	10	1	1	5	5	10	10	10	5	5	10	1	520	66.7
Vendor A Filter 3 (PES disk)	10	10	1	1	1	10	10	10	10	1	5	5	5	5	385	49.4
Vendor A Filter 2 (PES pleated)	10	10	10	10	5	5	10	10	10	10	5	5	10	1	635	81.4
Vendor A Filter 2 (Cellulose prefilter+PVDF)	5	10	5	1	10	10	5	10	10	10	5	5	5	1	490	62.8
Vendor A Filter 2 (Cellulose)	10	10	10	1	5	5	5	10	10	10	5	5	5	1	490	62.8
Vendor B Filter 4 (PES)	10	10	5	10	10	5	10	10	10	5	10	10	5	1	585	75.0
Vendor B Filter 4 (PVDF)	10	10	5	10	5	5	10	10	10	5	10	10	5	1	560	71.8
Vendor B Filter 5 (PES)	10	10	5	5	5	5	10	10	10	5	5	10	5	1	485	62.2
Vendor B Filter 5 (PVDF)	10	10	5	5	5	5	10	10	10	5	5	10	5	1	485	62.2
Vendor C Caps Filter 6 (CA)	10	10	5	10	10	5	10	10	10	10	5	5	5	5	605	77.6
Vendor C Filter 7 (PES)	10	10	5	5	10	10	10	10	10	10	5	5	5	5	580	74.4

If the filter suitability score was greater than 70 %, the filter was marked "green" and was tested in the experimental program. Filters with a score below 70 % were highlighted "yellow" and were not tested.

Table 10 Filter capsules for experimental program

Manufacturer	Material	Pore size	Single/dual layer	Filtration area [m^2]
Vendor B	PVDF	0.2 + 0.2 µm	Dual	0.02
Vendor B	PES	0.45 + 0.2 µm	Dual	0.02
Vendor C	Cellulose	0.45 + 0.22 µm	Dual	0.015
Vendor C	PES	0.45 + 0.22 µm	Dual	0.015
Vendor A	PVDF	0.2 µm	Single	0.01
Vendor A	PES	0.2 µm	Single	0.022
Vendor A	PES	0.5 + 0.2 µm	Dual	0.014

In addition to the selected filter capsules, the following 47 mm disk filters were also included in the evaluation for V_{max} and adsorption studies only:

- A filter with a membrane made from Nylon was tested as a benchmark since this material is commonly used filter for i.v. administration
- A filter with a membrane made of cellulose (Vendor A) to compare different cellulose materials

All selected filters were subjected to the outlined fluid characteristics and compatibility studies as shown in Fig. 2, and a summary of all results is provided in Table 11.

5 Overall Available Knowledge Space and Evaluation of a Reduced Product-Specific Filter Evaluation

A summary of all process parameter ranges from the sterile filtration platform is summarized in Table 12. Furthermore an evaluation whether a molecule-specific testing is required for the lead and backup filter is provided, as well as a possible control strategy for the respective parameter.

6 Conclusion

Based on the sterile filtration platform evaluation described above, the standard lead and backup filters are Filter 7 and Filter 6 capsules from Vendor C or the respective membrane type as a filter element for stainless steel housings in commercial manufacturing areas.

Available knowledge for process parameters and their respective impact on pCQAs allows a first assessment to reduce product-specific filter testing.

The design of product-specific filter compatibility evaluation studies and timing of the studies and validation activities during development of a new biologic will be defined as part of the overarching manufacturing process development strategy.

Table 11 Available knowledge space based after establishing the sterile filtration technology platform

Category	Process parameter	Data from sterile filtration platform (MAB-B, MAB-A, MAB-C)
Filter	Materials of construction	The information about the impact of the respective filter materials have been evaluated as part of the filter suitability assessment and description of filter materials
		The outcome of the studies according to Module I/II/III resulted in identifying Filter 7 and Filter 6 as lead and backup filter, respectively. The definition is mainly based on their fluid characteristics. All tested filter materials showed almost comparable filter compatibilities/impact on CQAs. No filter membrane material showed incompatibilities with the tested fluids (Figs. 3, 4, and 5)

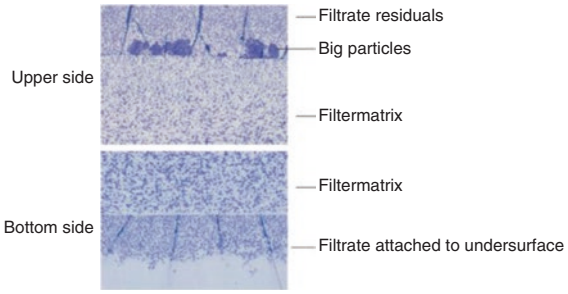


Fig. 3 Light microscopy image of membrane Filter 7 after filtration with MAB-A solution

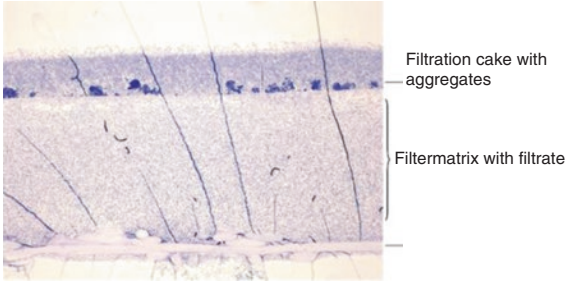
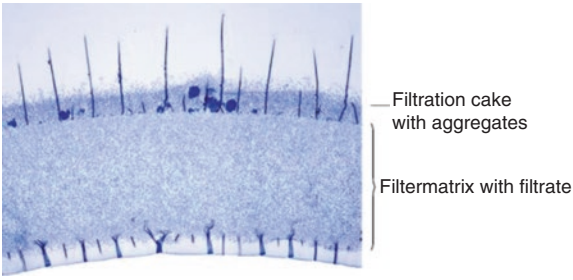


Fig. 4 Light microscopy image Filter 6 after filtration with MAB-A solution

(continued)

Table 11 (continued)

Category	Process parameter	Data from sterile filtration platform (MAB-B, MAB-A, MAB-C)
		 **Fig. 5** Light microscopy image Filter 1 after filtration with MAB-A solution
Process	Flushing (pre-run) volume DP bulk solution	Based on the capsule adsorption study and the disk adsorption study, the PES membrane showed the lowest protein adsorption, whereas PVDF showed the highest adsorption
		Furthermore, the protein adsorption is depending on the formulation composition. Adsorption studies should be conducted with manufacturing scale capsules. Filter soaking was not tested in the sterile filtration platform, but could be an option to reduce drug product filter flush volume
Filter	Pore size/ pre-filter	A pre-filter evaluation with PES, cellulose, and PVDF membranes for MAB-A did not reveal any relevant differences in filter capacity of unfiltered solution
		MAB-B: Data comparison shows higher flow rates for a solution that was passed through a 0.45 pre-filter prior to the 0.2 µm sterile-grade filter
		No gradual pore blockage occurred; consequently no V_{max} could be calculated. So, clear benefit in using a pre-filter is not supported by the data
		V_{max} calculations are very sensitive to minor flow rate changes and could either be performed over a longer period or smaller filter areas to obtain a more reliable data set, since solutions are very clean and very low blockage can be detected for the tested solutions, especially for larger pore sizes
Process	Filtration cycles	MAB-A and MAB-B: For filtrations up to five times, no impact on critical quality attributes could be detected by conducting a short-term stability study up to 3 months storage at 5 °C or 40 °C for 100 mg/ml

(continued)

Table 11 (continued)

Category	Process parameter	Data from sterile filtration platform (MAB-B, MAB-A, MAB-C)
		25× filtration cycles of MAB-A drug product solution lead to increased sub-visible particle >1 µm content, but the particle count remained constant in the subsequent short-term stability evaluation
Filter	Leachables/ extractables	Not tested as part of establishing the sterile filtration technology platform. Filters for experimental program were selected based on vendor information
Process	Filter flush volume WFI prior use	Filters should be rinsed and bubble-point tested (or blown out with high pressures) prior to autoclaving to expel excess water out of the filter. Residual water dilutes the drug product solution and therefore increases the flushing volume that must be discarded. Filter 4 capsules showed highest residual water contents
		Filter 7 and Filter 1 filters did not shed any particles when rinsed with water; sub-visible particles were comparable for all filters tested
Process	Filter contact time	The filter contact studies for all MAB solutions tested, covering 24 hours storage in filter capsules, showed no impact on CQAs
Process/ filter	Flow rate per membrane area (flux)	As expected, the V_{max} 0.2 µm DoE study showed for all tested solution that flow rates are increasing with increasing filtration pressure and decreasing with protein concentration. The highest flow rates can be observed for PES membranes from Vendor A and C as shown in the corresponding main effects plots in Figs. 6 and 7. The respective placebo solutions were tested for comparison and showed as expected a much higher flow rate (two to three-fold) compared to a protein solution with a concentration of 100 mg/mL

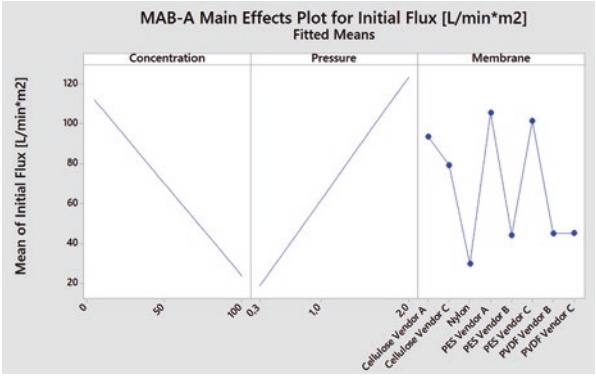
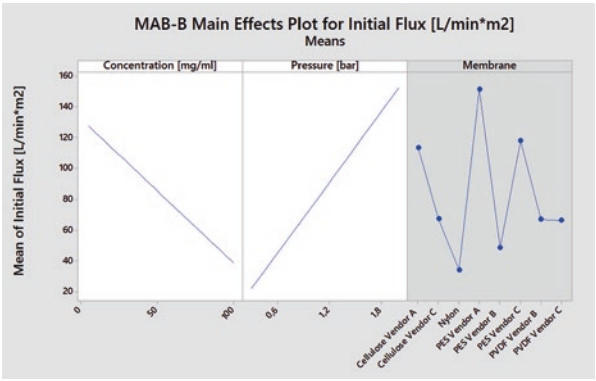


Fig. 6 MAB-A main effects plot for initial flux

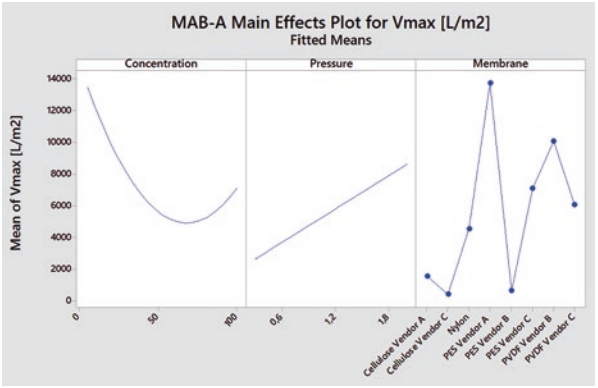
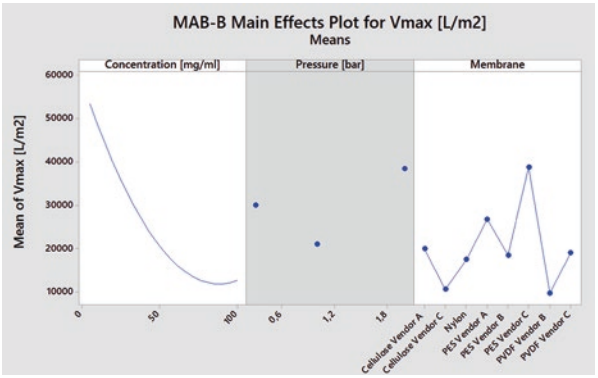
(continued)

Table 11 (continued)

Category	Process parameter	Data from sterile filtration platform (MAB-B, MAB-A, MAB-C)
		 **Fig. 7** MAB-B main effects plot for initial flux
Process	Filter integrity test (prior to and after filtration)	Filter contact studies for all MAB solutions using 24 hours storage in capsules showed no impact on the filter membrane as determined by bubble-point measurements
		Rinse volume to wash out residual drug product solution from the filter to conduct a water bubble-point test: Rinsing volume was highest for Filter 4 (PES) for all solutions tested
Filter	Filter filtration area	Disk filters with 13.5 cm^2 were used to determine V_{max}. To assure a robust scale-up to enable larger batch sizes capsules up to 0.022 m^2 were also tested in the sterile filtration platform evaluation, mainly for compatibility assessments and to also cover capsule component materials
Filter	Configuration/ geometry	Based on the outcome of the filter suitability testing, filter in single-use capsules (staked disk and pleated membranes) and plain disk filters were the most suitable for developmental studies in the lab and scale-up for clinical manufacturing
Process	Filtration pressure	The impact of pressure (and of the filter type) on flow rates is higher for low concentrated solutions as seen on the examples of MAB-A (Fig. 6) and MAB-B (Fig. 7): Flow rates are similar at low pressures but become more filter dependent at higher filtration pressures
		In general, higher pressures and lower concentrations lead to larger filter capacity (L/m^2) filter area (see Figs. 8 and 9). V_{max} is a pressure-dependent value, and the maximum throughput is highest at 2 bars filtration pressure. However, pressure has only little influence on V_{max} for the 5 mg/mL concentrated protein solution. For the 100 mg/mL concentrated solutions, V_{max} values at 0.3 bar are considerably lower compared to 2 bar (MAB-A, MAB-B)

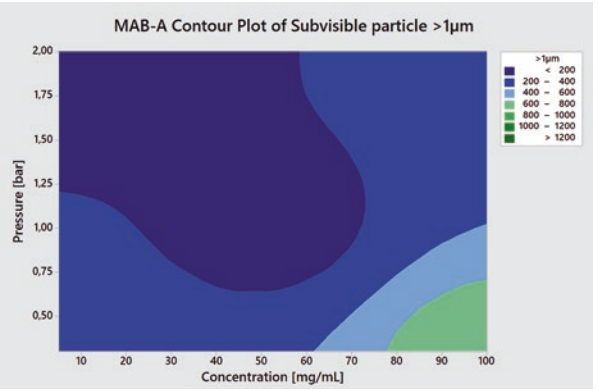
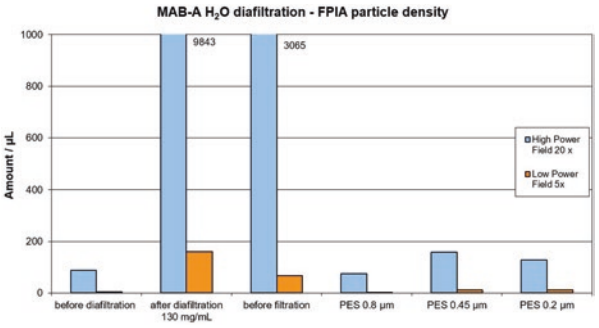
(continued)

Table 11 (continued)

Category	Process parameter	Data from sterile filtration platform (MAB-B, MAB-A, MAB-C)
		 **Fig. 8** MAB-A main effects plots for max. filter capacity (V_{max}) for all evaluated filters  **Fig. 9** MAB-B main effects plots for max. filter capacity (V_{max}) for all evaluated filters No relevant pressure-dependent difference in protein stability was observed for the test solutions filtered through the tested filters, except for the number of sub-visible particle >1 µm for MAB-A. The evaluation of all filters indicates an increase of sub-visible particle >1 µm with increasing protein concentration and decreasing filtration pressure as shown in Fig. 10

(continued)

Table 11 (continued)

Category	Process parameter	Data from sterile filtration platform (MAB-B, MAB-A, MAB-C)
		 **Fig. 10** Concentration- and pressure-dependent sub-visible particles formation >1 µm of MAB-A
Process	Pressure or peristaltic pump	Only pressure filtration was tested in the sterile filtration platform studies
Solution	Particle status (prior to filtration)	Stressed protein solution (e.g., stirred, diafiltered), block 0.2 µm filter membranes very rapidly. The higher particle load of the solutions (Fig. 10) results in pore clogging and lowers the capacity of a given filter significantly. Sub-visible particles >1 µm after diafiltration are significantly higher compared to particle contents seen prior to a regular manufacturing process. The V_{max} can considerably be increased with the use of a pre-filter. Suitable pre-filters for MAB-A solution after diafiltration are filters with a pore size of 0.45/0.5/0.65/0.8 µm. Cellulose filter configuration with a 0.45/0.2 µm membrane combination is the lead filter configuration for filtrations after diafiltration. Back-up filter is a PES filter 0.5/0.2 µm pore size filter configuration
		For all filters larger particles than the nominal pore size could be detected in the filtrate (Fig. 11). Particles seem to re-form upon passing the filter membrane (Figs. 12 and 13)  **Fig. 11** FPIA particle data for MAB-A prior to and after diafiltration in H2O

(continued)

Table 11 (continued)

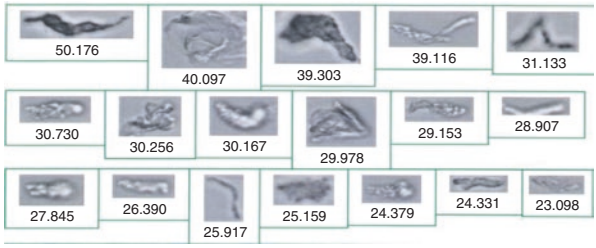
Category	Process parameter	Data from sterile filtration platform (MAB-B, MAB-A, MAB-C)
		 **Fig. 12** FPIA particle images for MAB-A after diafiltration and concentration to 130 mg/mL – HPF  **Fig. 13** FPIA particle images for MAB-A after diafiltration, dilution to 100 mg/mL and 0.2 µm filtration – HPF
Solution	Bioburden prior filtration	Not part of the sterile filtration platform studies
Solution	Filtration temperature (RT)	All experiments were conducted at 20–25 °C
Solution	Batch size (filtration vol.)	The max. capacity of a filter is often determined by V_{max} testing. Since drug product solutions usually do not contain high amounts of particles, because drug substances and excipient solutions are already sterile filtered prior to compounding. Therefore, only minimal pore blockage is detectable, and V_{max} showed high variability
		For most tested solutions, the V_{max} calculations failed the acceptance criteria for model validity (data correlation >0.99), especially for low concentrated solutions
		Therefore, V_{max} is not a suitable test method for the tested MAB solutions for the development of the sterile filtration platform

Table 12 Application of prior knowledge for molecule-specific selection of sterile-grade filters based on an established technology platform

Category	Parameter	Product-specific evaluation needed?	Standardized product-specific filter studies	Design space or prior knowledge	Control space	Control strategy
Filter	Materials of construction	No	N/A	*Established platform:* Lead filter: PES Back-up filter: Cellulose	Lead and backup filter 0.2 µm (with or without pre-filter)	Definition in manufacturing documents
Process/ filter	Flushing (pre-run) volume DP bulk solution	Yes	Adsorption study	*Prior knowledge:* Required flush volume is dependent on formulation composition. The Design space (min value) will be based on the product-specific testing	min value + safety margin	Definition in manufacturing documents
Process	Pore size/pre-filter	No	N/A	*Established platform: U*se of 0.2 µm pore size filters and 0.45/0.2 µm pore size dual-layer membranes	Definition and validation of lead and backup filter for each molecule/formulation	Definition in manufacturing documents
Process	Filtration cycles	Yes	Multiple filtration study	Molecule specific evaluation of multiple filtration cycles The design space (e.g., five cycles) will be based on the product-specific testing	Definition of maximal no. of filtration cycles	Definition in manufacturing documents
Process	Leachables/ extractables	Yes	Filter validation	Product-specific filter evaluation of leachables to comply with USP Oxidizable substance test and tested as part of material validation program (CFR 21, part 211.65) [13]	N/A	Definition in manufacturing documents
Process	Filter flush volume WFI prior use	Yes	Particle shedding study	Filter size-specific evaluation needed	Definition of minimal filter flush volume	Definition in manufacturing documents

Filter	Filter contact time	Yes	Contact study and filter validation	Product-specific filter validation and compatibility study needed	Definition of maximum filtration time	Definition in manufacturing documents
Process	Flow rate per membrane area	Yes	Measured with intended large scale filtration setup (e.g., incl. tubing and pump pressure tanks)	Product-specific flow rate	Definition of maximum flow rate	Definition in manufacturing documents
Filter	Filter integrity test (prior to and after filtration)	Yes	Bubble point test	Product-specific validation required	Must pass minimum acceptance criteria of established filter integrity testing	Definition in manufacturing documents
Filter	Filter membrane area	Yes	V_{max} study	Batch size-dependent definition and validation needed	Definition of minimum filter membrane area per filtration volume	Definition in manufacturing documents
Filter	Configuration/ geometry	No	N/A (defined by vendor)	Pleated single-use capsules as single or two filters in series	Definition of lead and backup filter type	Definition in manufacturing documents
Process	Filtration pressure	No	N/A	Established platform: 0.3–2 bar	Filtration pressure between 1 and 2 bars	Definition in manufacturing documents
Process	Pressure or peristaltic pump	N/A	N/A	N/A Dependent on manufacturing process	N/A	Definition in manufacturing documents
Solution	Particle status (prior to filtration)	No	N/A	*Established platform:* pre-filtered API and excipient solution for dilution	Pre-filtered API and excipient solution for dilution	Definition in manufacturing documents

(continued)

Table 12 (continued)

Category	Parameter	Product-specific evaluation needed?	Standardized product-specific filter studies	Design space or prior knowledge	Control space	Control strategy
Solution	Bioburden prior filtration	Yes	Bacterial retention as part of filter validation	Bacterial retention study as part of the filter validation And the maximal bioburden content of <100 cfu/mL	<100 cfu/mL	Bioburden testing and aseptic handling
Solution	Filtration temperature	No	N/A	15–30 °C	15–25 °C	Temperature-controlled manufacturing facilities
Solution	Batch size (filtration vol.)	Yes	V_{max} study and filter validation	Batch size-dependent definition and validation needed	Definition of minimum filter membrane area per filtration volume	Definition in manufacturing documents

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Chapter 24
Considerations and Challenges When Filling High-Concentration Monoclonal Antibody Formulations into Prefilled Syringes

Wendy Shieu and Yuh-Fun Maa

1 Introduction

Over the past decade, the use of devices in the biopharmaceutical industry has become more widespread due to their ease of use compared to traditional vial and intravenous fluid bag delivery systems as well as their ability to enable subcutaneous and intramuscular home administration for patients with chronic conditions [1]. One of the most common primary containers used with biopharmaceutical devices for subcutaneous delivery is the prefilled syringe (PFS). However, the drug product manufacturing process for PFSs can include several challenges not observed in traditional vial formats, such as:

- *Optimization of pump parameters for an ideal filling profile:* Due to the geometry of a syringe, PFSs are typically filled from the bottom-up, with the filling nozzle diving to the base of the syringe and then dispensing fluid as it retracts out. The motion of the nozzle must be well-coordinated with liquid fill (pump) speed in order to obtain a "clean" filling profile (i.e., no splashing, dripping, or bubbles forming). A "clean" profile is desired in order to minimize the occurrence of wet plunger stoppers (which can result in a breach of container closure integrity) and to avoid fill weight deviations due to droplet dripping at the end of the fill.
- *High-concentration monoclonal antibody (mAb) formulations (e.g., ≥100 mg/mL) with relatively high viscosities:* Due to deliverable volume limitations with subcutaneous delivery, the standard formulation for PFSs typically requires a higher-concentration mAb (e.g., ≥100 mg/mL) compared to formulations for

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vial formats in order to ensure that an efficacious dose can be delivered [2, 3]. However, these higher-concentration mAb formulations typically result in higher viscosities [3], which may impact filling performance, specifically in flow rate and in filling accuracy. Higher-concentration mAb formulations with higher viscosities are also more likely to have the issue of formulation drying and filling nozzle clogging, which is explained in more detail later in the chapter.

- *Tight requirements for fill weight and plunger stopper position:* Although there are no specific regulatory requirements on fill weight tolerances, a tighter degree of precision is generally desired for PFS products if the entire volume is administered during delivery in order to ensure a safe and efficacious dose [4]. In addition to fill weight, the placement of the plunger stopper into the syringe must adhere to tight requirements; the size of the air gap between the plunger stopper and the fluid should be minimized in order to decrease the amount of air injected subcutaneously, but wet plungers can occur if the plunger stopper is inserted too far into the syringe barrel. The position of plunger stopper placement can also affect downstream device assembly as well as the degree of plunger movement that may occur during air transportation [5].

These challenges should be properly addressed as part of process development prior to implementing the PFS processes on the production line. However, obtaining sufficient line time to develop and optimize these processes can be difficult and expensive. Therefore, the rest of this chapter will focus on designing small-scale experimental setups and studies for PFS filling manufacturing process development.

2 Development and Use of a Bench-Top Filling Setup for PFS Process Design and Optimization

A bench-top filling setup can be very useful when designing and optimizing a PFS filling manufacturing process since it can provide more flexibility compared to performing studies directly on the production line in terms of line time availability and material requirements. Additionally, a bench-top setup is more compatible with a high-speed camera system to allow for more in-depth analysis and identification of critical filling process parameters. However, manufacturing-scale studies may still be required to confirm the findings of small-scale studies.

An example of a bench-top filling setup is provided in Fig. 1. The filling system should consist of a pump, identical to the one used on the manufacturing line, and a linear actuator for mimicking the linear motion of the nozzle during filling of drug product. The setup in Fig. 1 highlights a Flexicon peristaltic pump (although it is compatible with other filling pumps) and a high-speed camera for monitoring experiments, which typically would not be possible on an at-scale manufacturing line.

Fig. 1 (Left) An example of a bench-top filling setup with a Flexicon PD12 peristaltic pump and a ROBO Cylinder® linear actuator controlled by a Volo Technologies controller. Connected to the linear actuator is a filling nozzle holder that is directly above a syringe holder. The Volo controller is interfaced with the Flexicon controller and sends it a signal to start pumping liquid. (Top right) The filling experiments can be monitored with a high-speed camera. In this example, a 20/20 hindsight is pictured. (Bottom right) An example of video playback of a filling experiment

3 Optimization of Filling Profiles

When filling syringes using a pump mechanism, there are two sets of filling parameters: filling nozzle movement and liquid flow modulated by the pump. The motion of the nozzle and the flow of the liquid must be synchronized to deliver a "clean" filling profile, where the nozzle retracts to maintain a constant distance from the liquid level, thereby having no contact with the fluid/liquid (too close) or causing undesired splashing/foaming/bubbles (too far apart). Manufacturing sites may use a fixed nozzle movement profile, especially if the motion is driven by a mechanical cam that is controlled by the filler's main drive speed. However, these speeds are often described as a percent (e.g., 50% main drive speed) and must be translated into velocity (e.g., mm/s) in order to develop an optimal filling profile using a bench-top setup. After the filling nozzle movement is translated into the bench-top setup, it is possible to optimize the pump parameters (i.e., velocity, acceleration) in order to align the nozzle movement with the fluid dispense via visual observation with a high-speed camera. Figure 2 depicts a closeup high-speed video of a fill performed using a bench-top setup.

Another consideration for a "clean" filling profile is to minimize dripping from the filling nozzle at the end of a fill. If the fluid drips from the filling nozzle while it is moving out of the syringe barrel, splashing may occur on subsequent fill cycle, and/or liquid droplets may land on the top of the barrel, potentially leading to wet plunger stoppers. Inconsistent dripping could also decrease fill weight accuracy.

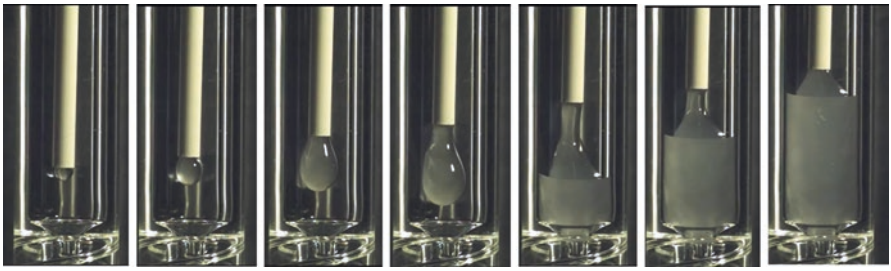

Fig. 2 High-speed video screenshots of a fill performed using a bench-top setup

Most pump systems allow for a reversal of the pump mechanism at the end of dispense in order to "suck back" (SB) any residual droplets left on the nozzle tip and minimize dripping. Some pump systems have a relatively simple reversing or "suck back" setting where the user can only enter a nominal number (e.g., 1–10) to control the degree of pump reversal, whereas others allow for the user to control the exact degree of reversal as well as the speed and acceleration for the reversing motion. A high-speed camera on a bench-top setting can be utilized to monitor droplet formation across multiple fill dispenses in order to verify that filling behavior remains consistent from fill to fill.

Although manufacturing sites often used a fixed nozzle movement profile, pump parameters must be individually optimized for each product due to the influence of individual fluid properties (i.e., viscosity, elasticity, surface tension between the fluid and the filling nozzle surface, etc.). For example, pump acceleration typically will need to be increased with increasing fluid viscosity in order to align with the same nozzle movement profile. This can be explained with the Hagen-Poiseuille equation for laminar fluid flow through a pipe (Eq. 1):

$$Q = \pi R^4 \Delta P / 8 \mu L \tag{1}$$

In this instance, the pipe is the tubing in series with the filling nozzle, which means that fluid flow is a function of pressure drop (ΔP), fluid viscosity (μ), nozzle and tubing radius (R), and nozzle and tubing length (L). For a given pipe/tubing (fixed R and L) and nozzle motion profile at a fixed Q, fluid viscosity must have a direct relationship with pressure drop, meaning that the pressure drop must increase as fluid viscosity increases in order to maintain Q. This increased pressure drop can be achieved by increasing the acceleration of the pump (i.e., $\Delta PA = ma$, where m is the mass of the fluid, a is the acceleration, and A is the surface area where the pressure is applied). Since acceleration is a finite setting, a limitation may eventually be reached where the pump cannot increase in acceleration enough to meet the desired nozzle movement profile. When this occurs, increasing the tubing/nozzle radius or piston size may have to be considered—depending on the pump system being used—but these options have the downside of potentially impacting filling precision. The bench-top setup can be utilized to perform filling precision studies, which are explained later in the chapter.

Fluid viscosity can also influence the amount of dripping that occurs at the end of the fill. Optimizing droplet formation at the end of a fill is the final step of a "clean" filling profile. To mitigate dripping, the pump can either be set at a higher SB at the end of the fill or a larger filling nozzle can be used. These observations can also be explained by the Hagen-Poiseuille equation (Eq. 1): A greater suction force (ΔP in the direction opposite of the liquid fill) is required to pull a more viscous fluid, but the suction force required for a larger nozzle (larger R) is lower. However, both parameters can influence other performance attributes besides post-fill dripping, including mitigating the issue of formulation drying at the filling nozzle tip, which is explained in the next section.

4 Mitigation of Formulation Drying at Filling Nozzle Tip during Prolonged Interruptions

Process interruptions may occur for uncertain durations throughout filling operation. During these interruptions, the fluid at the nozzle tip may dry, resulting in a complete or partial blockage of the nozzle. Nozzle blockage can cause an explosive fill due to pressure buildup immediately after the filling operation is resumed (Fig. 3).

The formation of a solid plug begins with the pinning of the liquid to the solid edge of the filling nozzle. Water evaporates at this liquid-substrate interface, and the remaining solids create a solid ring at the inner surface of the filling nozzle (Fig. 4).

Fig. 3 Screenshots from videos of attempting to dispense after nozzle clogging has occurred. (Left) Fluid stream bursts out of the right side of the nozzle. (Right) High-speed fluid stream bounces off the bottom of the syringe and up against the syringe barrel

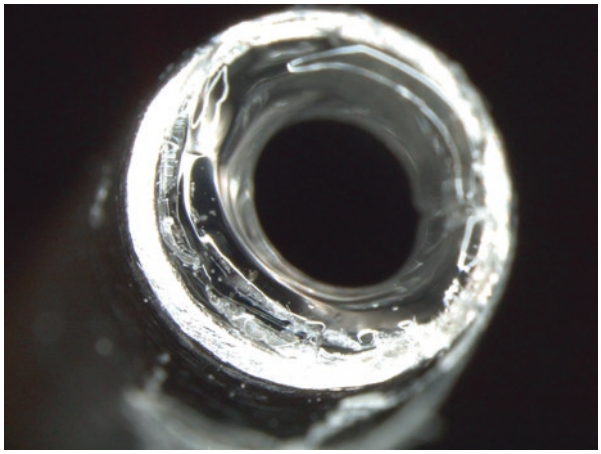

Fig. 4 Magnified image of the formation of a dried ring inside of a filling nozzle

As the liquid on the liquid-substrate interface dries, it must be replenished by liquid from the interior via capillary flow [6]. The dried ring will keep growing toward the center of the nozzle, leaving a smaller opening or clogging the nozzle completely.

This formulation drying phenomenon is more likely to occur with high-viscosity, high-concentration mAb products that are more commonly filled into PFSs. The more viscous liquid is more likely to result in a liquid plug at the filling nozzle tip. The higher solid content associated with higher-concentration mAb will typically result in the quicker formation of a hard protein plug due to the lower water content in the formulation. The relationship between viscosity and liquid plug formation is pictured in Fig. 5. Water (1 cP at 25 °C), a 200 mg/mL mAb solution (~10 cP at 25 °C), and a 60% sucrose solution (~60 cP at 25 °C) are filled through a glass nozzle using identical filling parameters. Figure 5 shows how each fluid looks like inside the filling nozzle after performing a dispense. Water, the lowest-viscosity fluid, barely forms a liquid plug at the tip of the nozzle, whereas the higher-viscosity solutions have obvious liquid plugs. The differences in viscosity influenced the thickness of the liquid film that adhered onto the inner surface of the filling nozzle. For water, the amount that had adhered onto the inner surface of the nozzle was too small to form a plug, whereas the higher-viscosity solutions had a thick enough layer to result in the accumulation of a liquid plug at the filling nozzle tip.

Many variables can influence the rate of drying and the likelihood of a complete clog of the filling nozzle. Many risk mitigation tools and methods that would not be possible to use on a manufacturing line can be utilized on a bench-top setup. Therefore, if a product is at risk of drying or has been observed to clog nozzles during actual manufacturing, it is recommended to use a bench-top setup to better understand the phenomenon and determine the best path forward for mitigation. Some parameters that have been determined to affect formulation drying phenomenon include (a) pump SB (also known as “suck back,” reversing, or retraction)

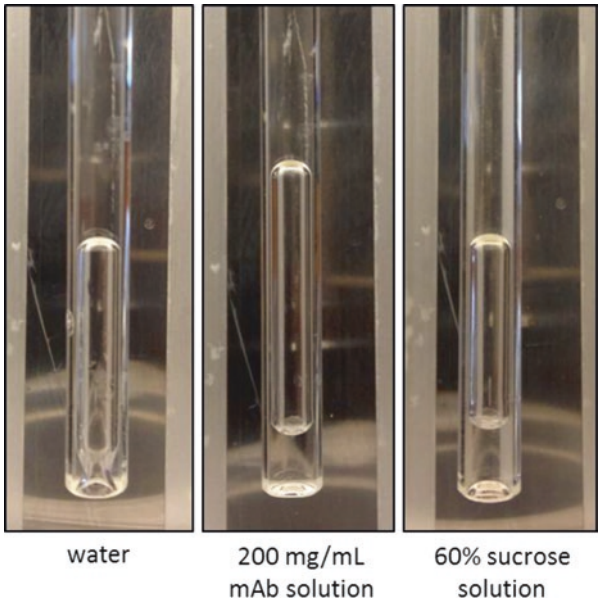

Fig. 5 Photos of water (1 cP at 25 °C), a 200 mg/mL mAb solution (~10 cP at 25 °C), and a 60% sucrose solution (~60 cP at 25 °C) after a fill dispense with a 3.0 mm inner ID filling nozzle

settings (e.g., degree, speed, and acceleration of pump reversal) [7, 8] and (b) filling nozzle characteristics (e.g., inner diameter and materials of construction) [7, 9]. The next section will explain how to assess and optimize these parameters.

4.1 Effect of SB Settings

The primary function of SB settings is to control how the pump reverses at the end of a fill dispense, but the extent of SB can hugely influence the phenomenon of formulation drying on the filling nozzle tip because the settings impact liquid plug formation [7]. Therefore, it can be possible to mitigate the formation of a liquid plug by optimizing the SB settings. Utilizing transparent filling nozzles (i.e., glass) instead of stainless steel nozzles allows visual observation of filling behaviors in a bench-top setup, providing insights into what is occurring during and after fill.

Figure 6 demonstrates the different patterns that can be observed when a liquid is reversed into a glass filling nozzle at different SB settings. Without exerting a SB (i.e., SB Setting 0 (SB 0) in Fig. 6), a liquid drop will hang at the tip; at too high of a SB setting (SB 7 in Fig. 6), the residual liquid that has adhered into the inner surface of the filling nozzle will create a liquid plug. Both cases can cause nozzle blockage after the liquid formulation dries. However, at the optimal setting (SB 3 in Fig. 6),

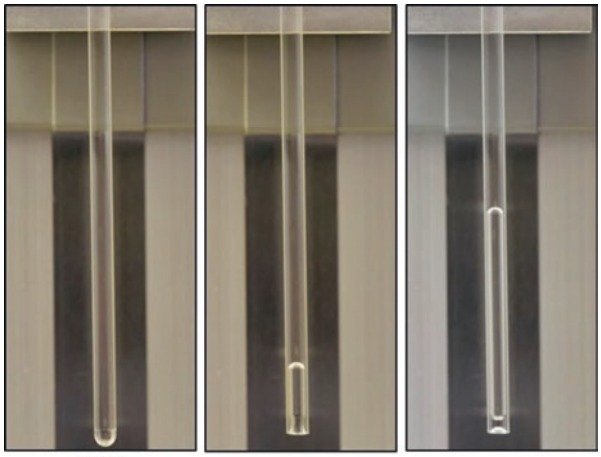

Fig. 6 Photos of water in a 2.4 mm ID glass filling nozzle after a fill dispense by a Flexicon PD12 peristaltic pump at various SB settings (0, 3, and 7). For the Flexicon PD12 peristaltic pump, SB is only controlled by a single nominal parameter that can be set from 0 to 10, where 0 is no pump reversal and 10 is maximum reversal

liquid will recede into the filling nozzle without forming a liquid plug since there isn't a significant enough residual liquid layer. When the liquid surface is away from the nozzle tip, water evaporation rate is significantly reduced due to the reduced airflow and humidity concentration gradient.

The user of the pump utilized in Fig. 6 can only control pump reversal via a single nominal setting from 0 to 10, where "0" is no pump reversal and "10" is maximum reversal. However, other pumps allow the user to control more parameters for pump reversal, including the exact degree of rotation of the pump (i.e., 0–360°), speed, and acceleration. If these parameters can be controlled, control of the SB phenomenon can be more finely tuned. For example, Hanslip et al. determined that utilizing slower pump speeds during pump reversal (<10 rpm; liquid flow rate <5 mL/min) was a key parameter in preventing liquid plug formation [8]. This observation is related to the flow velocity gradient in the pump tubing and filling nozzle. Typically during SB, there is a flow gradient from the edge to the center of the tubing, with the fastest flow occurring in the center of the tubing. In the filling nozzle, this results in residual liquid on the inner surface of the filling nozzle, resulting in a liquid film on the filling nozzle tip. Reducing the SB speed decreases this gradient, which decreases the likelihood of liquid film formation and results in a cleaner retraction.

In summary, one potential method of minimizing the risk of nozzle clogging during a prolonged manufacturing interruption is utilizing a bench-top setup to determine an optimal range of SB settings with a manufacturing representative filing nozzle. These settings should then be confirmed during an at-scale manufacturing run. Transparent filling nozzles can be used to visualize filling behavior for further characterization. In some circumstances, it may be difficult to determine optimal

settings for SB. For example, with a pump where the only setting that can be modified is the degree that the pump can be reversed, it may be more difficult to find an optimal setting for more viscous fluids. As viscosity increases, the optimal range for SB decreases since it becomes more difficult for the pump to reverse the droplet into the nozzle (i.e., too low of a setting), and once the liquid recedes into the nozzle, it becomes easier to form a liquid plug due to the tendency of higher-viscosity fluids to form a thicker residual layer on the inner surface of the nozzle (i.e., too high of a setting). Fortunately, SB settings are not the only critical parameters that can be adjusted to mitigate nozzle clogging.

4.2 Effect of Filling Nozzle Characteristics

Characteristics of the filling nozzle—such as inner diameter (ID) and materials of construction—can also greatly influence the phenomenon of nozzle clogging.

Effect of Filling Nozzle Characteristics: ID

Increasing the ID of the filling nozzle is one change that can be implemented to decrease the risk of nozzle clogging. Smaller ID nozzles are generally more difficult to fully retract fluids into a phenomenon which can once again be explained by a modified Hagen-Poiseuille equation (Eq. 1), where pressure (ΔP) is described as (Eq. 2):

$$\Delta P = \Delta F / A \tag{2}$$

where A is the surface area of the filling nozzle, described by (Eq. 3):

$$A = \pi d^2 / 4 \tag{3}$$

where d is the ID of the filling nozzle. Therefore, as the nozzle surface area (A) decreases, the amount of force (ΔF) required to retract a fluid exponentially increases. This relationship is further impacted with more viscous solutions because as viscosity (μ) increases, even more force (ΔF) is required to retract the fluid up the nozzle.

In addition to being more difficult for liquid to retract fluid, smaller ID nozzles also will clog more quickly compared to larger ones. As discussed earlier, the formation of a liquid plug starts on the inner surface of the filling nozzle as water evaporates from the liquid-substrate interface. The rate of growth of the dried ring is comparable between nozzles of different sizes, but less water needs to be evaporated to create a hard clog for smaller ID nozzles due to their smaller surface area (Eq. 3). Therefore, one mitigation strategy against nozzle clogging is to use larger ID nozzles. However, using larger ID nozzles is not always preferred since they could potentially impact filling precision because of potentially insufficient fluid flow.

Effect of Filling Nozzle Characteristics: Materials of Construction

Changing the materials of construction of the filling nozzle can be another consideration when attempting to decrease the risk of clogging. Hydrophilic materials, such as stainless steel, are more likely to experience liquid plug formation compared to hydrophobic materials, such as Teflon or PEEK [9]. Hydrophilicity and hydrophobicity are relative terms to describe the relationship between water or a water-based solution and a surface. A surface is considered hydrophilic if water is attracted to it; conversely, a hydrophobic surface is one that repels water. The degree of hydrophilicity/hydrophobicity can be determined by placing a water droplet on a surface and measuring the degree that it spreads out (i.e., the contact angle).

As previously described, liquid plug formation at the filling nozzle tip significantly impacts the likelihood of nozzle clogging, and this liquid plug is an accumulation of the liquid film that adheres to the inner surface of the filling nozzle during pump reversal at the end of a fill dispense. Since the degree of hydrophilicity impacts the adherence of a solution to a surface, a more hydrophobic material will be less likely to result in liquid plug formation. Although a liquid film will still be generated at the end of a fill dispense, it will be repelled by the hydrophobic surface and contract away from the surface as quickly as possible. Therefore, although a liquid plug is formed, it does not end up at the filling nozzle tip (Fig. 7). Its final location is based on the force balance of repulsive force from its interaction with the hydrophobic surface and the downward force of gravity (Fig. 8). For a hydrophilic surface, the attractive force of adhesion and the downward force of gravity ensure that any residual fluid on the inner surface of the filling nozzle will accumulate at the nozzle tip. As a result, utilizing a hydrophobic material for the filling nozzle can significantly extend drying times and decrease the likelihood of nozzle clogging.

In summary, optimizing SB settings, utilizing a larger filling nozzle, and utilizing a filling nozzle made out of a hydrophobic material are all potential methods to reduce the risk of formulation clogging. Depending on the product and the manufacturing line, one method may be preferred over another. For example, although using a hydrophobic filling nozzle is likely the most effective way to reduce the risk of nozzle clogging across multiple products, it may be difficult to implement a new product-contacting part into a GMP manufacturing line (assuming that a hydrophobic filling nozzle hasn't already been in use by the line), in which case optimizing SB settings might be preferred. However, optimizing SB settings will require more bench-top work since they will likely need to be defined on a product-specific basis due to SB settings being much more sensitive to formulation of physical properties (e.g., viscosity). The different advantages and disadvantages must be weighed against one another to determine the best path forward when faced with such a challenge.

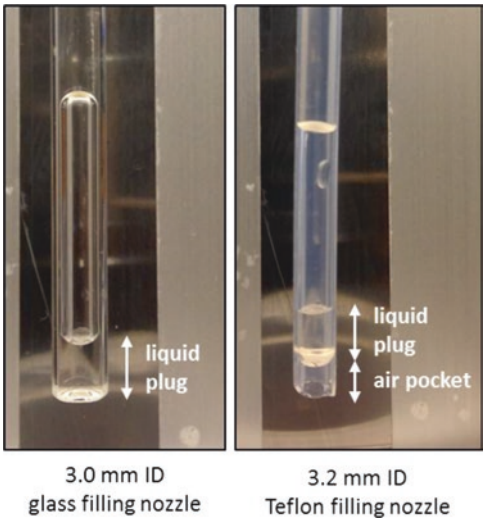

Fig. 7 Photos of a 200 mg/mL mAb formulation (~10 cP at 25 °C) after a fill dispense using identical pump parameters in a 3.0 mm ID glass tubing (left) and a 3.2 mm ID Teflon "nozzle" (right). In the hydrophilic glass filling nozzle, residual fluid adheres to the inner surface of the filling nozzle and accumulates at the nozzle tip. In the hydrophobic Teflon filling nozzle, residual fluid is repelled by the surface, and the liquid plug settles above the nozzle tip

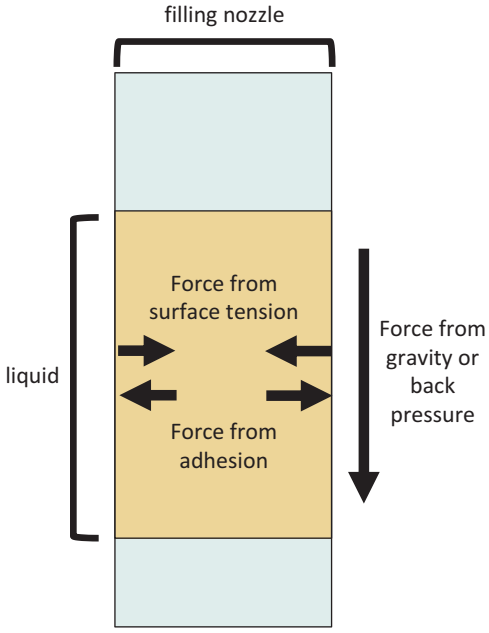

Fig. 8 A schematic depicting the force balances experienced by a liquid in a filling nozzle at the end of a fill dispense

5 Optimizing Filling Precision with Different Filling Systems

One of the often cited claims for the advantage of PFSs is their improved dose accuracy over vials as a result of the dose being premeasured and fully injected into the patient [10, 11]. Another cited claim is that PFSs require less excess volume ("overfill") compared to vials since PFSs do not require additional material for withdrawal and administration, which minimizes material waste [12, 13]. However, in order to meet these claims of improved dose accuracy as well as minimized overfill, filling precision for PFSs must meet a tighter range than that for vials.

In addition to the general expectation for tighter filling precision for PFSs, PFSs are also typically filled at small volumes (i.e., 2.0 mL or less). When considering filling targets in terms of percent accuracy, these smaller volumes are more difficult to fill precisely. On top of these complicating factors, there is currently no clear guidance on what is regulatory expectation in terms of PFS filling accuracy. Whereas vials have clearly stated guidance about allowable overfill [12, 13], PFSs do not. Therefore, it is up to manufacturers as well as product developers to determine what is considered to be an acceptable range for fill volume. Generally, manufacturers will try fill at a range as tight as possible, with an industrial standard of ±0.5% for fill volumes ≥0.5 mL and ±1% for fill volumes ≤0.5 mL [14].

5.1 Comparison of Filling Technologies: Piston Pump, Time-Pressure Filler, and Peristaltic Pump

Several filling options are available for manufacturers, and the one that is selected is dependent on how the balance of the desire for efficient output with filling accuracy requirements as well as manufacturing operation considerations (e.g., ease of use, cleanability, previous experience) is skewed for particular product. The three most common filling technologies utilized in PFS manufacturing are:

- The rotary piston pump
- The time-pressure filler
- The peristaltic pump

These three options will be discussed below followed by an explanation of how to utilize a bench-top setup to perform filling accuracy optimization studies for these different systems.

Piston Pumps

Piston pumps have commonly been utilized by manufacturers to meet tight requirements for filling accuracy. A piston pump is a positive displacement pump that pulls and pushes a fluid through its housing as a result of pressure changes caused by the piston movement. An example of a rotary piston pump is diagrammed in Fig. 9.

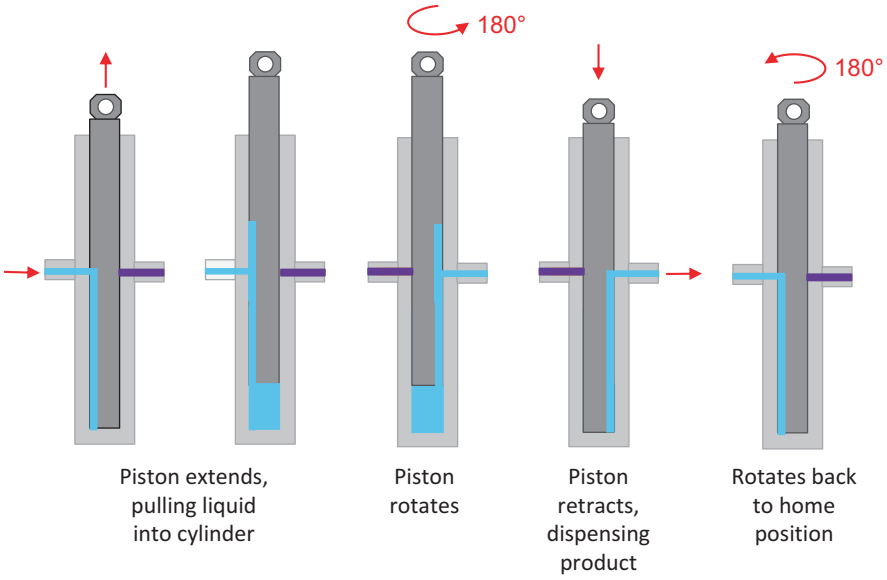

Fig. 9 A schematic depicting a single cycle of a rotary piston pump. The piston pump pulls and pushes fluid through its housing as the piston rotates

Valveless, ceramic, or stainless steel piston pumps are commonly used in biopharmaceutical manufacturing since they have fewer mechanical parts that directly contact the product. However, the pistons are very delicate and easy to damage [14], which can result in particle shedding or loss of precision. Piston pumps raise an additional concern about biopharmaceutical filling due to a very narrow clearance between the piston and housing. This narrow gap creates a high-shear zone that can result in protein particle generation since the product can fill the gap to serve as the lubricant for the piston and housing [14–16]. Despite these drawbacks, the piston pump remains one of the most commonly used systems for filling products of low fill volumes (<0.5 mL) and/or high viscosity.

Time-Pressure Filler

Time-pressure filling systems are also frequently used for biopharmaceutical manufacturing. A fluid is held under a slight overpressure in a surge vessel directly upstream of a pinch-valve system that controls the opening and closing of silicone tubing. The pinch-valve system is controlled by an automated program that opens the pinch-valve system for a specific amount of time based upon tank pressure and fluid properties. The fluid then flows through an orifice that regulates flow rate before being dispensed through a filling nozzle. An example of the mechanism of the time-pressure filler is diagramed in Fig. 10.

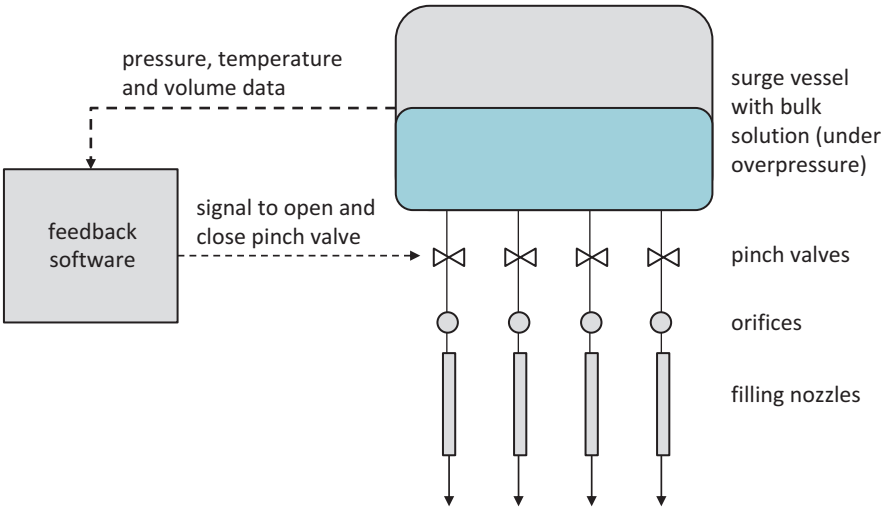

Fig. 10 A schematic depicting a time-pressure filling system. The time pressure system controls fluid dispensing through the automated opening and closing of pinch valves based on in-line sensor data

The time pressure system can be very precise but is highly dependent on how well-tuned the computer program compensates for variations that occur during filling, such as temperature or pressure [4]. The time-pressure filler is also more sensitive to viscosity compared to the piston pump and can have limitations with high protein concentration products since these products will typically require more compensation for changes in pressure and temperature. However, compared to the piston pump, the stress on the product is relatively gentle, and the fill times will be quicker since there is no recovery phase required between dispenses.

Peristaltic Pump

The peristaltic pump is a positive displacement pump that typically utilizes a rotating rotor with rollers to alternately pinch and unpinch a piece of flexible tubing. When a part of tubing is pinched closed, the fluid is forced forward; after the tubing is unpinched, the fluid is forced into its empty space. A schematic of a peristaltic pump is provided in Fig. 11.

The alternating pinching and pinching of tubing create pulsation of flow, which can impact filling accuracy. However, peristaltic pumps utilized for pharmaceutical manufacturing typically use two rotors with offset rollers in order to minimize pulsation [14]. Nonetheless, peristaltic pumps are generally less precise compared to piston pumps and time-pressure filler [4], and filling very small volumes ($\leq$0.3 mL) can be challenging [17] unless if smaller tubing sizes are used. However, utilizing smaller tubing sizes will exponentially decrease flow rate, meaning a balance of achieving desired filling precision while also achieving desired throughput must be

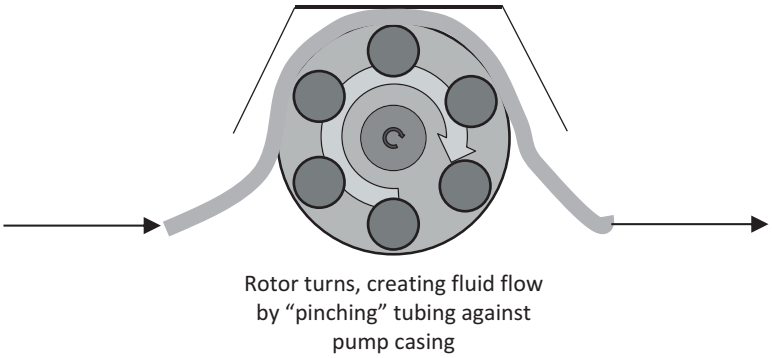

Fig. 11 A schematic depicting a peristaltic pump. The peristaltic pump uses a rotor with rollers to compress and decompress flexible tubing to create fluid flow

established. Peristaltic pumps are also more limited in filling more viscous liquids compared to the piston pump, and loss of filling precision can often be observed as viscosity increases [14, 17]. Additionally, attempting to use smaller tubing sizes to improve precision can be challenging as viscosity increases since the pump may not be able to generate the necessary pressures required to achieve consistent flow.

Another challenge with peristaltic pump filling precision is that the phenomenon of fill volume drift can be observed over time [4]. As the flexible tubing in the pump goes through more and more cycles of compression and decompression, it can stretch out and lose its shape as well as its ability to maintain its "memory" after compression. To minimize the effect of fill volume drift that may occur over time, regular in-process checks can be performed to ensure that fill volumes remain within tolerances. Different tubing also may have different attributes (i.e., wall thickness, durometer) that affect their mechanical properties and their relationship with the peristaltic pump rollers. As a result, many peristaltic pump manufacturers produce tubing that is specific for their pump.

Despite all of these drawbacks and limitations, the peristaltic pump is still considered a choice of selection when filling PFSs since it is the most user-friendly and renders minimal stress to the product compared to the piston pump. The only contact the product has with the system is through the flexible tubing in the pump that is disposed of after fill, which means that the system does not need to be cleaned between uses, and there is minimal shear to the product since low pressures are utilized to induce fluid flow [14].

5.2 Utilizing a Bench-Top Setup to Perform Filling Precision Studies

A bench-top setup can be useful for determining the optimal process parameters for achieving desired filling precision results for products of low fill volumes and high viscosity prior to manufacturing on an at-scale line since the bench-top setup allows

for the flexibility to test different pump and equipment parameters with minimal product volume requirements. Some considerations for how to perform these studies are detailed below.

Surrogate or Product?

Although a bench-top study may require a relatively small quantity of the bulk material compared to the manufacturing-scale study, in some cases such quantity may still be too large to come by, particularly for projects in early development. Therefore, it may be desired to use a surrogate solution. Viscosity, surface tension, and density of the product should all be considered when formulating a surrogate solution since all of these properties can influence flow characteristics. For high protein concentration formulations, viscosity is an especially critical parameter since it can be high enough to present challenges for peristaltic or time-pressure filling. If a study needs to look at filling precision under different temperatures (e.g., filling at 2–8 °C versus filling at ambient temperature), the surrogate solution's viscosity curve at these different temperatures should mimic the product's or multiple surrogate solutions at different viscosities should be tested.

Experimental Setup Design

A scale can be integrated into the bench-top setup pictured in Fig. 1 for filling studies. In the setup, the two main pieces of equipment are the pump system and the scale utilized for measuring weight dispenses. The pump system used must be identical to the one used in at-scale manufacturing since even pumps utilizing the same mechanism can be drastically different in filling precision based on the pump design or the level of control over parameters. This requirement is not too difficult for most rotary piston pumps and peristaltic pumps to meet since these systems can typically fit into a bench-top system relatively easily. However, a time pressure system will require significantly more space to accommodate all of the parts of the system (e.g., surge tank, sensors, controllers, etc.).

The scale used should be precise enough to capture 1% differences in filling precision, meaning when assessing 0.5 mL fill volumes, the scale should be able to capture 0.005 mL changes at a minimum. One should also consider whether or not 100% of dispenses should be analyzed or if performing periodic in-process checks is sufficient. Performing 100% checks provides the most information, but extra care must be taken to ensure that environmental influences (e.g., air flow around the balance) are minimized during the entire study. Performing in-process checks can be done manually and only requires the environment to be controlled when measurements are being taken.

The study's purpose must also be considered in the experimental design. For example, a study looking at the feasibility of precisely filling a product on a particular manufacturing line is different from a study looking to compare one thing against

another (e.g., comparing different tubing brands for a peristaltic pump). Feasibility studies should try to mimic system pressure drops as closely as possible since these pressure drops can influence the flow rate into and out of the pump, meaning that tubing lengths and height differences between the various components (e.g., bulk solution, pump system, and filling nozzle) should be considered in the setup. Comparison studies only need to maintain a consistent setup across the different items being compared since only relative differences are being studied.

Example of Filling Precision Data Analysis with the Peristaltic Pump

In this example, two different pumping conditions of a peristaltic pump are tested for a high-concentration mAb product with a viscosity of about 10 cP at 25 °C filled to a 0.3 mL target volume. The bench-top setup utilizes a pump and pump tubing that are identical to those used in manufacturing as well as a high precision scale that can measure down to 0.001 g. A total of 200 dispenses are performed for each experiment, with a dispensing cycle of 5 seconds. All fill weight data are recorded. Figure 12 summarizes the fill volumes of the first 100 dispenses, and its histogram is presented in Fig. 13. Data in Fig. 12 are scattering around the target fill volume of 0.3 mL for both pumping conditions, but their histogram profiles (Fig. 13) show distinctively different distribution patterns. Condition 2 has a much wider fill volume distribution, while Condition 1 has a Gaussian-like distribution skewed on the high side. Overall, Condition 1 appears to be a more optimal set of pump parameters though the higher skewed values indicate that further optimization is required.

Most of peristaltic pump manufacturers would report their filling accuracies in terms of standard deviation. It is important to note that one standard deviation simply means that 68.2% of values fall within that value for a normal distribution. Given the two non-normal distributions in Fig. 13, it may be misleading to apply

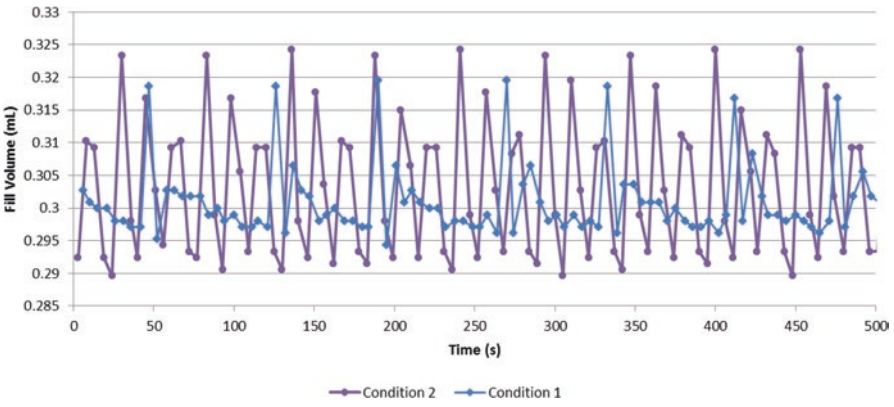

Fig. 12 Filling data for 100 dispenses of a target fill volume of 0.3 mL for two different pumping conditions with the peristaltic pump

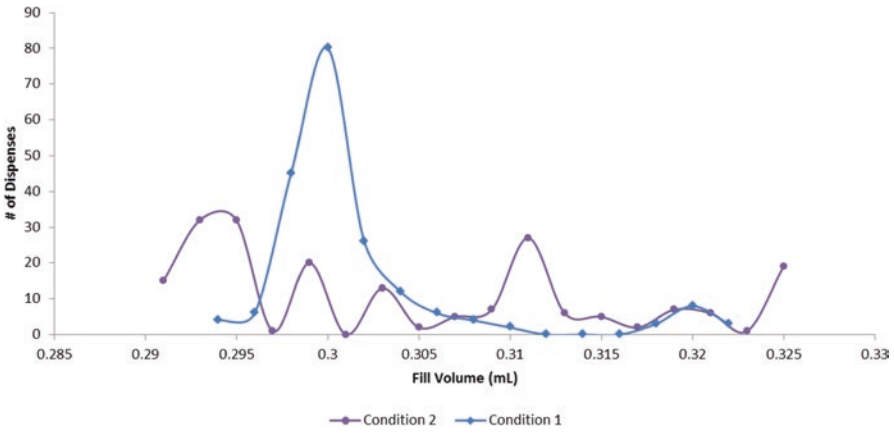

Fig. 13 Histogram of the filling data from Fig. 12. Condition 1 has a more Gaussian-like distribution, whereas Condition 2 has an irregular distribution

standard deviation as the measure of filling accuracy for peristaltic pump. Condition 1 has a standard deviation of ±2.0%, while Condition 2 has a standard deviation of ±3.7%. However, neither condition has a normal distribution. In the example of Condition 1, 87.5% of dispenses actually fell within ±2% of the mean fill weight, which is higher than one would have estimated when using standard deviation. The high deviations of Condition 1 skewed its standard deviation to a higher value although the majority of Condition 1's data points are within a tight range. An optimal set of filling parameters should result in a consistently normal distribution, but until that point is reached, it is important to analyze filling precision data holistically rather than immediately assuming standard statistical methods are applicable.

6 Plunger Stopper Insertion

The insertion of the plunger stopper or piston ("stopper" will be used henceforth) completes the PFS filling process. Stoppers must be properly positioned. Placing the stopper too close to the fluid can result in "wet" stoppers, where fluid is in contact with the ribs of the stopper and can potentially cause the breach of container closure integrity (CCI). However, placing the stopper too high would result in a larger air bubble. In cases where the PFS cannot be primed prior to use (i.e., when using an autoinjector), the air bubble is a safety concern when injected subcutaneously into the patient. A larger air bubble can increase the likelihood of stopper movement during air transport [5]. Stopper movement may also impact CCI, a concern that the FDA often recommends for assessment [18]. Furthermore, the stopper position can affect downstream assembly steps (e.g., placement of the stopper rod), so PFS product developers must ensure that the range of stopper position meets assembly requirements.

6.1 Comparison of Stopper Insertion Technologies: Vent Tube (Mechanical) Versus Vacuum (Pressure)

Stoppers can be inserted into syringes either through a vent tube (a mechanical mechanism) or vacuum (a pressure mechanism). In the vent tube method (Fig. 14), the stopper is compressed in a stainless steel tube and then pushed into the syringe barrel with an insertion rod. During insertion, air in the syringe is vented out around the compressed stopper, which allows for the stopper to stay in place after it expands to fit inside the syringe barrel. The position of the syringe barrel or insertion rod can be adjusted up or down to modify the stopper position. This venting method, featuring simple mechanical manipulations and high throughput, is widely accepted in the industry [19]. However, a misaligned or slightly damaged vent tube can scratch internal surfaces of the syringe barrel and/or create stainless steel particles that may mark the stoppers [20]. A damaged vent tube can also physically deform the stoppers or can cause the coating to wrinkle [19, 20].

The vacuum stoppering method can either occur in-line (pulls a vacuum on each syringe) or off-line (pulls a vacuum on a tub of syringes). For the in-line method, the stopper is collected in a cylinder, which then seals the syringe and pulls a vacuum (Fig. 15). The stopper is then pulled from the cylinder into the syringe due to

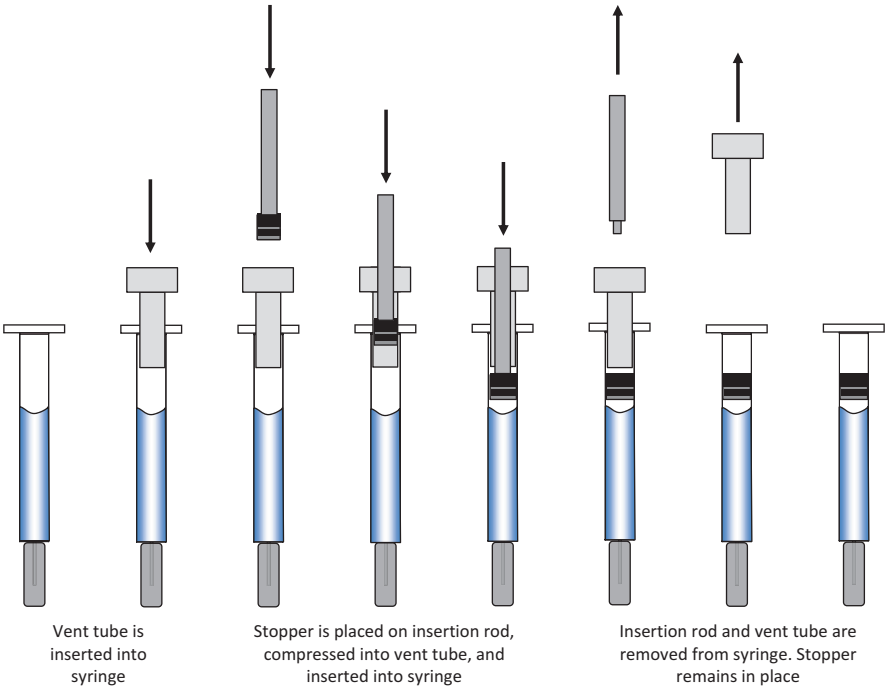

Fig. 14 A schematic depicting vent tube stopper insertion mechanism. The stopper is compressed into a stainless steel tube and then inserted into a syringe

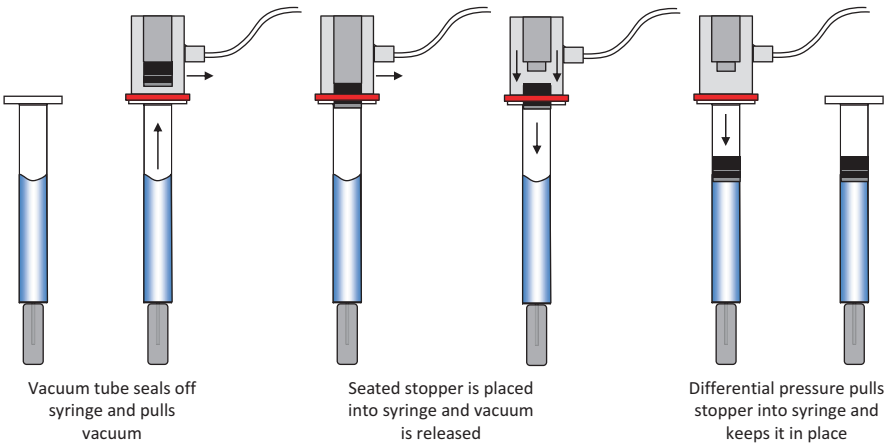

Fig. 15 A schematic depicting in-line vacuum stoppering mechanism. A vacuum is created in the syringe and pulls the stopper into the syringe due to differential pressure

differential pressure. The off-line method works similarly, except that an entire tub of filled syringes is placed into a chamber, with the stoppers seated on a tray above the syringes. A vacuum is pulled in the chamber, and the stoppers are lowered and mechanically pushed into the syringes by metal pins. When the chamber returns to atmospheric pressure, all the stoppers are pulled into the syringes by the pressure differential.

Compared to the vent tube, the primary advantage of vacuum stoppering is the complete removal of the air bubble from the syringe. In addition, vacuum stoppering does not cause stopper deformation or syringe surface scratches. However, the production speed of vacuum stoppering is slower than that of vent tube stoppering, and the off-line method runs into a higher risk of contamination due to the potential need for manual operator intervention to move syringes. Additionally, the lack of an air bubble can present challenges in visual inspection for particles [19]. The vacuum stoppering method may not be suitable for Luer-Lok syringes as the vacuum will draw out the air bubble at the tip cap, which could cause liquid splashing or stationary bubbles in very viscous solutions. If vacuum stoppering is desired in these situations, it may be necessary to employ vacuum filling, which will pull vacuum on the container and allow for the solution to fill the tip cap void.

6.2 Utilizing a Bench-Top Setup for Stoppering Studies

Stoppering studies require a completely different experimental setup from the filling studies outlined in previous sections. A bench-top stoppering setup should incorporate stoppering equipment and the stoppering mechanism (e.g., vent tube or vacuum) comparable to the manufacturing line.

Bench-top studies on the vent tube stoppering method may not be required because there are no specific parameters to optimize other than the vertical position of the syringe or the stopper insertion rods. However, the vacuum stoppering method involves more parameters, such as vacuum pressure or time, so it may be beneficial to perform bench-top studies to optimize these parameters to minimize the bubble height. Generally, a deep vacuum may be pulled during stoppering as long as there are minimal sources of gas within the syringe. However, for Luer-Lok syringes, pulling a deep vacuum may pull out the air bubble in the tip cap, which could result in splashing or stationary bubbles remaining in the solution. For these syringes, either vacuum filling should be performed in conjunction with vacuum stoppering or a vacuum stoppering cycle should be developed that minimizes bubble height while also mitigating the concerns of air expansion.

7 Conclusion

This chapter provided an overview of the unique challenges encountered during the PFS drug product manufacturing process involving high-concentration, viscous protein formulations. Some of these challenges include developing a "clean" filling profile, mitigating filling nozzle clogging during prolonged line interruptions, and meeting tight filling precision (particularly for peristaltic pump) and stopper insertion requirements. This chapter offered examples demonstrating that bench-top systems are effective in understanding and optimizing relevant filling parameters to overcome challenges mentioned above. Better understanding of these process challenges would help the readers design and establish a more robust fill-finish manufacturing process.

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Chapter 25
Peptide Drug/Device Combinations

Shahid Uddin

1 Overview of Peptide Therapeutics

The role of peptides in normal human physiology has led to a large amount of interest in the development of peptide therapeutics. But what are peptide therapeutic agents and how are they poised within the context of pharmaceutical patient management strategies? The aim of this section is to provide an overview of the history of peptides as drugs and the uses of peptides in clinical practice. This section provides an insight into how peptide therapy has developed over time, focusing on the advances underlying contemporary uses of peptides as drugs.

1.1 History of Peptides as Drugs

Peptides are defined as amino acids joined through amide bonds and range in length from three amino acids (e.g. thyrotropin-releasing hormone) to 100 amino acids [57]. Long length chains of amino acids are typically not considered peptides and will not be discussed in the present chapter. There are over 7000 naturally occurring peptides, many of which play a role in human physiology [31]. Peptides are essential in the regulation of homeostasis within the human body, performing a range of functions. One of the clearest examples of peptide homeostasis is the role of the peptide insulin in regulating blood glucose levels [80]. Insulin secretion from the pancreas acts on designated receptors to promote uptake of blood glucose into cells while also reducing the synthesis of new glucose and moderating metabolism of

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glucose. In patients with type 1 diabetes, insulin is not produced by the pancreas, leading to unregulated blood glucose levels; in this context replacement of insulin can provide therapeutic benefits to patients. This example is a classic instance of peptide therapeutics, and replacement of insulin has been used clinically since the 1920s [80]. However, many other peptides may be used therapeutically to manage endocrine and central nervous system disorders, infectious disease, and cancer.

Recent advances and approvals of drugs have led to an emergence of peptides as an innovative and growing therapeutic area. It is estimated that over 140 peptide therapeutics are undergoing clinical trials, while new peptide designs and approaches are being developed routinely [31]. Peptides may be formulated as small molecules (akin to many drugs in the marketplace), larger molecules, or as biologic agents. Biologics was a term that used to include blood or blood components, but this has progressed to include monoclonal antibodies, cytokines, tissue growth factors, vaccines against non-infectious disease targets, and gene transfer products [8]. These agents may have pronounced immunomodulatory effects and illustrate the diversity of peptide therapeutic approaches as a means to prevent immune-mediated disease or to enhance tissue growth, recovery, and protection against disease [8].

Compared with pharmaceutical agents, peptides are generally considered to have a predictable safety profile and tolerance in patients. Furthermore, peptide therapy is selective and potentially efficacious, particularly where naturally occurring peptides are replaced for therapeutic effect [31]. Therefore, interest in peptide therapy is growing with time and changing the pharmaceutical marketplace. Table 1 lists the peptide therapeutics marketed in the last few decades.

Table 1 Marketed therapeutic peptides

Trade name	Generic name	Target	Indication
Forteo	Teriparatide	P1TH1R agonist	Osteoarthritis
Fuzeon	Enfuvirtide	Protein-protein inhibitor	HIV
Prialt	Ziconotide	Calcium channel inhibitor	Pain
Byetta	Exenatide	GLP-1R agonist	Type 2 diabetes
Symlin	Pramlintide	Calcitonin agonist	Type 1 or 2 diabetes
Somatuline	Lanreotide	SST agonist	Acromegaly
Nplate	Romiplostim	Thrombopoietin agonist	Haematology
Egrifta	Tesamorelin	GHRF agonist	Lipodystrophy
Victoza	Liraglutide	GLP-1R agonist	Type 2 diabetes
Bydureon	Exenatide LAR	GLP-1R agonist	Type 2 diabetes
Surfaxin	Lucinactant	Uncertain	IRDS
Omontys	Peginesatide	Erythropoietin analogue	Anaemia
Signifor	Pasireotide	Somatostatin analogue	Cushing's disease
Kyprolis	Carfilzomib	Proteasome inhibitor	Multiple myeloma
Linzess	Linaclotide	Guanidyl cyclase 2C agonist	Irritable bowel syndrome (constipation)
Gattex	Teduglutide	Glucagon-like peptide analogue	Short bowel syndrome

Table taken from Dunn [25, 222]

Peptides offer an enormous potential for growth within the pharmaceutical industry; although many peptides have been developed, traditional peptide design has been modified to allow for a range of putative peptide products [70]. In particular, peptide biologics represent a growing field, including monoclonal antibodies, cytokines, and growth factors [8]. Furthermore, advances in the delivery and efficacy of peptide therapeutics hold great promise for expanding their use in practice. The limitations of peptides in current practice and the potential to overcome these limitations will be considered in the remainder of this section.

1.2 Limiting Factors When Using Peptides in the Clinic

Although peptide therapeutics has grown as a subdivision of the pharmaceutical industry, peptides have a relatively small market share at present [31]. The reasons underlying this observation are numerous, including the limitations of traditional delivery techniques of peptide therapeutics, a limited range of clinical targets, the relative cost of developing peptide therapy, and the practical use of peptides.

One of the characteristics of peptides as therapeutic agents is their molecular size: positioned between small molecules and proteins. Furthermore, the molecular characteristics of peptides differ significantly from either small molecules (most drugs developed) or proteins [31]. The size of peptides and their vulnerability to natural processes of enzymatic degradation and metabolism reduce the potential routes of administration and putative efficacy within the body. Most peptide drugs are administered through injection (intravenous, intramuscular, or subcutaneous) to ensure efficacy within the body and to avoid degradation via the oral route [88]. This may limit the convenience of the use of peptide therapy in the clinic.

As a general rule, naturally occurring peptides have a short plasma half-life, which can limit their therapeutic potential [31]. Half-life control forms an essential aspect of the homeostatic regulation of peptides as part of an endocrinological system, so strategies have to be devised to overcome this limitation [52]. Enzymatic cleavage is a common means for degradation of peptides, and prevention of enzymatic activity, potentially through alteration of cleavage site amino acid sequences, is one strategy to extend the life of peptides in the body. However, stability of naturally occurring peptides has been a major obstacle to the development of many peptides as viable therapeutic agents [31].

Additional imitations to peptide use in clinical practice include the limited range of targets available or peptide therapy and the cost of peptide therapy. Targets are typically limited to replacement therapy in many clinical contexts, thereby only covering a small range of conditions. There is the potential to develop peptides for many more therapeutic purposes – the supply of available agents does not match this potential, however. The relative cost of peptide therapy may also be higher than other forms of therapy, particularly as manufacturing techniques become more

advanced [52]. Therefore, limitations to peptide use in practice are numerous but represent challenges that can be potentially overcome using alternations in peptide formulation and advances in peptide modification and development.

1.3 Advances in the Use of Peptides as Drugs

Although there are limitations to the widespread use of peptides in clinical practice, advances in drug development and refinement of the peptide therapeutic approach have opened up multiple avenues for expansion of this therapeutic area. Initially, peptides were endogenously sourced, derived from animals and acting as replacement therapies for human diseases. This is the case with insulin, as well as adrenocorticotrophic hormone (ACTH), which was isolated from bovine or porcine pituitary glands in the 1950s [49]. Once sequencing of peptides became possible, synthetic peptides were manufactured during the 1960s and 1970s, leading to a rapid expansion in available agents, including oxytocin, vasopressin, calcitonin, and octreotide [49]. The genomic era has seen massive leaps in peptide therapeutic technology, with identification of receptors and novel agents activating receptors for potential therapeutic effects [7]. As manufacturing approaches of peptide therapeutics have advanced over time, so too have the potential applications of this type of therapy.

Increasing the pharmacological potency of peptides has also been a key research focus, and peptide modifications to promote cell entry and increase stability have been developed accordingly [7]. Although native peptides do not typically cross cell membranes, cell-penetrating peptides (e.g. penetratin) have been devised to overcome this limitation and expand molecular targets to include intracellular targets [61]. Balancing the potential to expand the range of targets of peptides with the increased volume of distribution and potential for lower potency of the peptide is an important factor for refinement [31].

Oral bioavailability of peptides has generally been poor, requiring routes of administration through injection [82]. Improving oral bioavailability is considered an important therapeutic hurdle, which would make peptide therapy simpler and more attractive to patients. Chemical strategies to overcome acidic and enzymatic digestion in the gastrointestinal tract have emerged, including features of peptide stabilisation, such as hydrophobic face construction, cyclisation, methylation of amino acid N-terminals, and introduction of intramolecular hydrogen bonds [31]. However, advances in injectable peptide delivery have also been pursued in order to improve the convenience of delivery and patient experience [82]. Therefore, changes to peptide stabilisation can have an impact on the attractiveness of these therapeutic options.

Peptide sequencing techniques have developed dramatically over time, allowing for an expansion of the available targets of peptides as well as the techniques used to synthesise peptide therapies [87]. Sequencing of peptides allows for accurate characterisation of the likely chemical properties of the peptide, including involvement in degradation pathways and likely shelf-life of the drug, as well as efficacy in targeting specific clinical conditions [7]. Sequencing techniques have

become more rapid and dynamic, allowing for high-throughput approaches to refining drug candidates and use strategic design approaches to drug synthesis [7]. Similarly, techniques used to synthesise peptides are advancing, reducing the cost and length of time required to take a peptide from the laboratory to the clinic [87].

The generation of peptide libraries has also facilitated coordinated research efforts on a global scale [51]. These libraries catalogue identified peptides and allow researchers to identify and optimise peptides for a range of clinical uses, including antimicrobials [5]. Libraries allow for rapid screening of peptides for use as drugs and can facilitate early stage drug development, making this strategy a powerful tool for expanding the repertoire of peptides available for clinical use [51]. These libraries may also include information on modifications and formulations of peptides and their corresponding pharmacokinetic and pharmacodynamic profiles, advancing the potential to formulate peptides for specific uses [26].

One of the most promising avenues of research is the potential to target peptides to cells or tissue, allowing for highly specific therapeutic effects [30]. Peptides interact with cell surface receptors in a highly specific manner, affording the opportunity to modify peptide sequences to target specific cellular or tissue receptors [30]. These peptides may be used alone for therapeutic purposes or may be associated with other drugs and delivery systems, facilitating tissue-specific drug delivery [42]. The combinations of advances in library catalogues of peptides and synthesis approaches have generated a massive interest in the potential for targeted activity with these drugs, opening up many avenues to future pharmacological development.

2 Formulation of Peptides

The formulation of peptides refers to the process of managing bulk raw materials and producing therapeutic peptides through a series of manufacturing and processing stages. The strategies employed in refining peptides and ensuring a viable clinical product are diverse and remain integral to the potential utility of peptide therapeutics in practice. Different formulation strategies may also have implications for the efficacy and pharmacokinetics of peptides produced, highlighting the need to balance the outcomes of different formulation strategies. This section will consider how peptide formulation is facilitated in practice, with a focus on newer pharmaceutical methods, as well as essential quality control processes used to ensure the viability of peptides for clinical use.

2.1 Pre-formulation Studies

The use of pre-formulation studies as an initial stage in evaluating bulk material is essential prior to formulation of peptides. These studies provide the basis for development of optimal dosage forms of the peptides and the design of a suitable delivery

system, with the overarching goal of achieving maximal stability and bioavailability [59]. Pre-formulation studies are often used in small molecule drug development, including the use of crystallography, nuclear magnetic resonance, and mass spectrometry to characterise bulk materials and determine the atomic-level structures of molecules [59]. The complexity of proteins and peptides, including the formation of higher-order forms of these molecules, complicates this process, but lower-resolution methodologies may be applied [85]. Gel electrophoresis and high-performance liquid chromatography can be used to analyse bulk materials, peptides, and the presence of any impurities allowing for refinement of the peptide product for subsequent formulation into a pharmaceutical agent [37].

2.2 Formulation Development

Following completion of pre-formulation studies, formulation development aims to characterise impurities in the product, including the presence of any degradation products [37]. Furthermore, the packaging and environmental conditions under which the peptide can remain stable should be investigated and optimised [3]. A combination of literature review and analytical methods can determine the likelihood of the presence of leachable elements from protein/peptide storage vessels. Specific challenges regarding the formulation of lyophilised or high-concentration formulations are also noted [3].

Buffer systems need to be selected carefully in order to prevent small pH changes from adversely affecting the stability or function of the peptide. Phosphate buffers are commonly used but are limited when applied to zinc insulin (zinc phosphate precipitations arise) or in peptides that require a low pH to maintain stability (e.g. gamma-interferon) [31]. For lower pH solutions, organic acid buffers, such as lactate, may be useful [76]. However, generally inorganic buffers are used in practice in order to achieve the desirable characteristics of being zwitterionic, excluded from the peptide domain, acting as a scavenger of free radicals and preventing mechanical stress in the peptide [31]. For instance, histidine buffer has a pH of 7.4 and is commonly used for monoclonal antibody preparations [72, 75]. Buffers also need to be considered in terms of how the solubility of the protein or peptide is affected [31].

In addition to the buffer system used, the pH of the formulation can affect stability and bioavailability; often a compromise is needed to prevent deamidation reactions but minimising oxidation reactions [31]. The solution pH and use of excipients may also affect the solubility of the peptide formulation [54]. Ideally, solubility should be achieved where the maximum amount of peptide is dissolved without precipitation in a medium. This may be predicted, in part, from the structure of the peptide, although other methods are needed in practice to optimise solubility. This includes extrapolation of peptide solubility based on polyethylene glycol precipitation values, a time-consuming process, or light scatter solubility assessment [54].

Similarly, the selection of solvents, preservation agents, and container are all important during peptide formulation, as these affect stability, solubility, and the

bioavailability of the peptide solution [31]. Polyhydric alcohols, including glycerol, can stabilise peptides solutions. Preservatives may be added to stabilise molecules for a longer shelf-life, although these should be considered cautiously and in-line with regulatory requirements. Containers, including glass or composite materials, should be selected to increase stability and minimise the potential for alterations of peptides, as well as for practical use in the clinical setting.

2.3 Pharmaceutical Excipients

Another important part of the peptide formulation process is the use of pharmaceutical excipients, non-medicinal substances added to facilitate stability and desirable characteristics to facilitate drug delivery in the body [54]. Common excipients for peptide therapeutics include albumin, amino acids, carbohydrates, chelating and reducing agents, cyclodextrins, surfactants, salts, alcohols, and glycol. These excipients have varying biochemical roles but all act to alter the chemical environment, reducing the rate of peptide degradation or enhancing the stability of peptides in specific tissues [33].

Excipients may also play a role as solid supports and linkers, which assist in peptide synthesis and in stabilising the peptides once formed [33]. Solid supports include resins, which are stable and inert, often comprising polystyrene beads cross-linked with divinylbenzene, although many other solid supports are used in contemporary peptide therapeutic formulation [74]. Linkers may be used to attach amino acids of the peptide to the resin or solid support, and the characteristics of these linkers may influence their functionality. Cleavage of linkers often occurs under acidic conditions, allowing for pH-based control over formulation of peptides once stabilised [74].

Finally, the use of excipients as protecting groups has been observed as essential to ensuring amino acids are protected (as well as side chains) from degradation or alteration during peptide synthesis and storage [54]. Two commonly used protecting groups are fluorenylmethyloxycarbonyl (Fmoc) and tert-butyloxy-carbonyl (tBoc), of which many molecules can be used to protect different amino acid residues [84]. Introduction of protecting groups is a complex process and requires careful consideration of the effects of the protecting groups on subsequent synthesis reactions and formulation of the peptide.

2.4 Aggregation in Protein Formulations

Numerous processes and unintended reactions within peptide and protein therapeutic solutions can affect the synthesis and formulation of an effective drug. Aggregation of proteins occurs under numerous environmental conditions and is governed by the intrinsic structural or chemical features of the protein, as well as the

external environment [31]. The consequences of protein aggregation may be a reduction in biological activity or the potential development of immunogenicity, which can limit the therapeutic use of proteins, as well as peptide agents [86]. Aggregation of proteins and peptides may occur in an orderly fashion, often with linear aggregate formation (as seen with amyloid proteins in Alzheimer's disease) [20], or in a disorder manner, termed amorphous aggregation [58]. In both instances, aggregates can serve as seed nuclei for the generation of larger aggregates and visible particles, which can have damaging effects on the cellular environment [86].

Aggregation is dependent on the environmental conditions where the protein or peptide is located. These conditions include temperature, pH, the presence of solvent compounds, and the presence of additional environmental stressors [72]. These conditions affect the intrinsic molecular bonds within peptides and proteins, affecting secondary, tertiary, and quaternary structures, potentially resulting in unfolding, dimerisation, and then formation of oligomers of peptides [81]. The susceptibility of different peptides to aggregation depends on the molecular characteristics of the peptide. Different peptides or proteins may have more desirable environmental resilience, depending on the intended use of the agent and the association of additional drugs or adjuvants [81].

Control of aggregation is essential in preventing loss of biological efficacy of the peptide, as well as preventing immunogenicity, characterised by immunological reactions to the aggregates [14]. The use of protecting groups and microwave heating are techniques associated with the prevention of aggregation in synthesis techniques. Scavenger agents within the working solution can also be used to prevent aggregation, by removing substances that promote aggregation of modify the binding characteristics of peptides [14, 31]. However, these techniques are diverse and individualised for specific peptides, adding complexity to this discussion and suggesting the need for transparent synthesis strategies where aggregation is managed appropriately.

2.5 Peptide Bond Formation (Coupling Methods)

Peptide bonds (amide bonds) form the basis for joining amino acid residues together in order to form peptides [11]. These bonds are formed between a C-terminal (carboxyl group) and N-terminal (amino group) of different amino acids. Although peptide bonds form naturally, facilitating these bonds during peptide synthesis is essential to produce the desired end product. Essentially, the strategy for bond formation involves the presence of amino acid residues, a peptide bond forming reagent, and a target activating group on the amino acid to be joined [11].

Peptide bond forming reagents are numerous, but the most common agents are carbodiimides, symmetric anhydrides, and acid halides [72]. Carbodiimides are water-soluble molecules with the general formula RN = C = NR [9]. These molecules are advantageous in that they hydrolyse to form urea, which does not interfere with peptide synthesis reactions. Carbodiimides activate the carboxyl group of an

amino acid allowing for formation of peptide bonds under certain conditions [9]. Symmetrical anhydrides are carboxyl acid anhydrides that are transient but persist in solution long enough to complete peptide bond formation [72]. Mixed and N-carboxy anhydrides may also be used in peptide synthesis, facilitating the formation of peptides and protected amino acids in solution or solid phase [72].

2.6 Synthesis Approaches

Numerous synthesis approaches have been employed in peptide therapeutics, and a brief discussion of these approaches and their key differences should be considered. The first synthesis of oxytocin occurred in the 1950s using a classical approach, termed solution-phase synthesis (SPS), or synthesis in solution, which remains the main synthesis techniques used in contemporary peptide therapeutics [9]. The principle of this method is to add amino acids to a central amino acid or group or amino acids in sequence, with all reagents in a solution (i.e. homogeneous phase) [9]. The SPS approach is considered beneficial for large peptide synthesis, as the control over soluble elements of the solution can be greatly enhanced by refining the technique [9].

Solid-phase synthesis is an alternative to SPS and is commonly based on the Fmoc/tBu strategy in association with activated carboxyl groups and the use of modified polystyrene resins [21]. Essentially, the C-terminal amino acid is anchored to the solid supporting resin, often using a linking agent, allowing for the removal of the N-terminal protecting group [67]. When performed in sequence, this process protects the side chains from alteration and provides a sequential approach to peptide synthesis as each amino acid is introduced in turn [67]. This process is illustrated in Fig. 1.

Hybrid synthesis involves the use of a solid-/solution-phase approach, whereby peptides are synthesised on solid supports within a solution [9]. For instance, small peptides (e.g. up to ten amino acids) can be produced in solid phase on resins, and then segment condensations are completed in solution to construct the entire sequence of the peptide [2]. This combined approach can overcome the disadvantages of solid-phase approaches, including extensive cost and limits to the size of peptides produced, while taking advantage of the ability to utilise peptides that are not amenable to bacterial expression, required for synthesis in solution [2].

The synthesis of cyclic peptides is an area of specific interest, as cyclic peptides are generally more stable, have greater resistance to degradation, and have longer-lasting (depot) effects in the body [11]. The synthesis of cyclic peptides requires the formation of disulphide bridges of amide bonds between sulfhydryl groups or other groups [90]. These cyclic peptides may be formed using chain-to-side chain, head-to-side chain, side chain-to-tail, or head-to-tail strategies; the choice of technique depends on the peptide structure and the cyclisation position [17]. Most commonly, disulphide bridges are formed between two amino acid elements through a variation of solid- or solution-phase techniques.

Finally, the depsipeptide method for peptide synthesis is designed to overcome the challenges of folding and aggregation with other techniques [21]. Despipeptides

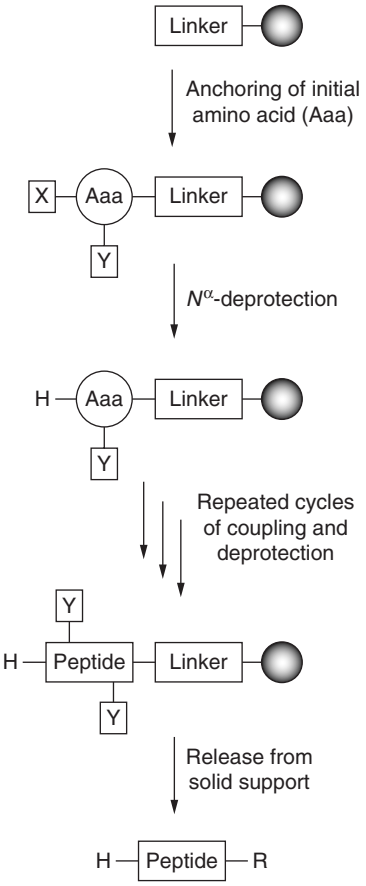

Fig. 1 The principle of solid-phase peptide synthesis. X, temporary protecting group; Y, semi-permanent side-chain amino acid (Aaa) protecting groups; R, C-terminal functionality [67]

are *O*-acyl isopeptides and are ester isomers of the intended peptide sequence, which are more advantageous during synthesis as they are (1) easier to assemble, (2) easier to purify, and (3) can be easily converted to parent amides [21]. This method is generally employed for challenging peptide sequences prone to folding and aggregation using other methods, with a solid-phase basis [21].

2.7 Separation and Purification (Chromatography)

Once peptides have undergone synthesis, it is essential that the resulting solution is separated and purified to remove contaminants and substances that may affect the degradation potential of the peptide product [9]. Chromatography techniques are typically used for this purpose, allowing for separation of molecules based on numerous characteristics, including charge or molecular size, depending on the type of chromatography employed.

Gas, ion-exchange, and affinity chromatography techniques may be employed allowing for separation of peptides and proteins based on molecular size, charge, polarity, solubility, and/or covalent interactions [72]. Selection of appropriate chromatography techniques is dependent on laboratory resources and technician experiences as well as the presence of specialised classes of proteins and the need for amino acid residue distribution and modifications.

2.8 Characterisation with Mass Spectrometry

Following separation and purification of the peptide agent, mass spectrometry can be used as an accurate means of characterising the final product [9]. Ion mobility mass spectrometry (IMMS) has emerged as a powerful analytical tool and is increasingly used to characterise peptides and proteins in complex samples [27]. The principle of ion mobility is the separation of ions based on their size and charge ratios, as well as considering interactions of ions with a buffer gas [41]. This technique allows for an accurate and sensitive way of separating proteins and peptides within a complex mixture, as well as allowing for careful characterisation of all present elements [19]. Five stages are used during IMMS: sample introduction, compound ionisation, ion mobility separation, mass separation, and ion detection [41].

The potential use of IMMS for peptide therapeutics includes the ability not only to separate complex mixtures but also to characterise peptides or proteins within complex mixtures. The complementary approaches of ion mobility spectrometry and mass spectrometry allow for combination into IMMS, which serves as an adjunct to traditional structural techniques. For instance, IMMS can identify rotationally averaged cross-sectional area, which may not be achievable using other techniques, as well as the conformational dynamics of the peptide solution, as well as appreciating folding mechanisms and aggregation profiles of proteins and peptides [48]. High separation selectivity during bioanalysis has been observed [41], emphasising the role of IMMS in peptide therapeutics characterisation.

The reliability of structural interpretation and identification of ions relies on careful calibration of the IMMS equipment and consideration of variables within the analytical process, including gas pressure, gas compositions, temperature, and modes of separation [13]. Therefore, as IMMS technology continues to advance, calibration and regulation of this analytical procedure is needed to ensure consistency in results and utility in drug manufacturing.

2.9 Stability Testing

Stability of peptides is a principle concern for drug manufacturers, as stability can be indicative of the lifespan of the drug in storage and during clinical use [10]. It is essential to determine stability characteristics of any peptide agent to understand the lifespan of the drug.

Many aspects of the environment can affect stability, and stability testing involves monitoring the effects of pH, temperature, humidity, and light exposure on peptide structure, function, and efficacy [9]. Characterisation and stability testing of oral peptide agents' procedures highlight the need for rigor when investigating drugs at this stage of development, but criteria for stability will likely evolve as drugs move from preclinical to clinical development [10]. The complexity of formulations and the use of excipients to facilitate oral delivery of peptides raise an intriguing challenge to stability testing in the future, and standards and testing regimens will need to follow the example of small molecule development to ensure drug longevity and patient safety [6].

3 Delivery System Considerations for Peptide Therapeutics

This section illustrates the role of delivery systems in the development of peptide therapeutics. The route of administration and the delivery method of peptides are heavily dependent on the pharmacokinetic properties of the drug, and the implications of delivery system design are numerous. This section highlights the main delivery techniques used in current practice while highlighting novel strategies and developments for the future of this therapeutic field.

3.1 Pharmacokinetics of Peptides

Pharmacokinetics covers a range of characteristics of a putative drug or molecule when introduced into the body [66]. The term encompasses a range of features of the drug, including bioavailability, volume of distribution, clearance characteristics, half-life, stability, and concentration characteristics (i.e. peak concentration and trough concentration). As noted in previous sections, peptides are prone to degradation and have a short half-life, which impacts on their overall pharmacokinetic profile, making them less suited for pharmaceutical purposes than small molecules [72]. For instance, peptides with a large molecular weight, susceptibility to digestive enzymes, low permeability through the intestine, and hydrophilicity (features common to most peptides) can yield a low potential for distribution throughout the body and the achievement of biological concentration to elicit a therapeutic effect [66].

The pharmacokinetics of peptide agents has implications for administration and device design. Most importantly, the route of delivery is largely determined by the extent to which the peptide drug can survive in the body – degradation due to proteolytic enzymes and acidic conditions can limit oral delivery of many agents [31]. Furthermore, short half-lives of peptides in the body suggest the need for rapid delivery, close to the target organ, often favouring parenteral (i.e. injected) delivery [9].

3.2 Delivery Approaches

Delivery of peptides has seen a massive increase in diversity and design over the past few decades, underlining advances made in the formulation of effective products [80]. The main routes of administration include parenteral, transdermal, oral, inhalation, intranasal, and ocular. Each administration route is associated with unique device characteristics designed to optimise effective dosage and reduce or minimise patient side effects [6]. Each of these routes of administration is associated with distinct advantages and disadvantages in practice and has implications for the design of device used to administer medications [7].

Drug delivery approaches must be carefully considered and should ensure that pharmacokinetic factors are reflected in the delivery route of the drug [86]. Table 2 illustrates the range of delivery technologies used for intra- or transdermal peptide delivery and oral peptide delivery. The delivery approaches of peptides may be related to their specific drug type, i.e. the differentiation between small molecules and biologics. Biologics are often regulated with greater scrutiny than small molecules, and their use as intravenous or injectable agents (e.g. vaccines, monoclonal antibodies, and cytokines) may be preferred to oral routes to enhance delivery and minimise instability [8].

Structural modifications of peptides are diverse and may be used to modify degradation and half-life characteristics in complex ways but potentially compromise the efficacy of the drug [72]. This principle applies to peptide delivery, which may be facilitated by polymers of peptides that are biodegradable or non-biodegradable, the use of enzyme inhibitors, the use of permeation enhancers, and consideration of strategies used to target individual tissues or organs (e.g. transport across the blood-brain barrier).

Delivery systems must be designed with the specific qualities of peptides for which they are intended to deliver. A range of characteristics influence the design of a delivery system, including pharmacokinetics of peptides, available delivery approaches, site of action of the drug, and the clinical use of the drug [7]. Only where peptide stability can be ensured can a specific route of administration be considered for widespread use in practice [87]. Modifications to peptide formulations may yield impressive benefits in stabilising and improving the pharmacokinetic profile of the drug once delivered, but the device characteristics may still provide limitations to the dosage needed and the therapeutic effect [85]. For instance, inhaled peptides used in the management or respiratory conditions may be prone to deposition in the oropharynx, particularly where inhaler technique is suboptimal, limiting the therapeutic efficacy of the delivered dose and increasing the risk of local side effects [83]. Hence, delivery system design and use by the patient can significantly influence how a well-formulated peptide drug impacts the clinical status of the patient. All these factors therefore need to be considered in the context of peptide delivery.

Protection of the peptide against enzymatic or environmental degradation can be achieved using delivery of peptides combined with polymers, designed to either

Table 2 Peptide delivery strategies undergoing or receiving approval, p. 44 [50]

Company	Details	Technology	Reports/claims
Intra- and transdermal delivery of peptides			
3 M	Solid and hollow microneedle patches	sMTS, hMTS	hPTH, PTHrP
Corium	Dissolvable peptide microneedle patch	MicroCor	hPTH
Isis biopolymer	Iontophoresis	IsisIQ	Collagen-stimulating peptides
NanoPass	Intradermal microneedle injection system	MicronJet	Proteins, vaccines
Pantec Biosolutions	Laser-assisted ablation	PLEASE	Triptorelin
Phosphagenics	Topical	Targeted Penetration Matrix	Insulin
TheraJect	Dissolvable peptide microneedle patch	TheraJectMAT	hPTH
Vaxxas	Microprojection patch	Nanopatch	Vaccines
Vysteris	Iontophoresis	SmartPatch	Peptides
Zosano	Solid coated microneedle patch	ZP Patch	hPTH
Oral delivery of peptides			
Access	Oral, receptor-mediated uptake	CobOral	Insulin, hGH
Aegis	Buccal, oral	Intravail	AFREP, octreotide
ArisGen	Buccal, oral	ArisCrown	Exendin, hPTH, insulin
Biodel	Sublingual film tablet	VIAtab	Insulin
Proxima Concepts	Oral, enteric-coated capsule	Axcess	Calcitonin, hPTH
Chiasma	Oral, oily suspension of enhances	TPE Technology	Octreotide
Emisphere	Oral, passive transcellular uptake	Eligen	Calcitonin, insulin, GLP-1, PYY
Merrion	Oral, enteric-coated tablet	GIPET	Insulin, GLP-1, GnRH analogue
Midatech/ MonoSol	Buccal film, nanoparticles	PharmFilm	Insulin
NanoMega Medical	Oral, nanoparticles	–	Insulin
NOD Pharmaceuticals	Oral, nanoparticles	NOD	Insulin
Oramed	Oral, enteric-coated tablet	–	Insulin, exenatide
Unigene	Oral, enteric-coated tablet	Peptelligence	Calcitonin, hPTH, CR845

resist or undergo biodegradation, including conjugation with carriers or polymers, adsorption to carriers, or encapsulation in carrier systems [16]. The principle is that these polymers will facilitate delivery of peptides to target tissues within the body, either by persisting or degrading in a controlled manner, allowing release of the peptide into the bloodstream or target tissue [65]. Polymeric nanoparticles have been used widely in pharmaceuticals for this very purpose and may be designed to release peptides or proteins gradually over time, up to weeks or months [65]. However, this is a complex process, and application to peptide therapeutics is promising, but not complete (Fig. 2).

Enzyme inhibitors may be introduced with the peptide as a means of avoiding degradation upon oral delivery or delivery through other routes [22]. Soybean trypsin inhibitor, FT-448, is a leading inhibitor against chymotrypsin degradation and can enhance peptide absorption as well as prevent degradation when co-administered with peptides in animal models [12]. Other enzyme inhibitors have been trialled for use with insulin and other peptides, with mixed results. Enzyme inhibitors may also disrupt the absorption of normal dietary peptides and may have toxic effects over time [72].

However, although degradation by enzymes remains one of the major challenges to peptide use in therapeutics, peptides are also limited by their poor permeability across membranes and structures [55]. Permeation enhancers have been proposed and include modifications to the peptide structure, to facilitate entry into cells and

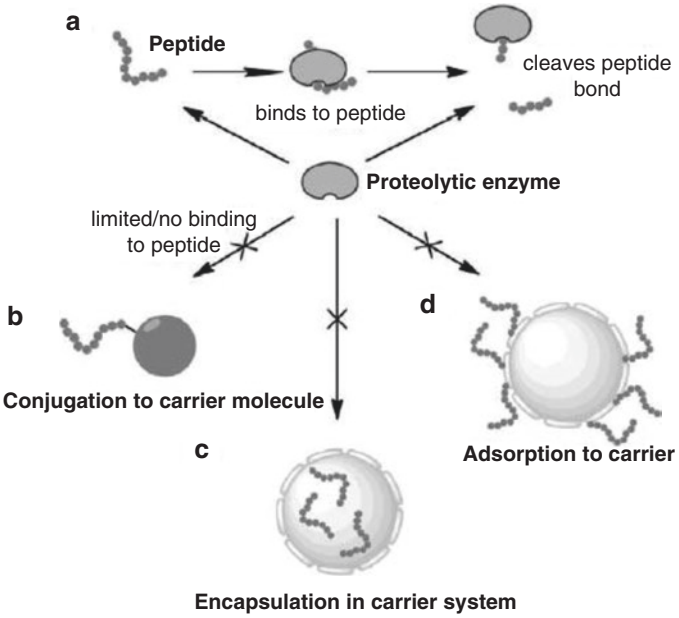

Fig. 2 Conjugation, adsorption, and encapsulation of peptide therapeutics to reduce proteolysis and degradation. (a) Free peptides are rapidly degraded, but the use of carriers (b–d) can block degradation [33]

across membranes [65]. Chitosans are polymer derivatives of chitin, which are known to enhance absorption of macromolecules in the gut, while not being absorbed themselves, potentially limiting side effects of their use [55]. Chitosans have been used in conjunction with insulin, atenolol, and vasopressin and have shown increased permeability and absorption of these peptides, with a good safety profile [62]. Furthermore, lectins and certain types of toxin, which have natural roles in facilitating cross-membrane transportation of macromolecules, have shown promise in enhancing permeation of peptides [62].

Delivery across the blood-brain barrier is a specific challenge for peptide delivery and applies to agents that would be considered to have a primary mode of action on cerebral structures [12]. Liposomes may be used to enhance transport across the blood-brain barrier in animal models, although subsequent liver accumulation of these carriers is a concern [60]. More research is needed to ensure safe and effective transport into the brain prior to human studies.

3.3 Parenteral Peptide Drugs

One of the key delivery strategies for peptides and proteins used for therapeutic purposes is the ability to control the release of the agents, allowing for long-term use without repeat administrations. Furthermore, optimisation of the parenteral use of the peptide, including enhancing stability and targeting specific tissues, is an important feature of modern delivery methods [72]. Microspheres represent one strategy to encapsulate peptides and control their release over time while avoiding degradation [73]. The type of microsphere used in practice is dependent on the polymer used and the sphere-forming method, including the use of phase separation, emulsions, spray-drying, and cryogenic techniques [47]. Typically, the microsphere product is a dry powder that is suspended in the delivery device (e.g. syringe) prior to injection [47]. A similar approach to peptide delivery is the use of injectable implants, essentially polymers inserted subcutaneously and permitting controlled release of the drug over time [1]. Implants can protect peptides from degradation and may be combined with gelling agents to improve their efficacy and length of drug delivery [1]. Concerns over the toxicity and limited lifespan of implants have impeded this area of research and development, although phospholipid-based phase separation gel technology appears to be a low toxicity approach with great promise, as demonstrated in octreotide delivery [89].

Liposomes and nanoparticles have generated a great deal of interest as nanosized drug delivery mechanisms, affording optimal pharmacokinetic and drug release control in parenteral peptide systems [56]. Liposomes are phospholipid-enclosed bilayer spheres, which can be used to transport drugs and peptides and have been shown to improve delivery of anticancer drugs in animal and human studies [4]. Similarly, nanoparticles are colloidal carriers of peptides, fabricated from lipids or polymers, with uniform drug distribution within a matrix [12]. Nanoparticles contain a cargo peptide within a lipid (solid) core, surrounded by

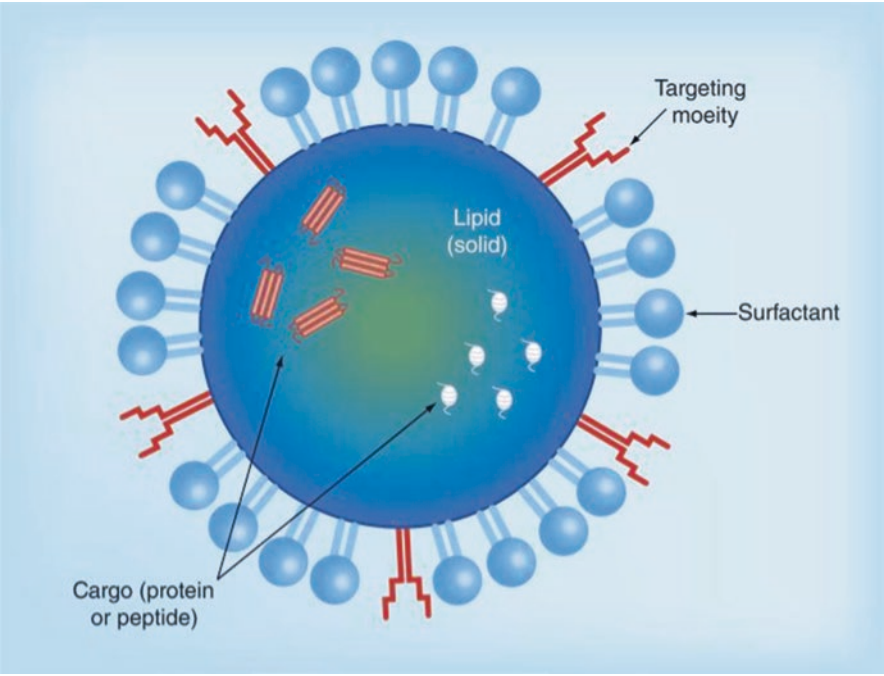

Fig. 3 Solid lipid nanoparticle. The solid lipid core contains the cargo peptide and is surrounded by surfactant, with or without targeting moieties to guide delivery to specific tissues [12]

targeting receptors/ligands and surfactants, allowing evasion of degradation and targeting to specific tissues (Fig. 3). Although both approaches offer the opportunity to overcome degradation and target specific tissues, through promotion of membrane entry, few clinical studies have verified the use of these technologies with peptide delivery [65].

Other approaches to peptide delivery across the skin include the use of microneedles, iontophoresis (electrical charge mediated drug transfer), and patches of drugs applied to the skin (Table 2). The microneedle system (Fig. 4) can involve the use of hollow or soluble microneedles and may rely on skin porations and then drug patch application, needle dissolution in the skin, or infusion of drug formulations through hollow needles. These approaches are actively being explored as patient-friendly approaches to deliver drugs (e.g. human parathyroid hormone (Table 2)). Although a potentially promising route of delivery, there are a couple of key limitations to this approach: (a) immunogenicity (an immune response to the peptide in the transdermal space) and (b) a limitation in the volume of drug product which can be delivered and absorbed in the subcutaneous space.

Vaccines are another interesting area of peptide therapeutics, as peptides may be a preferred vaccination strategy than traditional strategies [53]. The use of proteins and whole or partial microorganisms in vaccines leads to a high antigenic load and the delivery of many substances that may provoke unintended immunological

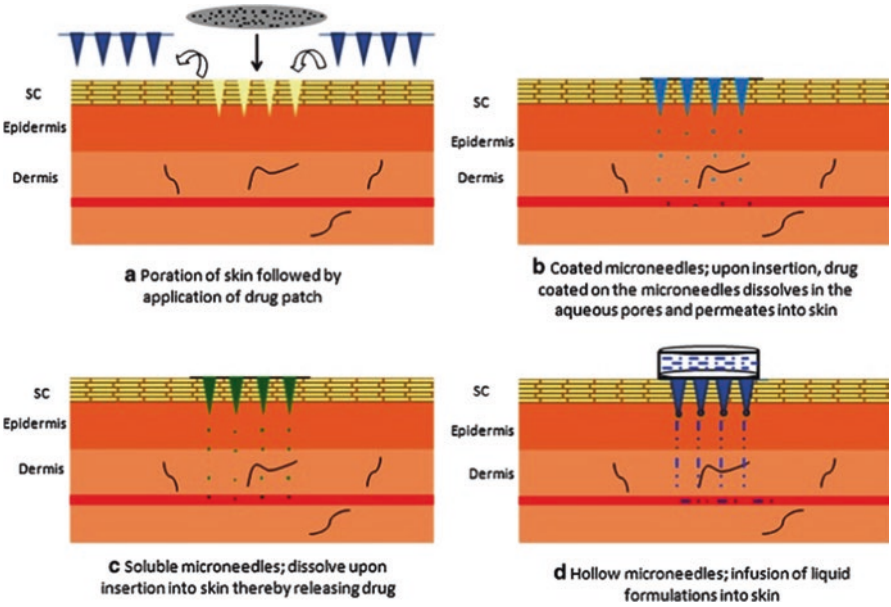

Fig. 4 Microneedle drug delivery systems. (**a**) Solid microneedles cause skin poration, and a drug-loaded patch is placed onto the skin; (**b**) drug-coated solid microneedles are inserted into the skin; (**c**) drug-encapsulated soluble microneedles are inserted into the skin; and (**d**) hollow microneedles allow for liquid formulations to be infused into the skin [40]

reactions [53]. In this application, the potential immunological response is exactly the intended action. Peptide vaccines can avoid this antigenic load, potentially increasing efficacy and reducing the potential for adverse reactions. Emulsions, liposomes, and polymer-based systems have been applied to peptide vaccine development, but efficacy remains weak compared to traditional standards. Adjuvant development is essential to maximise the potential of peptide vaccination for a range of conditions [53].

3.4 Intranasal and Enteral Delivery

The intranasal delivery of peptides has been considered an attractive option for drug delivery due to the potential to bypass first-pass hepatic metabolism and enter the bloodstream rapidly [24]. The use of intranasal drug delivery is considered particularly relevant to central nervous system therapeutics, as the olfactory neurons provide a direct route to this system [64]. Microspheres and liposomes have been used to facilitate intranasal delivery of peptides, but results remain limited in many regards [9]. This may be due to the challenges of accurate dosing with intranasal

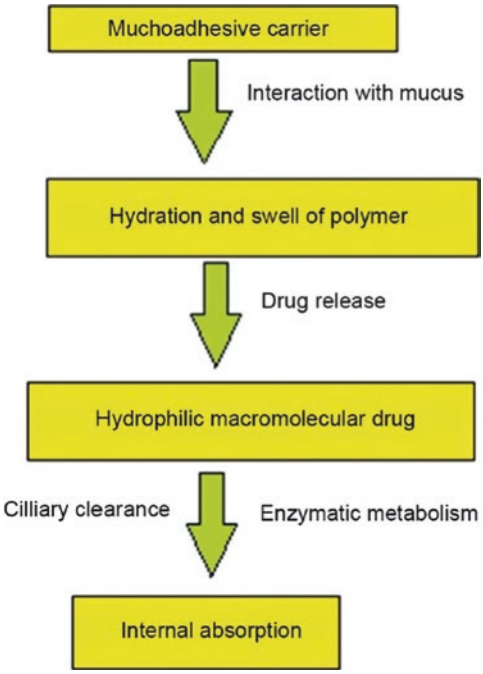

Fig. 5 Schematic mucoadhesive drug delivery for intranasal delivery [18]

methods, including the need for larger doses than delivered via the parenteral route, as well as the presence of degrading enzymes within the nasal cavity [24]. The principle of passing drugs through the nasal mucosa includes consideration of how drugs interact with mucus, can effectively avoid mucociliary clearance, can be effectively released, and can be absorbed (Fig. 5).

Intranasal efficacy for peptides has been achieved at levels comparable to those seen with parenteral peptide administration when using transmucosal delivery agents, including alkylsaccharides [64]. Furthermore, penetration enhancers with or without protease inhibitors offer attractive intranasal delivery methods for peptides [78]. Indeed, chitosan-based delivery methods and alkylsaccharides have both been shown to have applications in the nasal delivery of drugs and peptides in particular [35]. Nanotechnology approaches also hold great promise, despite the lack of clinical breakthroughs in recent years [78]. Chitosan nanoparticle delivery of intranasal peptides appears to maximise the transport of drugs from the nose to brain, compared with simpler chitosan formulations, suggesting that this approach may be worth exploring in the future [15].

Enteral delivery, including delivery through the oral route, has seen similar advances but remains problematic compared with parenteral delivery due to bioavailability issues, the need for higher drug concentrations or dosages, and the issue of degradation. Microemulsions, liposomes, nanoparticles, and microspheres have all been proposed to facilitate delivery of peptides via the oral route [9]. Some of these

strategies have progressed to clinical trial phase and are worthy of greater discussion. For instance, the delivery of insulin using the POD technology approach has been developed by Oramed Pharmaceuticals, Inc. [63], whereby the oral insulin formulation combined with protease inhibitors and absorption enhancers in enteric-coated capsules. This approach is associated with glucose-lowering effects in clinical trials, but safety of the approach needs further validation [83].

3.5 Challenges in Delivery of Peptides

There are multiple challenges to the delivery of peptides for therapeutic effect in the human body. Principally, peptides are easily degraded through enzymatic and chemical processes within the gastrointestinal system, and oral administration of drugs requires significant modification to the formulation to ensure any form of efficacy [34]. The typical route of administration is the use of subcutaneous or intramuscular formulation, which bypasses gastrointestinal enzymes and has a more stable pharmacokinetic profile [31]. However, it is important to note that this route of administration is associated with a range of challenges, some specific to the peptide injected and many ubiquitous across all forms of peptide therapy.

One of the challenges with multidose protein or peptide formulations is the ability to maintain peptide stability and prevent contamination. Most peptide formulations are available in single-dose forms, but multiple-dose forms have the advantage or amenability to dose titration or dose combination. However, preservatives are required within the multidose formulation in order to prevent contamination with microbes and/or microbial growth that may occur during container closure/opening or transient loss of integrity. Bactericidal agents (e.g. 0.1–0.2% phenol or cresol) may be used within the formulation to ensure control of bacterial contamination [23], while specific limitations on the size of the container and the amount of uses permitted can reduce the risk of contamination during use. The amphiphilic nature of peptides encourages adsorption onto materials such as glass, rubber, and plastic, which can reduce the quantity of active materials during processing and storage, and therefore compatibility with primary containers and closures should be evaluated [6, 68]. Specific testing protocols may be applied to determine the effectiveness of stopper mechanisms and/or preservatives, which vary according to geographical region and national standards.

However, it is important to note that the addition of preservatives to the peptide formulation inevitably modifies the stability of the drug. This may lead to product aggregation or precipitation and can affect the shelf-life of the product substantially. Surface binding sites are general finite in nature, and the use of human serum albumin or surfactant agents can effectively prevent active peptide binding during storage, while surfactants may also act to stabilise formulations by preventing denaturation and the tendency of hydrophilic reactions to cause adsorption [39]. Some surfactants can cause reduced stability in peptides, including polysorbate sur-

factants that contain oxidative impurities [6]. Manufacturers should rigorously explore these possibilities and take appropriate remedial actions.

Another consideration which can arise in subcutaneous delivery of peptides is the potential for local toxicity and irritation at the site of application of parenteral peptide therapeutics. Lipohypertrophy is a common complaint among individuals who inject insulin and results from specific effects of insulin on subcutaneous fat (lipodystrophic reactions) that cause a swelling to appear in commonly used injection sites [32]. When these sites are continually used, the absorption of the drug may be erratic, and glycaemic control may be substantially reduced [29, 38]. Although rotation of injection sites and associated patient education is essential in preventing this complication, it is important that device designs are consistent with minimising this risk and optimising drug delivery [32].

4 Conclusion

This chapter has provided an insight into a complex and emerging class of drugs: peptides. Peptide therapeutics is a broad field, and although traditionally dominated by insulin and hormone delivery in states of deficiency, increasingly complex mechanisms are being established through which peptides may exert biological effects. This includes the potential for biologic agents, such as monoclonal antibodies, growth factors, cytokines, and vaccines, all of which can have profound effects on disease courses.

The formulation of peptides remains a complex challenge to maximising the therapeutic potential of these agents. Peptides generally have a poor bioavailability and unstable pharmacokinetics when delivered orally, and they are routinely degraded quickly as part of a natural homeostatic mechanism. Modifications to peptide structure, as well as encapsulation in various devices or delivery methods, can overcome some of these limitations. However, the use of various devices and the development of novel delivery strategies must be cost-effective and should minimise the risk of harm or side effects to the patient.

The delivery of peptides through intranasal, transdermal, intradermal, and oral routes has been achieved in practice, and many delivery methods are being devised to optimise therapeutic effects. In the future it will be vital to optimise delivery strategies to enhance patient adherence and acceptability of therapeutic peptide treatment. Furthermore, the use of nanoparticles and emerging technologies represents a unique opportunity to regulate peptide use in the body and provide a means of achieving modifiable, responsive, and controlled release of peptides over time. This revolutionary approach to drug delivery could minimise the need for repeat administrations while facilitating natural homeostatic mechanisms to release peptides over time. Safety, convenience, and cost all need to be considered in these approaches, as formulation approaches look set to advance with the promise of peptide therapeutics.

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Part II
Drug-Device Combination Products

Chapter 26
Development Challenges and Opportunities for Drug/Device Combination Products

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1 History

The original hypodermic syringe patent was issued to Robb S Porter in 1914. For decades after, subcutaneous and intramuscular drug delivery was performed using a glass vial and hypodermic syringe. It was not until the early 1980s that several European companies came to appreciate the confluence of patient convenience and technological capabilities in design, materials, and manufacturing.

Interestingly enough, it was insulin delivery that first presented itself as an opportunity to improve patient convenience. Given insulin's low therapeutic index (a high-alert medication where both underdosing and overdosing can be associated with serious adverse outcomes), it was a high-risk proposition should they have faltered. It required courage and vision to embark on the development of novel delivery devices, pen needles, elastomers, and cartridge manufacturing. The term "combination product" had not yet been coined. Human factors and risk analysis methodologies were in their infancies, and there was a paucity of global guidance regarding design verification and the device user interface.

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The vial and syringe suffered from a number of user challenges. Most vials of medicinal product, including insulin, required refrigeration. This challenged the notion of convenience and portability given that insulin therapy often required dosing outside of the home (cartridges, in contrast, would later be approved for room temperature storage after first use). Furthermore, dose preparation required the introduction of an equivalent volume of air into the vial. The user would then balance the syringe needle in the vial while they withdrew the desired dose by "eyeballing" the volume against the graduations on the syringe; a method that suffered from parallax, the meniscus, and inherent limitations on visual acuity, thus challenging accuracy of the delivered dose.

Pen injectors and the systems to follow transformed drug delivery beyond function. Success now required an appreciation for two additional concepts: fit and form. Critical component dimensions ensuring accuracy (e.g., inner diameter of 1.5 mL cartridge and pen-injector leadscrew calibrated to move 0.0106″ for each unit of U-100 insulin) had to be identified and controlled (i.e., fit), while the overall user experience required thoughtful ergonomic designs enabling ease of use (i.e., form). The pursuit of convenience in the self-administration of medicinal products promised improved compliance and quality of life with the concomitant result of better health outcomes and lower costs.

System designs evolved rapidly in the 1980s with the introduction of multidose 1.5 mL and 3.0 mL glass insulin cartridges to be used with multidose prefilled and reusable pen injectors, largely from Novo, Nordisk, and Hoechst. Novo launched the reusable 1.5 mL NovoPen I in 1985, the first multidose pen injector. Hoescht was second with their 1987 introduction of the OptiPen I. The merger of Novo and Nordisk in 1988 produced the first prefilled insulin pen (Novolet). Lilly's 1.5 mL insulin cartridge entered the market in the early 1990s (e.g., regional availability with Haselmeier's Diapen in Germany/EU and global availability with like Becton Dickinson's reusable BD Classic and Owen Mumford's reusable AutoPen). The 3.0 mL cartridges and pens were soon to follow. Novo was the only company at the time that manufactured all three components (pen, cartridge, and needle).

These various products and companies highlighted two basic organizational models (i.e., vertical and horizontal configuration) for system development. As an example, in a vertical configuration for pen-injector systems, a single company produces the needle, cartridge, and pen injector, allowing them to better control performance and quality of the overall system. In pursuing a more horizontal approach, a company might manufacture insulin cartridges, while other partner companies manufacture the devices and/or pen needles. Being second or third to market, the horizontal approach likely benefits a company as it facilitates the "catch up" in terms of time and expertise. Given the potential for multiple partners and suppliers in a horizontal approach, contractual agreements governing timely communication of design and manufacturing changes became critical for success. Absent that, the risk of quality and safety concerns would be difficult to anticipate and resolve proactively.

Independent of supply chain configurations, some of the early issues experienced in the market taught hard lessons for all players involved regarding the need for more guidance and dedicated development processes and infrastructure. For example,

system component compatibility concerns surfaced when a *company* attempted to manufacture a cartridge for use in a *competitor's* pen without understanding the critical length dimensions. This caused a failure of the cartridge to engage the pen's internal clutch mechanism (due to insufficient cartridge length, i.e., the fit) resulting in failure to deliver the insulin dose, which could go undetected by the patient.

In another example, system component compatibility concerns surfaced when a *company* attempted to manufacture a cartridge for use in a *partner's* pen without an understanding of the critical geometries and their measurement – the fit. This created sustained stresses to the pen's cartridge housing leading to external component cracking, potential dose inaccuracy, patient complaints, and frustration.

The absence of clear user needs, interface requirements, and assessment methods placed the dose accuracy burden on the end-user when dosing with one of the earliest pen injectors – the form. Given the device's theory of operation, users were responsible for keeping count, for example, whereby a 20-unit dose required counting of 10 repetitive 2-unit injection strokes to complete the full dose.

The lack of design verification and product durability testing guidance became evident when another reusable pen injector suffered a recall due to fatigue in its dial clicker. Claims suggesting that the dose could be dialed by counting clicks were undermined when the clicker became inaudible over time and repeated use. Reliance on counting, in general, can lead to a number of undesirable dosing errors.

Finally, the absence of human factors guidance in the mid-1990s was evident in yet another pen injector, which suffered from a compound use error (i.e., multiple use steps completed out of order) whereby the user could, theoretically, dial a small dose and receive a life-threatening overdose. Serious adverse events were experienced in the market resulting in a recall, spelling the end of that product.

While not to dismiss the important advancements in early drug delivery system development, these difficult lessons demonstrate the value and importance of international standards (refer to Chapter 30), regulatory guidance (refer to Chapters 27, and 28), dedicated engineering resources and project management, robust quality systems, human factors (refer to Chapters 29 and 31), and comprehensive risk management practices (refer to Chapter 29).

2 Power of Global Guidance

Nothing has been more influential in institutionalizing the philosophical and technical importance of the system, the user interface, and design verification of drug-device combination products than the global standards from ISO Technical Committee 84 (Refer to Chapter 30). These standards are tightly linked to ISO 14971 (Risk Management) and ISO 13485 (Design Controls) discussed in Chapter 30.

A standard is a document that provides requirements, specifications, guidelines, or characteristics that can be used consistently to ensure materials, products, and services are fit for their intended purpose. In particular, ISO standards ensure that

products and services are safe and reliable and of good quality. They are strategic tools that reduce costs by minimizing waste and errors, while increasing productivity. They help companies access markets, level the playing field for developing countries, and facilitate free and fair global trade. This facilitates "global relevance" given the breadth of the stakeholder process and the fact that these standards are based on consensus. ISO standards are not statutory instruments (not "the law") but where regulatory bodies expect compliance they effectively become compulsory. When harmonized globally, wherever possible, language is written broadly enough to allow applicability as widely as practical. The desired characteristic of an International Standard is that it can be used and implemented as broadly as possible by affected industries and other stakeholders in countries around the world. They are flexible in approach and have the following characteristics:

- Take into account global regulations and market needs
- Do not restrict free trade
- Are performance-based rather than design prescriptive
- Facilitate innovation
- Require less frequent revision
- Are globally adopted, minimizing the need for regional or national guidance

3 Drug Delivery Systems (Drug-Device Combination Products)

The drug delivery systems of interest here are consumer-based and ambulatory in that they are "hand-held" or "body-worn" and are typically intended for patient self-administration into subcutaneous tissue (inhalers and nasal systems being obvious exceptions). While home or ambulatory use of such devices facilitates the convenience and compliance argument, it also raises the bar for risk acceptability given the potential for misuse as a function of the education/reading comprehension levels of impacted demographics. Examples of these combination products include:

- Prefilled syringes (PFS)
- Pen injectors
- Auto-injectors (i.e., a pen injector or prefilled syringe outfitted with automated insertion, delivery, or retraction functions)
- On-body delivery systems (OBDS: referred to as bolus injectors or large-volume delivery (LVD) devices)
- Patch pumps
- Needle-free injectors (not typically a combination product)
- Inhalers and nasal systems (while not subcutaneous delivery, standards and processes discussed herein apply)

In general, these combination products come in single-dose and multiple-dose formats. Single-dose devices are typically manufacturer-filled, fixed-dose PFSs and

PFS-based auto-injectors where dosing frequency can be weekly, bi-weekly, or monthly (e.g., immunosuppressants). Multiple-dose devices come in both prefilled and reusable devices and are either fixed-dose or variable-dose designs used on a daily basis (e.g., insulin). Except for inhalers and needle-free injectors, most mechanical and electromechanical combination product delivery systems are needle-based. Some concerns for these various formats include the following:

3.1 Multiple-Dose Reusable Devices

- Container changes and priming requirements (depending on accuracy specifications) can introduce patient risk if the user fails to establish contact between the container plunger and the device drive mechanism.
- Removal of mechanical devices from the market once their life expectancy is reached is problematic given reliance on user for compliance with labeling.
- Medication errors if the wrong formulation in a similar-looking/fitting container is used with the device.

3.2 Multiple-Dose Prefilled Devices

- Environmental concerns for disposal of many more devices
- Sensitivity to cost of product sold given a device is used for each container
- Increased complexity of parts due to need for additional features related to automated handling and assembly of higher volume products (including management of tolerances given higher cavity injection molding tools)
- Larger tolerances resulting from higher production mold cavitation
- Impossibility of testing 100% of the devices prior to release.

3.3 Single-Dose Auto-injectors (AI) and Prefilled Syringes (PFS)

Typically, the AI is comprised of a PFS wrapped with a delivery mechanism that might automatically insert the needle, deliver the dose, and retract the needle once the dose is complete. Table 1 highlights several differences between the AI and the user-controlled or manual PFS:

With the number of monoclonal antibodies (mAbs) and other biologic entities entering clinical trials, a key trend toward higher doses and thus higher injection volumes presents opportunities and challenges for combination product development. In general, mAbs are recognized to have a wide therapeutic window, and the trend has been to explore higher doses to achieve maximum efficacy. While formu-

Table 1 Differences between the AI and manual PFS

Property	AI	Manual PFS
Injection technique	Flat	Pinch hold or pinch release
Needle length	5–7 mm	12 mm to achieve 5–7 mm depth
Needle angle	90 degrees	45 degrees
Injection depth	Constant	Variable
Injection speed	Higher (faster)	Lower (slower)
Cost	Higher	Lower

lators have been successful at increasing mAb concentrations to keep injection volumes around 1 ml, many of these new biologics in development become too viscous or are unstable (e.g., aggregation) at higher concentrations and thus require delivery volumes much higher than typical 1.0 mL prefilled syringes. As a consequence, delivery times (greater than 10–15 seconds) can challenge a user's ability to comfortably hold and deliver (e.g., viscosity increasing glide force) the dose. These new therapeutics and related formulation constraints (including the need for refrigeration) further highlight the level of innovation required and the challenges for developing new classes of combination products (e.g., OBDS, 2.25 mL prefilled syringes/auto-injectors) and the growing role of automation.

However, automation comes with its own set of challenges. While many users desire automation for reasons of simplicity and preference or to address physical limitations like neuropathy, hand size, and hold time, some patients may prefer to "participate" in the actual injection as it can serve as sensory confirmation of drug delivery progress and completion. As such, users many not have "faith" in what is now automated when it was once their responsibility. Also, while automation strives to simplify operation for the user, it tends to belie the requisite technical complexity inside. Here, developers must appreciate the potential consequences of automation where what may have been use error is now a malfunction and patient noncompliance is now company liability should these systems fail (e.g., electromechanical failure, internal failure to trigger delivery, spring forces unable to overcome plunger glide forces).

3.4 Platform Designs

Platform designs are not based on an existing device. They start from a "clean sheet of paper" and have product lifecycles of 5–10 years. The basics of these core designs can be modified to become follow-on variants that achieve a specific purpose. Device designs derived from the core "platform" can be referred to as iterations with reduced development timelines of 1–2 years. These variants may change a few parts or may be substantially different while still relying on the same core design or mechanism (e.g., 1–3 mL auto-injector with same theory of operation). The nature of the therapeutic and the targeted demographics are important inputs in making those decisions. The advantage of iterating a platform design is that they can keep

the device constituent part of the combination product off the critical path given the opportunity to leverage an existing design and manufacturing infrastructure. This can assuage concerns from the medical organization when weighing the risk of introducing the combination product into the clinical plan earlier compared to later (e.g., delay the AI and use a bridging strategy to compare AI to PFS at a later time) such that risk to the drug program is minimized. The challenge of platforms is "exposure" in that multiple therapeutics or formats could be placed at risk when core design or manufacturing issues arise in the market.

4 Organizational Challenges

To address the challenge of developing combination products, a dedicated device group, a development process model, an integrated organizational structure, and device quality system are required, independent of whether that structure is vertical or horizontal. The development model must be capable of providing innovation at a pace that can match or exceed the schedule of the overarching therapeutic to be marketed.

Ultimately, the question is where should central control and project management for device development reside? The reality is that these programs, at their core, are complex engineering project management efforts and are likely best located within a device development organization. They should not be "special projects" within the drug development organization or run out of the packaging development group or the impacted therapeutic business unit. That said, for traditional pharma companies, devices are rarely stand-alone considerations either and must instead be fully integrated into the individual drug molecule development strategies. These are important considerations given the fact that many of the combination product development efforts originate from within large pharmaceutical and biotechnology organizations whose primary focus and expertise are drug development.

4.1 Drug and Device Organization Integration

Device development models come in many forms and phases. Nevertheless, a core structure should have several basic elements. While the components and activities will not be solely owned by the device group, their integration with the effort is critical to developing novel delivery technologies in an expeditious and compliant fashion. A version of the basic device development process mode is listed below:

- Advanced development
  - Market opportunities and customer needs identified through corporate intelligence, technical expertise, and marketing
  - Legal management of intellectual property to protect innovation and manage freedom to operate

- Development/commercialization/registration
  - Rapid conversion of innovative ideas in dedicated programs
  - Design and prototype development.
  - Packaging and labeling development
  - Pilot product and design verification
  - Human factors and regulatory strategy
  - Clinical plan development
- Marketed products
  - Manufacturing scale-up/validation of high-quality products
  - Distribution and sales/call center training
  - Product monitoring processes (adverse events and product complaints) and continuous improvement

Device development milestones must be integrated and aligned with drug development milestones. Common milestones in the drug development model that are key to coordinating with the device program would likely include:

- Candidate selection
- First human dose
- First effective dose
- Product decision (drug product proof of concept)
- Marketed formulation decision
- Phase IIB/III clinical trials
- Registration
- Approvals
- Global launch

Figure 1 shows a hypothetical schematic of how drug and device development processes and milestones might be aligned in integrating the development of drug-device combination products.

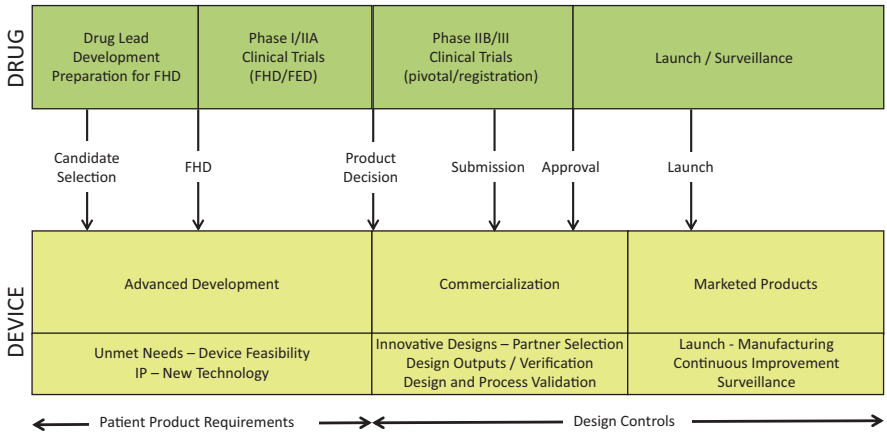

Fig. 1 Hypothetical alignment of drug and device development milestones

Alignment of these two models might, for example, co-locate formulation chemists with advanced development engineers such that spring forces in an auto-injector are matched with the physical properties of the to-be-marketed drug or biologic formulation. Likewise, the medical organization should engage early to be positioned to make informed decisions regarding the early introduction of the representative to-be-marketed combination product into the clinical development plan. Again, absent that engagement, the medical organization might be reluctant to "place the molecule at risk" by introducing a new and "unproven" device design. Moreover, the medical organization plays the critical role in assessing the overall residual risk of the final product such that they own the final risk/benefit determination (in collaboration with governance) and whether postmarketing clinical follow-up (PMCF) studies are required. Early engagement allows for more informed decisions such that the business is more comfortable advancing the final combination product toward validation and launch.

Early involvement from the marketing group is also critically important. By understanding the challenges and timeline impact to managing change in the development and manufacturing processes, they will be less prone to requesting, for example, modifications to the device that are naively perceived to be "simple" or "aesthetic" late in the process (e.g., requesting component color change to resins impacting injection molding tool design or process validations). Such fundamental gaps in understanding can damage core relationships and create finger-pointing and significant program delays.

4.2 Project Management/Technical Leadership

Drug development lifecycles (7–12 years) are considerably longer than device development cycles (2–6 years). As such, the same device program leadership and core resources are more likely to stay engaged throughout a program without "missing out" on other career opportunities or feeling stagnant in a specific role. This can provide continuity and predictability in delivering the asset, which is important given the pace of innovation, competitive pressures to meet schedules, and the reality that the device constituent part may be the competitive advantage compared to the competitor's product (i.e., differentiation based on convenience of user interface).

Due to these urgencies and expectations from the business, project management must be prepared to manage uncertainty. That can be accomplished through the delicate balancing act of carrying contingency or parallel designs forward long enough to integrate the best ideas of each into a final synergistic design without prolonging timelines and resource utilization so as to be prohibitive. Simply put, contingencies can help optimize scope without unreasonably impacting cost and time.

Therefore, relative to the device constituent part, central project management and communication leadership should reside within the development group. Marketing and other corporate project management resources, unlike the core engineering and technical roles, are more likely to move on to other opportunities at a pace that can damage continuity of the device program. It should not matter which

part of the broader organization (e.g., the business side) is providing the capital to the extent that "control" goes to those "writing the checks." Rather, core project management should remain with the device engineering group developing the delivery system. In fact, focus and potential separation from the broader organization can be helpful. However, that should not ignore or diminish the value of integration and communication between all functional disciplines for both the drug and device constituent parts. The basic elements of sound project management include:

- Appreciation for the relationship of time, scope, and cost.
- Decision-making empowerment from senior leadership.
- Corporate commitment at senior levels.
- Commitment to not operate out of an environment of crisis (i.e., time only).
- Informed partner selection and the establishment of trust and respect.
- Management of project risks with clear mitigation strategies for key risks.
- Clearly defined project teams across all partners.
- Deep technical engineering expertise.
  - Model early, Model often: Encourage team to test the design and the system (all constituent parts) as much early as possible. The later you do simulations (e.g., highly accelerated lifecycle testing (HALT)), the harder it is to reconcile the final product with discrepancies coming from the theoretical simulations and related assumptions. Let the modeling direct the team early.
  - Contingency designs: Carry alternative designs forward in parallel in an effort to de-risk a single design solution. Merge best of both ideas at the appropriate time to optimize scope while avoiding excessive cost.
- Operate from a single integrated project schedule for all partners.
- Smaller team structure with broader responsibility.
- Team leaders empowered to make decisions and coordinate with their respective leadership.
- Overall team leadership should come from the sponsor. That leadership must establish clear roles and responsibilities and clear communication across the program.
- Right-sized meetings based on relevant issues to be resolved.
- Adoption of core terminology and methods for all partner interactions and documentation.

4.3 Regulatory Challenges

While it is largely accepted globally, given primary mode of action, that drug-device combination products are regulated as drugs, regulatory treatment of drug-device combination products is not harmonized internationally (e.g., US 21 Code of Federal Regulations (CFR) Part 4, EU Medical Device Regulation (MDR), related

device guidance). Drug branch leadership in combination product review and regulation is often inefficient when the innovation is largely device-related. It can be frustrating for sponsors when the drug experts, with or without device consultation, facilitate "inefficient" reviews of the device constituent part.

That said, a global regulatory framework for combination products is evolving and should address the following:

- Device and drug quality system integration (e.g., GMP and QSR)
- Safety reporting for the combination product as a whole
- Postapproval changes to the device constituent part
- Review timelines for the device constituent part
- Human factors coordination
- Timely introduction of combination products in clinical trials
- Bridging requirements for clinical to the to-be-marketed device (see Chapter 33)

In the prior century, most needle-based combination products were designed around existing and well-characterized drugs (e.g., insulin and growth hormone) using relatively mature containers (i.e., 1.5 mL and 3.0 mL glass cartridges). Products were rarely required to be tested in clinical trials even if they were prefilled and regulated as drugs. Human factors (HF) and other device-specific characterizations were also not required for the most part.

In this century, however, there is much more competition across many more therapeutics with vastly different physical properties (e.g., viscosity, volume, pH) impacting device design and customer satisfaction beyond simply force to inject. Novel primary containers, formulations, and devices are now being developed in parallel. Human factors and product disposal considerations are consuming enormous resources. Regulatory authorities demand more clinical trials where the to-be-marketed device is wanted for initiation of Phase IIb or Phase III trials. A sponsor, however, may desire, as noted previously, to use the PFS in Phase III while delaying introduction of the AI until launch or later in clinical trial extension arms. This can be met with regulatory resistance given comparability (bridging) concerns (see Table 1 for comparison of PFS and AI features). Frequent, early, and constructive conversations with the regulatory agencies can be helpful in resolving these debates and providing clarity to the path forward.

4.4 Human Factors Challenges

Human factors or usability evaluations are intended to generate strong evidence that a product is safe and effective when used by the intended populations in the intended use environments and in foreseeable use and misuse scenarios (refer to Chapter 31 and ISO 62366, Application of usability engineering to medical devices). The goal for the medical organization and governance is to conclude that any *residual risk* remaining after validation:

- Cannot be further reduced by design modifications to the user interface employing state-of-the-art technology
- Is outweighed by the benefits

These are important expectations in ensuring patient safety. Successful human factors programs, however, do not necessarily mandate results producing zero errors or observations as noted in the last bullet above. Nevertheless, confusion or disagreement around acceptable outcomes from human factors studies between sponsors and regulators can result in considerable program delays. Engagement with regulators and well-crafted protocols and reports are key in establishing expectations and minimizing program risks.

4.5 Needed Improvements in Regulatory Interactions

Program complexity is increasing as a function of the technologies and therapeutics such that better integration of drug and device development is warranted. With regard to quality system application to combination products, the spirit of the device regulations should be applied to the device constituent part (e.g., QSR), while the spirit of the drug regulations should be applied to the drug constituent part (e.g., GMP). Moving forward, it is important that sponsors engage with regulators, request the appropriate representation for meetings, and consider making the following points:

- Representative combination product designs are sufficient for clinical trials because, unlike drugs, we should expect minor device iterations even in Phase III.
  - Some design elements will change and can be changed without impacting drug/treatment exposure or delivery profiles.
  - Change can be managed with bridging studies using performance-based standards and engineering surrogates like those of TC 84 (see Chapters 30 and 32).
- Reviews should employ a least-burdensome approach. For example, in the United States, if the drug constituent part is an existing and well-characterized product while the device constituent part is new, a 510(k) review approach might be most appropriate even when the review is owned by the drug review branch.
- Review Centers (e.g., CDER vs. CDRH) must be constrained to their expertise. With regard to human factors:
  - Single source of HF requirements should be driven by the device constituent part.
  - One HF plan should be sufficient for platform designs supplemented per demographic/therapeutic specifics.
  - Sponsors need clarity on when and why to include both trained and untrained participants.
  - PK/PD studies are not appropriate for HF evaluations.

  - Take-home studies are not appropriate for HF evaluations as patients do not self-report use errors.
  - Foreign data and global AE/PC data may be acceptable for usability assessments.
  - Regulator reviews of formative and summative HF protocols prior to initiation of clinical trials may not be necessary.

- Use of device master files (DMF) for platform technologies.
- Regulators demanding late-phase instructions for use (IFU) revisions should be limited without strong rationale given impact on design controls, risk analysis, and timelines.

These challenges, if unresolved, can cloud predictability and create significant variability in review and approval timeframes. Again, the least burdensome risk approach is preferred.

4.6 Future Opportunities and Challenges

The level of innovation underpinning the Internet of Things (IOT) is a technological tsunami. Sensors of all types (e.g., motion, biological, environmental, gesture) and applications are proliferating across multiple product classes, not the least of which is healthcare. Vast amounts of data (e.g., blood sugars, heart rhythm, location, altitude, maintenance alerts) are becoming available for analysis and decision-making. Mobile applications are making this data available to the masses in terms of individuals and corporations. These advances are helping to better engage patients with healthcare professionals (HCPs) to improve quality of life and health outcomes as patient care migrates from hospitals and doctors' offices to self-administration in the home. For example, the use of an OBDS preprogrammed to deliver a critical medication 24 hours after discharge from the hospital saves the patient the time and cost of another visit to the hospital (refer to Chapter 36). It might also help prevent accidents should patients experience side effects from the treatment while returning home. Devices like OBDS and multidose pen injectors can be outfitted with measurement and communication capabilities and connected to mobile medical applications (MMAs) to facilitate data collection and patient alerts. Alerts might include last-dose memory, dose history, occlusion alarms, and related functionality issues as well as dosing recommendations. This connectivity comes with the responsibility to protect patient identifiable data that, if ignored, can lead to patient and business harm.

With advances in battery technology and power management, untethered (i.e., not connected to the grid) gadgets are capable of greater levels of functionality and connectivity over longer periods of time enhancing patient mobility. The impending arrival of 5G mobile networks and wireless systems (global telecommunication standards) and developments in artificial intelligence/machine learning, cloud-based storage, and distributed data structures like blockchain are creating opportu-

nities and security capabilities whose impact on society is yet to be fully appreciated in terms of both good and ill. In particular, the implications for individuals' privacy and cybersecurity are at the fore. Refer to AAMI TIR57 (Principals of Medical Device Security – Risk Management) for a detailed discussion.

These opportunities cannot blind sponsors from the responsibilities that come with this level of patient engagement. For example, patient-entered information, including opinions and statements, if entered as free text on an MMA, represents a potential diluvian flow of data that must be reviewed and acted upon to manage compliance requirements. What portion of that data constitutes a potential product complaint? What portion, in context with other collected data, represents a reportable adverse event (e.g., is a blood sugar of 500 mg/dl stored in the cloud a reportable hyperglycemic event if associated with a specific insulin)?

In a world of globalization, how do companies and developers arrive at common practices and procedures to address the mobility of patients in terms of the data that traverses national borders and unique and sometimes uncoordinated approaches to patient protection (e.g., understanding the US approach to privacy compared to General Data Protection Regulation (GDPR) in the EU)? What happens with current or inchoate cybersecurity methodologies with the approaching dawn of quantum computing? As Wayne Gretzky asked: "Are we skating to the puck or to where the puck is going to be?"

Devices capable of communicating with an MMA and the cloud carry considerable risk in terms of data breaches and "hacking." The standards and security protocols to protect these devices and patients are still being developed and harmonized, while the concerns for bad actors are a problem that exists today. Bad actors want to exploit the data for financial gain and, in some instances, for harm (e.g., a connected infusion pump hacked to create a "run-away" pump).

Are the risk management and design controls implemented for "traditional devices" appropriate or sufficient for digital devices and the bidirectional data flows they create? Regulators consider some software systems to be a medical device based on functions provided (e.g., dose selections designed to improve glycemic control). What constitutes a reportable malfunction if the functions provided do not translate to improvement? Unlike most "manufactured" products, software, when released into the market with a bug or anomaly, reflects a defect rate of 100%.

Are current measures and protections sufficient? What additional policies and procedures are indicated to evolve quality systems to the point where software as a medical device (SaMD) and cloud-based data analytics are compliant and robust?

5 Conclusion

Competent and timely development of drug delivery systems designed for self-administration requires a thoughtful organizational model, dedicated and centralized engineering project management, global design verification standards, and regulatory

engagement. Advances in sensors, wireless communication technologies, mobile medical applications (MMA), and cloud-based data paradigms are empowering patients and healthcare providers. However, these opportunities are not without risk.

Because innovation and the rules that govern are evolving in parallel, it is important for sponsors to review current systems, engage with regulatory authorities, and participate in industry and related groups (e.g., ISO, AAMI) to help shape the rules with which they must comply.

Chapter 27
Evolving Regulatory Landscape for Combination Products: US Perspective

Suzette M. Roan, Bonnie Scott, and James Boiani

1 Combination Products Defined

Combination products are defined in the USA by 21 U.S.C. 513(g) and 21 CFR 3.2 (e). Each component of a combination product (i.e., drug, device, or biologic) is called a "constituent part," and the combination could be drug-device, biologic-device, drug-biologic, or drug-device-biologic. The constituent parts may be physically or chemically combined, co-packaged, or separately distributed with specific labeling for their combined use:

- *Single-entity combination products*: two or more regulated components combined together to produce a single entity. *Examples: monoclonal antibody in a prefilled syringe or autoinjector; drug-eluting stent*
- *Co-packaged combination products*: two or more separate products packaged together in a single package/unit. *Examples: lyophilized drug vial in a kit with prefilled diluent syringe*
- *Cross-labeled combination products*: two or more components packaged separately but each labeled to indicate use specifically together to achieve the intended product effect. *Examples: topical solution and photodynamic therapy light*

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The regulatory requirements travel with each constituent part, and each constituent needs to meet the requirements for the constituent part and retains its legal status when combined. The primary mode of action (PMOA) of the combination product, in addition to how the drug, biologic, and/or device constituent parts are combined, will define the regulatory submission pathway(s) and labeling requirements for the combination product. For drug-device combination products, the PMOA is determined by which constituent part plays the primary role in producing the intended effect of the product. For example, a prefilled syringe has a drug PMOA, where the drug provides the therapeutic effect with the device being supportive by aiding in drug delivery. Alternatively, with a drug-eluting stent, the device has the PMOA as the physical effect of propping open an artery is the primary role and the drug plays an ancillary role, e.g., by reducing risk of clotting or improving healing . In the USA, the primary mode of action determines which Center will have primary jurisdiction over regulation of the combination. CDER has primary jurisdiction over drug and many biological PMOA products, CBER has primary jurisdiction over some biologic and device PMOA products, and CDRH has primary jurisdiction over most device PMOA products.

2 Combination Product Regulations and Key Policy Events

As innovators continue to look at ways to marry new device technology with drugs and biologics to improve outcomes, FDA is likely to receive a growing number of combination product submissions. These submissions have historically presented, and continue to present, certain regulatory and administrative challenges for the Agency, due in part to the fact that different combination product components (i.e., drugs, devices, and biologics) are typically reviewed by different FDA Centers, each with different policies, precedents, and cultures. However, FDA has devoted significant attention to these challenges in recent years, pursuing various efforts to improve the consistency and transparency of the combination product review process. Although the Agency is aware that there is still work to be done to ensure a more seamless regulatory pathway for these products (and prepare for new combination product review challenges that are bound to arise as these products and their underlying technologies evolve), the progress made to date and the Agency's drive toward improvement are encouraging.

2.1 Historical Overview

When combination products started coming on the scene in the 1970s, after Congress passed the Medical Device Amendments of 1976 (establishing a new and distinct risk-based regulatory framework for devices), there was no specific FDA framework in place to regulate these products. This left FDA to consider combination

products on a case-by-case basis and created significant regulatory uncertainty among sponsors. The landscape began to change, however, in 1990, when Congress passed the Safe Medical Devices Act ("SMDA").[1] The SMDA amended the Federal Food, Drug, and Cosmetic Act of 1938 ("FDCA") to expressly recognize the existence of products that "[constitute] a combination of a drug, device, or biological product" and create a mechanism for determining which Agency Center would be assigned the responsibility of regulating a particular combination product. The assignment process was based on the product's PMOA.[2] Soon after, in 1991, FDA promulgated product jurisdiction regulations (set forth at 21 C.F.R. part 3), which implement the SMDA and detail the "request for designation" ("RFD") process for determining the regulatory assignment of combination products where such jurisdiction is unclear or in dispute.[3]

Also, in 1991, the Agency Centers (CDER, CBER, and CDRH) entered into Intercenter Agreements, which described how the Centers would work together to manage the regulatory process for combination products.[4] At the time, these agreements provided important, though non-binding, guidance related to product jurisdiction. While the guidance in these agreements is still instructive for sponsors, they are much less useful today as combination product technologies have evolved significantly and new laws, regulations, and guidance have emerged.[5]

Another major landmark for combination product regulation came in 2002 with the enactment of the Medical Device User Fee and Modernization Act of 2002 ("MDUFMA"), which established the Office of Combination Products ("OCP") within FDA. OCP identifies its primary roles as follows:

- To serve as a focal point for combination product issues for FDA staff and industry
- To develop guidance, regulations, and standard operating procedures to clarify the regulation of combination products
- To classify products as drugs, devices, biological products, or combination products and assign an FDA center to have primary jurisdiction for premarket review and postmarket regulation where the jurisdiction is unclear or in dispute.

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[1] 21 U.S.C. § 353(g)

[2] This assignment process was refined by the Food and Drug Administration Modernization Act of 1997 ("FDAMA"), which provided a mechanism for sponsors to request that FDA classify a product as a drug, biological product, device, or a combination product. In response to such request, FDA is required to provide its determination regarding the appropriate product classification or review Center within 60 days. If FDA fails to do so, the sponsor's request is considered the final determination of the Agency. 21 U.S.C. § 360bbb-2.

[3] 56 Fed. Reg. 58754 (November 21, 1991)

[4] FDA, Intercenter Agreements, https://www.fda.gov/CombinationProducts/JurisdictionalInformation/IntercenterAgreements/default.htm

[5] In 2006, FDA noted the increasingly limited usefulness of the Intercenter Agreements and stated that rather than updating these documents to improve regulatory transparency, it would pursue various other approaches to provide stakeholders with information about the jurisdictional process. 71 Fed. Reg. 56988 (Sept. 28, 2006).

- To ensure timely and effective premarket review of combination products by overseeing the timeliness of and coordinating reviews involving more than one agency center
- To ensure consistency and appropriateness of postmarket regulation of combination products
- To facilitate resolution of disputes regarding the timeliness of premarket review of combination products
- To update agreements, guidance documents, or practices specific to the assignment of combination products
- To develop annual reports to Congress on the Office's activities and impacts
- To provide training to FDA staff and regulated industry on combination product regulation.[6]

Having a specific FDA office in place dedicated to combination product review issues spurred the issuance of a number of combination product-focused guidance documents and regulatory updates from the mid-2000s to the mid-2010s, which, overall, helped provide much-needed regulatory clarity to industry. These guidance documents and regulatory updates included, for example:

- 2004 draft guidance on the current good manufacturing practice ("cGMP") requirements applicable to combination products (such requirements were eventually finalized and memorialized in a 2013 final rule issued by FDA (21 CFR part 4), and FDA cGMP guidance for combination products was finalized in 2017[7])
- 2005 amendments to the Agency's product jurisdiction regulations to clarify the definition of the terms "mode of action" and "PMOA" and establish an algorithm to assist the Agency in assigning combination products to an Agency Center for regulatory oversight where the Agency cannot determine with reasonable certainty which mode of action provides the most important therapeutic action of the combination product (and require sponsors to base their recommendations for Center oversight of their combination products based on the PMOA definition and the assignment algorithm, as appropriate)[8]
- 2006 guidance on "Early Development Considerations for Innovative Combination Products"[9]

[6] FDA, Office of Combination Products, https://www.fda.gov/AboutFDA/CentersOffices/ucm2018184.htm (last updated Oct. 4, 2018)

[7] FDA, Current Good Manufacturing Practice Requirements for Combination Products: Guidance for Industry and FDA Staff (2017), https://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM429304.pdf

[8] 70 Fed. Reg. 49848 (Aug. 25, 2005). In May 2018, FDA issued a proposal to further amend the product jurisdiction regulations to: "(1) [c]larify the scope of the regulations; (2) streamline and clarify the appeals process; (3) align the regulations with more recent legislative and regulatory measures [(including the 21st Century Cures Act, as discussed below)]; (4) update advisory content; and (5) otherwise clarify the rule, including updating it to reflect Agency policies and practices." 83 Fed. Reg. 22428 (May 15, 2018).

[9] FDA, Early Development Considerations for Innovative Combination Products: Guidance for

- 2011 guidance on "How to Write a Request for Designation (RFD)"[10]
- 2013 draft guidance on "Submissions for Postapproval Modifications to a Combination Product Approved Under a BLA, NDA, or PMA"[11]
- 2016 draft guidance on "Human Factors Studies and Related Clinical Study Considerations in Combination Product Design and Development".[12]

On top of these regulatory and guidance-based developments, significant statutory changes affecting combination product regulation were enacted in December 2016, with the passage of the 21st Century Cures Act ("Cures Act"). Before summarizing these updates, we explore in the next section some of FDA's combination product-focused initiatives that were launched in the year leading up to the enactment of the Cures Act.

2.2 *FDA's Focus on Combination Products Leading Up to the Enactment of the Cures Act*

Dr. Robert M. Califf, who became FDA's Commissioner of Food and Drugs in late February 2016, recognized the importance of combination product innovation, and the unique regulatory challenges that these products presented. During his brief, 11-month tenure in the Commissioner role (which included the months leading up to the enactment of the Cures Act), he made improving the combination product review process an Agency priority.

Even before Dr. Califf became Commissioner (he previously served as FDA's Deputy Commissioner for Medical Products and Tobacco), he was intently focused on combination product issues. For example, an October 2015 FDA Voice blog post co-authored by Dr. Califf emphasized that FDA could do more to ensure the premarket review process for combination products runs smoothly, and noted that improvements were underway.[13] The blog post also referenced a FDA focus group study and resulting report (Intercenter Consult Process Study Report) which found

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Industry and FDA Staff (2006), https://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm126054.pdf

[10] FDA, How to Write a Request for Designation (RFD): Guidance for Industry (2011), https://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM251544.pdf

[11] FDA, Submissions for Postapproval Modifications to a Combination Product Approved Under a BLA, NDA, or PMA: Draft Guidance for Industry and FDA Staff (2013), https://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM336230.pdf

[12] FDA, Human Factors Studies and Related Clinical Study Considerations in Combination Product Design and Development: Draft Guidance for Industry and FDA Staff (2016), https://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM484345.pdf

[13] FDA, The Merging of Medical Products: Enhancing review of therapeutic and diagnostic combination products, FDA Voice (Oct. 15, 2015), http://wayback.archive-it.org/8521/20180925234325/https://blogs.fda.gov/fdavoice/index.php/2016/12/combination-products-review-program-progress-and-potential/

that differences between Centers with respect to communication, policies, practices, and systems created challenges during combination product reviews. In addition, the blog post discussed the steps FDA planned to take to address these findings including, for example, issuance of additional combination product guidance, facilitating staff in requesting and monitoring intercenter consults, and improving internal standard operating procedures related to combination products.

As detailed below, a number of new FDA combination product initiatives and process improvements were, in fact, launched as 2016 progressed. By early December 2016, even though the passage of the Cures Act was still on the horizon, with the initiatives described in the following sections in motion, FDA felt that it had already made solid progress in improving the combination product review process, and released a document summarizing the Agency's progress and next steps.[14]

Lean Management Process Mapping

In March 2016, FDA released a blog post discussing the Agency's plan to launch a "lean management process mapping" approach to create a more cohesive and collaborative system for the review of combination products.[15] FDA noted that it expected two key deliverables from this mapping: (1) a "current state" map showing existing sources of delay or redundancy to allow the Agency to target areas for improvement and (2) a "future state" map showing a streamlined, efficient process to eliminate the identified delays/redundancies.

Although this lean management approach sounds promising in theory, and has been employed successfully elsewhere in the Agency, FDA has not released further information regarding this initiative or the proposed deliverables to date.

Combination Products Policy Council

In April 2016, FDA announced the creation of the Combination Products Policy Council, a senior-level forum charged with (i) ensuring better coordination and consistency in combination product policy across the Agency and (ii) resolving disagreements among the Centers, OCP, and/or sponsors on issues related to combination products, including those related to medical product classification, clearance/approval, and cross-labeled products.[16] Specifically, the Council's stated goals are to:

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[14] FDA, Combination Products Review Program: Progress and Potential, FDA Voice (Dec. 2, 2016), http://wayback.archive-it.org/8521/20180925234325/https://blogs.fda.gov/fdavoice/index.php/2016/12/combination-products-review-program-progress-and-potential/

[15] FDA, "Leaning in" on Combination Products, FDA Voice (Mar. 7, 2016), http://wayback.archive-it.org/8521/20180926002206/https://blogs.fda.gov/fdavoice/index.php/2016/03/leaning-in-on-combination-products/

[16] FDA, Developing a Consensus Voice: The Combination Products Policy Council, FDA Voice (April 6, 2016), http://wayback.archive-it.org/8521/20180926001826/https://blogs.fda.gov/fdavoice/index.php/2016/04/developing-a-consensus-voice-the-combination-products-policy-council/

- Modernize the intercenter consultation process and related aspects of combination product and cross-labeled product review
- Promote development of innovative, safe, and effective combination products and cross-labeled products
- Promote alignment in addressing challenging medical product classification issues

Importantly, however, FDA has clarified that the Council will not meet directly with sponsors.

The Council is chaired by the Deputy Commissioner for Medical Products and Tobacco and includes representation from CBER, CDER, CDRH, OCP, and the Office of Special Medical Programs. Further detail about the Council's responsibilities are included in its charter.[17]

In January 2017, FDA established a docket to solicit stakeholder feedback on combination product policy issues that require Agency clarification. In the Federal Register Notice soliciting such feedback, FDA noted that it envisioned that combination product policy topics considered by the Council would generally meet one of the following criteria:

- "A novel combination product policy issue requiring senior management input;
- An identical issue on which FDA seems to have taken inconsistent combination product policy positions;
- An existing combination product policy position that should be reconsidered in light of scientific or regulatory advances; or
- A combination product policy that may be triggered by a specific combination product, but that will be applicable to other combination products."[18]

Improved Intercenter Consult Request ("ICCR") Process Pilot

Timely and consistent intercenter consults are critically important during the combination product review process, as they allow collaboration and the exchange of expertise between the lead review Center and the other Centers. In August 2016, FDA began piloting an improved ICCR process, focused on four key areas:

1. Establishing timelines, specific to Center and submission type, for identifying products as combination products and issuing and completing consults needed to support the review
2. Developing a tiered consult approach that streamlines interactions across Centers and identifies a clear process for identifying the right experts for a consult
3. Defining clear roles and responsibilities for the Lead Center, the Consulted Center(s), OCP, and the Combination Product Council for review of a combination product submission

[17] FDA Combination Products Council Charter (last updated April 13, 2017), https://www.fda.gov/downloads/AboutFDA/ReportsManualsForms/StaffManualGuides/UCM528113.pdf

[18] 82 Fed. Reg. 4349, 4350 (Jan. 13, 2017)

4. Creating a standard, semiautomated, user-friendly ICCR form that is managed electronically to ensure (a) users always have the most updated version and (b) all forms, and thus all intercenter combination product consults are tracked through a single system.[19] FDA also noted its plan to collect data during implementation of the pilot to help refine the process.

In June 2018, FDA released a Staff Manual Guide ("SMG") describing the procedures for when and how to request, receive, process, and track the progress of ICCRs between the Centers.[20] The process detailed in that SMG was developed based on the results of the ICCR process pilot launched in 2016.

Pre-RFD Process

As mentioned above, combination products sponsors can submit a RFD to obtain a binding FDA determination regarding product classification and/or which Center will have primary jurisdiction for the premarket review of their products. In August 2016, FDA described a new pathway for sponsors to get an "informal" designation for their products – the Pre-RFD process (we note that the substance of this process was not technically "new," as many sponsors had already been reaching out to OCP for this type of informal feedback for quite some time).[21]

2.3 Impact of the Cures Act on Combination Product Regulation

Signed into law on December 13, 2016, an overarching aim of the Cures Act was to foster innovation and accelerate the timeline for new medical products to reach patients in need. Significantly, Section 3038 of the Cures Act amended certain portions of the FDCA related to the regulation of combination products.[22] Provided below is a summary of some of the key changes:

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[19] FDA, Piloting an Improved Intercenter Consult Process, FDA Voice (Aug. 1, 2016), http://wayback.archive-it.org/8521/20180926000213/https://blogs.fda.gov/fdavoice/index.php/2016/08/piloting-an-improved-intercenter-consult-process/

[20] FDA, FDA SMG 4101, Combination Products Inter-Center Consult Request Process (eff. June 11, 2018), https://www.fda.gov/downloads/AboutFDA/ReportsManualsForms/StaffManualGuides/UCM283569.pdf

[21] FDA, Making Continuous Improvements in the Combination Products Program: The Pre-RFD Process, FDA Voice (Aug. 11, 2016), http://wayback.archive-it.org/8521/20180926000006/https://blogs.fda.gov/fdavoice/index.php/2016/08/making-continuous-improvements-in-the-combination-products-program-the-pre-rfd-process/

[22] 21st Century Cures Act, Pub. L. No. 114-255, § 3038 (2016) (codified at 21 U.S.C. § 353(g))

- *Impact of Chemical Action on the PMOA Determination*. Section 3038 prohibits FDA from determining that the PMOA of a combination product is that of a drug or biologic "solely because the combination product has any chemical action within or on the human body."[23] This is an improvement from an industry perspective as it permits greater Agency flexibility with regard to the PMOA determination, leaving room for sponsors to convince FDA that the presence of chemical action does not necessarily mean that CDER or CBER are the most appropriate centers to review a combination product. Further, this provision seemed to be in direct response to the Prevor decision[24] and a perception that the Agency was defaulting to designation of products for CDER review when a better case could be made for CDRH review.
- *Sponsor/Agency Collaboration*. Section 3038 allows a combination product sponsor who disagrees with FDA's lead center assignment to ask FDA for, and requires FDA to provide, a "substantive rationale" for the assignment and any underlying evidence upon which it relied.[25] It also provides additional opportunities for collaboration between sponsors and FDA for discussion regarding combination product review issues, such as premarket approval or clearance requirements, and requirements related to postmarket modification or good manufacturing practices that would apply to the combination product at issue.[26] FDA is required to issue final guidance no later than December 13, 2020 that describes in more detail the process for pre-submission interactions between combination product sponsors and FDA, including best practices for pre-submission interactions, and the information that needs to be included in a combination product pre-submission meeting request.
- *Number of Applications*. Section 3038 provides that sponsors are not prohibited from submitting separate applications for the constituent parts of a combination product (e.g., an NDA and a 510(k) application for a drug-device combination product) "unless [FDA] determines that a single application is necessary."[27] Similarly, Section 3038 further states that FDA "shall conduct the premarket review of any combination product under a single application, whenever appropriate."[28] While these provisions seem to offer sponsors some degree of flexibility with respect to their submission strategy, the discretion remains with FDA to determine whether a single application is necessary/appropriate, so the ultimate impact is likely to be limited.
- *Reliance on Prior Findings of Safety and Effectiveness/Substantial Equivalence*. The Cures Act also helps alleviate the burden for the review of combination products with approved constituent parts as it expressly allows sponsors to rely

[23] 21 U.S.C. § 353(g)(1)(E)

[24] Prevor v. FDA, 67 F.Supp.3d 125 (2014)

[25] 21 U.S.C. § 353(g)(1)(F)

[26] 21 U.S.C. § 353(g)(2)

[27] 21 U.S.C. § 353(g)(6)

[28] 21 U.S.C. § 353(g)(1)(B)

upon prior findings of safety and effectiveness or substantial equivalence for the approved constituent part. Specifically, sponsors of such combination products would only need to provide to FDA the data the Agency determines is necessary for clearance or approval, including any incremental risks and benefits posed by the combination product, taking into account the Agency's prior findings regarding the approved constituent part.[29] This is a positive change for industry, especially to the extent a sponsor's combination product includes a constituent part manufactured by another sponsor.

2.4 Changing Regulatory Landscape for Combination Products Post-Cures

In the wake of the Cures Act, the regulatory landscape for combination products has continued to evolve, with FDA (i) making a series of combination product-related commitments as part of its sixth reauthorization of the Prescription Drug User Fee Act ("PDUFA VI") and (ii) releasing a number of combination product-focused guidance documents.

PDUFA VI Commitments

Under PDUFA, FDA is authorized to collect fees from certain drug and biologic product sponsors to help fund the drug approval process and keep it running as expeditiously as possible. The law must be reauthorized every 5 years. In August 2017, PDUFA was reauthorized through September 2022 as part of the enactment of the Food and Drug Administration Reauthorization Act ("FDARA").

FDARA incorporates by reference PDUFA VI reauthorization performance goals and procedures for fiscal years 2018–2022[30] (commonly referred to as the "PDUFA VI goals letter"). The PDUFA VI goals letter is a product of discussions between the Agency, industry, and other public stakeholders. Included in the section of the letter related to "Enhancing Regulatory Science and Expediting Drug Development" are the following drug-device and biologic-device combination product-related commitments:

- FDA to develop staff capability and capacity to more efficiently review combination product submissions
- FDA to streamline the intercenter consult review process
- FDA to develop processes and procedures for conducting review of combination products, specifically, human factors assessment, quality assessment of combination products, and patient-oriented labeling

[29] 21 U.S.C. § 353(g)(3)

[30] FDA, PDUFA VI Commitment Letter. https://www.fda.gov/downloads/ForIndustry/UserFees/PrescriptionDrugUserFee/UCM511438.pdf

- FDA to publish and maintain key points of contact for combination product review
- FDA to establish submission procedures for human factors protocols and performance goal timelines for review of the protocols
- FDA to provide staff training related to development, review, and approval of combination products
- FDA to contract with a third party to assess current practices for combination product review
- FDA to publish draft guidance relating to bridging studies and patient-oriented labeling

These commitments are encouraging and indicate that the Agency agrees with industry that more streamlined oversight of combination products should continue to be a regulatory priority.

Post-Cures Combination Product Guidance

Along with FDA's PDUFA commitments, the Agency has issued several guidance documents related to combination products following the December 2016 enactment of the Cures Act. While there are certainly some places within these documents where FDA may not have given industry all of the regulatory clarity it had hoped for, overall, industry has appreciated FDA's efforts to explain its current thinking and add transparency to its processes. Provided below is a listing of some of the most significant combination product-related guidance documents and other publications issued by FDA between early 2017 and early 2019 (some other documents that fit into this category have already been described above, e.g., the final cGMP guidance and the draft Premarket Pathways guidance):

- December 2016 final rule setting forth postmarketing safety requirements for combination products. The purpose of the final rule was to clarify and ensure consistent postmarketing safety reporting requirements while avoiding duplicative reporting. In addition, draft guidance was issued by FDA in March 2018 addressing how sponsors could achieve compliance with the final rule.[31] Importantly, the draft guidance pushed back the compliance deadline with respect to the final rule. A more in-depth description of the final rule is included later in this chapter.
- September 2017 final guidance addressing cases where a combination product may be classified as either a drug or a device.[32]

[31] FDA, Postmarketing Safety Reporting for Combination Products: Draft Guidance for Industry and FDA Staff (2018), https://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM601454.pdf; *see also* FDA, Compliance Policy for Combination Product Postmarketing Safety Reporting (2018), https://www.fda.gov/RegulatoryInformation/Guidances/ucm601456.htm.

[32] FDA, Classification of Products as Drugs and Devices & Additional Product Classification Issues: Guidance for Industry and FDA Staff (2017), https://www.fda.gov/downloads/

- February 2018 final guidance on the Pre-RFD process, which covers the information that should be included in a Pre-RFD and expected review time frames.[33] While sharing some similarities with the RFD process, the less formal Pre-RFD process allows for greater interaction and submission of materials beyond the 15-page limit (inclusive of the request and all attachments) that RFD submissions must adhere to, which is especially valuable if the sponsor is relying on unpublished study reports. The Pre-RFD process may be preferable for sponsors who wish to engage with FDA in a more interactive way and may be particularly useful for sponsors who have products in early stages of development or who are trying to decide whether to develop a specific product. It should be noted that a Pre-RFD does not lead to final Agency action, meaning that FDA is technically not bound by a Pre-RFD decision, and a sponsor could not challenge the decision (as it could with an RFD). However, all the same individuals in OCP and Centers who would be involved in an RFD would also be involved in a Pre-RFD review, and FDA recognizes that the success of the program comes from ability of sponsors to rely on Pre-RFD findings, so as a practical matter, it can be expected that FDA would abide by its Pre-RFD decisions.
- March 2018 FDA Staff Manual Guide Section 4103 describing the process to ensure subject matter experts and policy staff from the different centers are engaged in a timely manner to help develop and clear regulations and guidance documents concerning combination products.[34]
- June 2018 Federal Register Notice explaining FDA's proposal regarding satisfying 21 CFR § 4.4 through "alternative or streamlined mechanisms" and listing types of combination products and manufacturing processes for which "good manufacturing processes" may be adopted that vary from the requirements set forth in § 4.4.[35]

3 Increasing Technical and Submission Expectations for Combination Products

Along with the introduction of rules and guidance relating to how combination products would be regulated as discussed in the previous section, the expectations for the design and development, submission documentation, and routine control of drug delivery system combination products have been expanding.

RegulatoryInformation/Guidances/UCM258957.pdf

[33] FDA, How to Prepare a Pre-Request for Designation (Pre-RFD): Guidance for Industry (2018), https://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM534898.pdf

[34] FDA, Staff Manual Guide 4103, Expectations and Procedures for Engagement Among Medical Product Centers and Office of Combination Products on Regulations and Guidance Pertaining to Combination Products (2018), https://www.fda.gov/downloads/AboutFDA/ReportsManualsForms/StaffManualGuides/UCM602810.pdf

[35] 83 Fed. Reg. 27609 (June 13, 2018)

Table 1 Historical approach to review of injection and inhalation combination products

Injection	Inhalation
• BLA/NDA reviews primarily focused on the drug product • Injectors viewed as "functional secondary packaging" • Primary container (e.g., syringe) treated only as a container closure system: compendial requirements sufficient • Device suitability established through conformance to standards	• BLA/NDA reviews primarily focused on the drug product • Chemistry reviewers assessing device materials, with a significant focus on drug and air contacting materials • Extractables and leachables key to demonstrate safety and control • Device review includes biocompatibility consult

3.1 Expanding Combination Product Industry

Over the past two decades, there has been a significant increase in the number and types of drug delivery combination products developed. For example, early and simple injectable combination products such as prefilled syringes paved the way for pen injectors, autoinjectors, and on-body delivery systems. The sophistication of other combinations, such as transdermal patches and inhalers also continued to grow.

Drivers for this increase include delivery of more complex molecules, e.g., monoclonal antibodies, and shifting the point of care from the healthcare setting to the home, with patient performing self-injections. The market is expected to continue to grow, for example, "the prefilled syringes market for subcutaneous administration of biologics to increase from about 1.6 billion annual units in 2014 to 3.6 billion units by 2025."[36]

Regulatory expectations for combination products in the early years were unclear, with both the FDA and industry "learning" together. The historical approach to injection and inhalation combination products is presented in Table 1.

3.2 Combination Product cGMPs

The finalization of 21 CFR Part 4[37] "Current Good Manufacturing Practice Requirements for Combination Products" in January 2013[38] was a key event that set off a cascade of increasing expectations for combination products. The rule was

[36] *Subcutaneous Biologics: Products, Technologies and Delivery Systems*, Roots Analysis Limited, 2015

[37] As the initial publication of 21 CFR 4 only included Subpart A—*Current Good Manufacturing Practice Requirements for Combination Products*, with Subpart B marked as [Reserved], it became common for FDA and industry to refer to Subpart A as Part 4. Subpart B (§4.100-4.105), *Postmarketing Safety Reporting for Combination Products* was finalized in December 2016, is commonly referred to as PMSR. Therefore, references in this chapter to 21 CFR 4 or to Part 4 without the subpart designation refer to 21 CFR 4(A), §4.1-4.1, inclusive.

[38] Notice of Publication of 21 CFR Part 4, Current Good Manufacturing Practice Requirements for Combination Products [Final Rule]. 78 FR 4307. 22 January 2013

Table 2 21 CFR 4 streamlining approach

Drug GMP-based		Device QSR-based	
820.20	Management responsibility	**211.84**	Testing and approval or rejection of component drug product containers, and closures
820.30	Design controls	**211.103**	Calculation of yield
820.50	Purchasing controls	**211.132**	Tamper-evident packaging requirements for the OTC human drug products
820.100	CAPA	**211.137**	Expiration dating
820.170	Installation	**211.165**	Testing and release for distribution
820.200	Servicing	**211.166**	Stability testing
		211.167	Special testing requirements
		211.170	Reserve samples

published with an effective date of July 22, 2013. The concepts contained in the rule were initially proposed in a draft guidance in October 2004,[39] whereby a manufacturer would designate the base GMP system and then add on those additional processes necessary to demonstrate compliance with those chapters which would be applicable to the combination. Part 4 provides that manufacture of single-entity or co-packaged combination products shall comply with all applicable cGMP requirements for the constituents contained within the combination product or adopt a streamlined approach. The streamlined approach requires the manufacturer to select the base quality system (e.g., drug GMP, device QSR) and then show compliance with additional specified provisions, as described in Table 2.

Compliance with the cGMP requirements includes both establishing/enhancing the quality system infrastructure at the company and application of the requirements to the device constituents of combination products and to the combination products, commensurate with the scope of responsibilities of the manufacturer. Accordingly, a facility that manufactures only one type of constituent part and not combination products only needs to comply with cGMP requirements for the constituent part.

As is common with combination products, this seemingly straightforward and logical GMP rule resulted in extensive dialogue with the FDA, as manufacturers were implementing the regulation within their quality systems. Manufacturers posed specific questions about how best to implement the rule to the Office of Combination Products in public forums as well as through direct dialogue with FDA.

Although drug and biologic GMP and device QSR chapters covered many of the same topics, the manner in which the regulations are drafted differs, which added to the complexity of implementing Part 4. Drug and biologic GMP regulations focus on requirements for the product, whereas the device QSR focuses on the responsible party. As an example, refer to Table 3 for a comparison of a few of the chapters not specified in the streamlining approach, as these topics were deemed "comparable"

[39] 69 FR 59239

Table 3 Drug/biologic cGMP and device QSR drafting comparison

Drug/biologic cGMP	Device QSR
211.130. Packaging and labeling operations There shall be written procedures designed to assure that correct labels, labeling, and packaging materials are used *for drug products*; such written procedures shall be followed...	**820.120. Device labeling** Each *manufacturer* shall establish and maintain procedures to control labeling activities...
211.100. Written procedures There shall be written procedures for production and process control designed to *assure that the drug products* have the identity, strength, quality, and purity they purport or are represented to possess. Such procedures shall include all requirements in this subpart	**820.170. Production and process control** Each *manufacturer* shall develop, conduct, control, and monitor production processes to ensure that a device conforms to its specifications

and "adequately addressed" by the base GMP system.[40] Emphasis has been added to highlight the differences in the frame of reference for the regulations.

Part 4 is written from the same frame of reference as the device QSR, focusing on requirements of the responsible party. Companies adopting a streamlined approach to Part 4 need to merge the expectations for the two quality systems, taking into account how to interpret the responsibilities and obligations, considering the different frame of reference in the drafting of the GMP requirements. This difference in drafting approach increased the areas of confusion with regard to obligations for components used in combination products, as further described in Section "Purchasing Controls for Combination Products".

When the final rule for Part 4 was published, the FDA responses to the comments on the proposed rule contained some key elements which had a significant impact on many combination product manufacturers. A few of the most significant include:

- *No new requirements*: The Preamble included clarification that the rule did not create any new obligations and was therefore applicable to all combination products, as explained in response to Comment 4: "This rule does not create new cGMP requirements, but rather attempts to clarify how to apply them to combination products. Compliance with all applicable cGMP requirements is required for all products and appropriate to ensure consistent manufacture of products that meet the safety and effectiveness and quality standards that form the basis for product marketing authorization, regardless of when a product was first marketed or approved."[41]
- *Prefilled syringe is a combination product:* There had been a long-running informal dispute among FDA and some members of industry who asserted that the syringe of a prefilled syringe is a container closure and not a device constituent part (thinking that traces its origins back to times before implementation of modern design controls). FDA took the opportunity in the Preamble to explicitly state

[40] See response to Comment 21, 78 FR 4307 at 4316.

[41] 78 FR 4307 at 4310

that a prefilled syringe is a combination product, as explained in response to Comment 8: "A syringe is a device used to deliver another medical product (e.g., a drug) (see, e.g., 21 CFR 880.5860). Accordingly, a prefilled syringe is a combination product and subject to this rule."[42] The FDA elaborated further in response to Comment 15: "for example, if a facility is manufacturing a finished combination product, a prefilled syringe for instance, from device components and drug components, that facility is subject to both the QS regulation and drug cGMPs."[43]

The impact of these two premises on combination product manufacturers was dramatically underscored by the first combination products Warning Letter,[44] issued in January 2014 relating to an inspection that occurred the month before 21 CFR 4 became effective. The warning letter cited design control (21 CFR 820.30) and purchasing control (21 CFR 820.50) deficiencies relating to a prefilled syringe and manual needle guard combination product, a lyophilized vial and diluent with vial adapter combination product, and a syringe with autoinjector combination product.

FDA issued the voluminous final cGMP companion guidance[45] for 21 CFR 4 in January 2017.[46] A few of the key aspects contained within the final guidance for combination product cGMPs include:

- Clarifications regarding terminology/applicability – manufacturer, component vs. constituent part, drug container vs. delivery devices, convenience kits
- Emphasized that design controls requirements relates to the combined use of the constituent parts, and not just the device portions of the combination product
- Expectations for coordination of cGMP compliance across facilities
- Outlined expectations for manufacturers responsible for only one type of constituent part, whereby the manufacturer needs to only comply with cGMP requirements for the constituent part, but is expected that the quality system should take into account considerations for the combination product as a whole
- Robust sections on how to implement streamlining approaches, including useful insights for both drug cGMP and device QSR-based systems
- Extensive scenarios provide guidance on how to comply for co-packaged and single-entity products

Additionally, AAMI published TIR 48:2015, *Quality Management System (QMS) Recommendations on the Application of the U.S. FDA's cGMP Final Rule on Combination Products*,[47] which includes considerations when adopting a stream-

[42] 78 FR 4307 at 4311

[43] 78 FR 4307 at 4314

[44] Warning letter issued to Amgen, Inc. (WL # 11-14) dated January 27, 2014. https://www.fda.gov/ICECI/EnforcementActions/WarningLetters/ucm385288.htm

[45] 82 FR 3336

[46] See Footnote 7

[47] Quality Management System (QMS) Recommendations on the Application of the U.S. FDA's cGMP Final Rule on Combination Products (AAMI TIR 48:2015). Association for the Advancement of Medical Instrumentation. 14 August 2015

lined approach along with detailed tables to guide the transition from QSR or drug GMP to a streamlined combination product quality system. The TIR also contains recommendations regarding application of design control and risk management to combination products, as well as several informative figures related to the interpretation of the Part 4 rule and the development process.

In June 2018, FDA issued a list of alternative or streamlined mechanisms for complying with the current good manufacturing practice (cGMP) requirements for combination products[48] to comply with its statutory mandate from the Cures legislation.

Key Terminology Clarifications

As manufacturers implement Part 4 into their quality systems and develop their products in accordance with Part 4, the following key terminology are important to understand.

Manufacture the "definition of the term 'manufacture' in part 4 is intended to include all of the activities considered within the scope of manufacturing for drugs, devices, biological products, and HCT/Ps."[49] A comparison of the definitions across combination products, drugs, devices, biological products, and HCT/Ps is provided in Table 4.

Constituent part and components the responsibilities in Part 4 are applicable to constituent parts and combination product manufacturers. Therefore, classification of the entity that each manufacturer is responsible for within a given program is important to appropriately define the scope of Part 4 obligations for that manufacturer. A constituent part is defined as a drug, device, or biological product that is part of a combination product[50] and a device constituent part incorporated into a combination product would be "suitable for use or capable of functioning, whether or not it is packaged, labeled, or sterilized,"[51] Related to single-entity injectors, often the device constituent is not formed until the combination product is assembled. In contrast, components[52] include sub-assemblies that are further processed to make a finished device and therefore component manufacturers are not subject to Part 4. Purchasing controls are the mechanism by which the quality of the components are assured under the QSR. See Section "Purchasing Controls for Combination Products" for additional information.

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[48] 83 FR 27609

[49] See footnote 45

[50] 21 CFR 4.2

[51] 21 CFR 820.3(l)

[52] Device Components means any raw material, substance, piece, part, software, firmware, labeling, or assembly which is intended to be included as part of the finished, packaged, and labeled device, 21 CFR 820.3 (c).

Table 4 Comparison of definitions for manufacture and manufacturer

Term	Product type	Definition
Manufacture	Combination product	Designing, fabricating, assembling, filling, processing, testing, labeling, packaging, repackaging, holding, and storage [21 CFR 4.2]
	Drug	Manufacture, processing, packing, or holding, packaging and labeling operations, testing, and quality control [21 CFR 210.3(b)(12)]
	Biologic	All steps in propagation or manufacture and preparation of products, filling, testing, labeling, packaging, and storage [21 CFR 600.3(u)]
	HCT/P	Any or all steps in the recovery, processing, storage, labeling, packaging, or distribution of any human cell or tissue, and the screening or testing of the cell or tissue donor [21 CFR 1271.3(e)]
Manufacturer	Device	Any person who designs, manufactures, fabricates, assembles, or processes a finished device, contract sterilization, installation, relabeling, remanufacturing, repacking, or specification development, and initial distributors of foreign entities performing these functions [21 CFR 820.3(o)]
	Drug	Anyone who is engaged in manufacturing, preparing, propagating, compounding, processing, packaging, repackaging, or labeling of a prescription drug [21 CFR 205.3(d)
	Biologic	Any legal person or entity engaged in the manufacture of a product subject to license under the act: "Manufacturer" also includes any legal person or entity who is an applicant for a license where the applicant assumes responsibility for compliance with the applicable product and establishment standards [21 CFR 600.3(t)]

Design Controls for Combination Product

The application of design controls for combination products has been a significant focus for industry in the years following the publication of Part 4. The interfaces between the drug product development and device design controls as applied to combination products involves new ways of working for manufacturers. The learning curve for combination product manufacturers included bridging the language gap between drug product and device vocabulary. The same term has different meanings in the two systems. For example, the term "validation" refers to validating test methods or manufacturing processes as part of drug product development, but it is also used for validating a device design typically through simulated or clinical use as part of device design validation.

Design controls provide for a standardized, systematic, prospective, iterative model for device design and development to ensure that the device is safe and effective. Design controls need to be in place for both the device constituent parts and the combination product. At a high level, when compared to the approach for

development and qualification of a container closure system, the application of design controls is not that different. Many of the same assessments are performed, and much of the same data is used to satisfy container/closure requirements as are used to satisfy design control considerations.

One area of confusion relating to applying design controls to combination products is when a manufacturer combines previously developed components and/or constituents into a combination product system. An example of this is a manufacturer including a vial adapter and dosing syringe in the packaging with the vial of drug product. For these type of products, there is no expectation for the combination product manufacturer to retrospectively design those purchased components or constituents; however, the manufacturer should "understand the constituent part's existing design specifications thoroughly in order to perform design controls properly for its use in the combination product."[53] These expectations are aligned with the expectations for suitability for dosing devices used to deliver a medicinal product.[54,55] While the technical expectations did not change significantly, the manner in which a company were to document conformance with these expectations did have an impact on the industry. Although the regulation and guidance documents for Part 4 indicated that there were no new requirements, there were changes in the development processes that manufacturers needed to follow, as the assessment of suitability/performance needed to be completed as part of the design control process for the combination product.

Remediation for Products Not Developed Under Design Controls

In the years following publication of the Part 4 final rule, industry conferences with combination products content included sessions on remediation strategies to develop procedures and close any gaps which may exist for combination products not developed under design controls. The common message from FDA at industry conferences was that manufacturers should document the DHF gaps through use of their CAPA process in their quality system, and appropriately remediate.[56,57] Manufacturers

[53] See footnote 7, page 25

[54] ICH M4Q(R1), The Common Technical Document for the Registration of Pharmaceuticals for Human Use: Quality, Sept. 2002, https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/CTD/M4_R1_Quality/M4Q__R1_.pdf

[55] FDA, Container Closure Systems for Packaging Human Drugs and Biologics, Chemistry, Manufacturing and Controls Documentation, May 1999, https://www.fda.gov/downloads/drugs/guidances/ucm070551.pdf

[56] Burns, M. *FDA Updates: Addressing Combination Product Challenges*, presented at 2016 PDA/FDA Joint Regulatory Conference, Sept. 13, 2016

[57] Tejero, M.I *Compliance with Applicable Medical Device Regulations of Combination Products: CDRH Expectations*, presented at CASSS CMC Strategy Forum: Combination Products for Biopharmaceuticals: Emerging Trends in Development, GMPs and Regulatory Expectations,

were also encouraged to reach out to the FDA with any questions regarding bringing their DHF into compliance. As presented by Lead Consumer Safety Officer, Isabel Tejero at the 2015 CASSS CMC Strategy Forum, steps to bring an existing design project into compliance with 21 CFR 820.30 include:

- Use the CAPA system to evaluate the non-compliance, find the cause, and address it (assess both operational and systematic functions).
- Evaluate other design projects subject to design controls for compliance.
- Retrospectively create the DHF as complete as possible, using risk to determine the timing, extent, and rigor of the activities needed.
- Identify and execute design control activities necessary to fulfill the gaps that were not able to be closed with existing data, as well as to mitigate any additional identified risks
- Document activities.[58]

Industry representative also shared their experiences remediating design history files at conferences,[59] and this shared learning supported the collective knowledge of the industry with regard to bringing their documentation into compliance. A retrospective design history file typically involves a compilation of historical documentation, with summary documents and matrices linking the historical documentation together for the purpose of demonstrating compliance. These design history files also contain risk analyses and provide a baseline for future changes of the combination product. The application of a risk-based approach allowed the manufacturers to focus on the possible harms for the patients and end users and the knowledge of performance of the product from the current market experience to determine the appropriate extent of design control documentation that would be needed, as well as considerations related to whether there are likely future changes to the device to provide manufacturers with a means to prioritize the order of design history files to remediate.

FDA provided clarity regarding their expectations in the cGMP companion guidance, whereby manufacturers were encouraged to review their premarket submissions, the product risk profile, and postmarket experience for the combination product to inform decisions on whether additional testing and documentation is required.[60]

January 26, 2015

[58] See footnote 57.

[59] For example, David Anderson, *Preparing Design History Files for Legacy Combination Product*, presented at 2016 PDA/FDA Joint Regulatory Conference, Sept. 13, 2016. Also, see Sandra Boyd, *The Journey to 21 CFR Part 4 Compliance*, presented at 2017 Xavier Health Combination Products Summit, September 2017.

[60] See footnote 46, Section IV.A.2.

Purchasing Controls for Combination Products

The purchasing controls[61] section of the QSR provides that "Each manufacturer shall establish and maintain procedures to ensure that all purchased or otherwise received product and services conform to specified requirements." Purchasing controls are of vital importance for combination products, as the majority of combination products involve partnership with external parties. Outsourcing design or utilizing an existing platform changes the "design" process, whereby the activities do not start with rough drawings and concepts, but instead start with selection of components and device constituents to be incorporated into the combination product. Purchasing controls assure rigor during the technology evaluation and selection and are integral for managing changes throughout the product life cycle.

Although device component manufacturers are not subject to device QSR or Part 4 (see Sect. 3.2), the purchasing control requirements for device constituent and combination product manufacturers provide the mechanism whereby GMP requirements are applied to control the purchased device components, as the definition for "product"[62] includes device components.

3.3 Impact of Technical Guidance on Combination Product Regulatory Expectations

Several key guidance documents have shaped the technical expectations for combination product development and submission requirements. As FDA and industry gained more experience with the different technology types, the extent and scope of the expectations included in guidance has expanded. Congruent with the expansion in the number of combination products, and influenced by the increasing complexity of the delivery systems and the molecules being administered, as well as the shift of the use environment to the home and the transition of the user from healthcare professionals to the patients performing self-injections, FDA began requesting more evidence from sponsors. A summary of some of these increased expectations is presented in Table 5, along with perceived influencing elements that were associated with the additional expectations.

The following sections provide specifics of the expectations as they relate to the different combination product types and key technical areas.

[61] 21 CFR 820.50

[62] 21 CFR 820.3(r). *Product* means components, manufacturing materials, in- process devices, finished devices, and returned devices

Table 5 Summary of key influencers leading to increased FDA expectations

Influencers	Increased expectations
Injection of more complex molecules (e.g., monoclonal antibodies)	Potential for impact to drug product quality due to shear stress and combination product assembly/manufacturing Potential for impact to aggregation and particulates due to interactions with container materials (e.g., silicone, tungsten)
Use of higher concentration formulations with increased viscosity	User interaction forces (e.g., syringe glide forces) Storage-related impacts to performance (e.g., injection time changes)
Increasing use of self-administration in patient populations that hadn't traditionally performed injections	Combination Product human factors (e.g., CDER DMEPA adding staff capability and expectations for human factors validation to be complete prior to use in pivotal clinical trial) Expectation that the to-be-marketed presentation be used in pivotal clinical trial, or supported by appropriate bridging

Container Closure Systems

The CDER/CBER container closure guidance[63] was finalized in May 1999 and provided manufacturers with the expectations for CMC (chemistry, manufacturing, and controls) documentation related to the container closure system (CCS) for both clinical trial and marketing applications. While this guidance does not mention the term "combination product," it does contain a strong framework for the key categories of expectations based on suitability for the intended use. Many of the suitability expectations outlined in this guidance are relevant to drug delivery systems and can be considered as design inputs for the combination product:

- *Protection* – the CCS should adequately protect the dosage form from factors that can cause degradation in the quality of the drug product contained within the CCS over the shelf and in-use life for the combination product.
  - *For example, protection from moisture, light, and microbial contamination*
- *Compatibility* – the CCS should not interact sufficiently with the drug product to cause unacceptable changes in the quality of either the dosage form or the component.
  - *For example, product and container/label interaction (sorption, leaching, extracting)*
- *Safety* – the CCS should be composed of materials that are considered safe for use with the dosage form and the route of administration.
  - *For example, safety and biocompatibility of materials*

[63] See footnote 55.

- *Performance* – where the CCS has a performance feature in addition to containing the product, the assembled container closure system should be shown to function properly. Container closure system functionality and/or drug delivery performance ensure that the system operates as designed. Tests and acceptance criteria regarding dosage form delivery and container closure system functionality should be appropriate to the particular dosage form, route of administration, and design features, including through the shelf life and in-use life of the combination product.
  - *For example, demonstration of reproducible and accurate dose delivery*

These suitability expectations are aligned with the submission content expectations for the pharmaceutical development section on container closure systems, 3.2.P.2.4, as outlined in ICH M4Q(R1).[64]

Inhalation Products

FDA issued the initial draft of the Metered Dose Inhaler (MDI) and Dry Powder Inhaler (DPI) guidance in November 1998[65] and the Nasal Spray and Inhalation Solution, Suspension, and Spray Drug Products guidance in July 2002.[66] These guidances included detailed sections outlining the quality expectations for the combination product. Examples of the expectations contained in these early guidances include:

- Aerosol characteristics, such as spray pattern, plume geometry, and particle size distribution
- Device characteristics, such as flow resistance, delivery reproducibility
- Material composition, extractables and leachables
- Characterization studies to assess the impacts performance of the product under use scenarios, such as cleaning, shaking, dosing orientation, and robustness of the device

In April 2018, FDA issued a second draft of the MDI/DPI guidance,[67] which updated the expectations for these combination products, to reflect the current standards and requirements, to enhance understanding of appropriate development approaches aligned with the quality by design (QbD) paradigm. This update also included a detailed section on combination products, including clarifying that

[64] See footnote 54.

[65] 63 FR 223

[66] FDA, Nasal Spray and Inhalation Solution, Suspension, and Spray Drug Products — Chemistry, Manufacturing, and Controls Documentation, July 2002, https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070575.pdf

[67] FDA, Metered Dose Inhaler (MDI) and Dry Powder Inhaler (DPI) Products - Quality Considerations Guidance for Industry, draft – revision 1, April 2018, https://www.fda.gov/downloads/Drugs/.../Guidances/ucm070573.pdf

design controls apply to these products. The draft guidance provides commentary to assist pharmaceutical manufacturers familiar with the QbD approach[68] leverage those practices to satisfy the Part 4 obligations. In particular, the draft guidance provides following the FDA perspectives:

- Quality Target Product Profile (QTPP) is similar to design inputs.
- Pharmaceutical studies conducted to verify that the drug product critical quality attributes (CQA) are met may also address design verification and design validation.

The industry response[69] to this analysis of QbD and design controls included concerns over the discussion regarding the drug and device concepts and how they relate to each other and that these concepts should be addressed in general combination product guidance, in lieu of a specific MDI/DPI guidance. These CPC comments recommended that the "document should be more careful in applying these terms (like CQAs) for device constituent parts where these may be considered 'essential design outputs' but the process for identifying and controlling these attributes will likely be handled differently than for drug attributes. Optimally, this terminology alignment would be consistent across various types of delivery devices (not just MDI and DPI products), and given it does not exist in other guidances issued to date, it would be better placed in a more general guidance that applies more broadly to drug/device combination products rather than this specific MDI and DPI products."[70]

Injection Products

During 2013, FDA issued a draft technical guidance for glass syringes that deliver drug and biological products[71] and also finalized the technical considerations guidance for pen, jet, and related injectors intended for use with drugs and biological products.[72] Both of these guidance documents included expectations for content to be included in premarket submissions and outlined functional and performance testing expectations. While the guidance documents contain valuable informative content, several of the expectations in these guidances caused challenges for industry:

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[68] See ICH ICH Q8(R2), Pharmaceutical Development, www.ich.org.

[69] Comments to FDA Docket FDA-2018-D-1098, http://combinationproducts.com/wp-content/uploads/2018/06/CPC-MDI-DPI-Draft-Guidance-Comments-18June2018.pdf

[70] See footnote 69 at page 2.

[71] FDA, Glass Syringes for Delivering Drug and Biological Products: Technical Information to Supplement International Organization for Standardization (ISO) Standard 11040-4, Draft Guidance, April 2013, https://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM346181.pdf

[72] FDA, Technical Considerations for Pen, Jet, and Related Injectors Intended for Use with Drugs and Biological Products, June 2013, https://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM147095.pdf

- Recommendation to provide engineering component and assembly drawings for injectors in submissions raised questions with regard to what level of reporting would be required for changes to these drawings.
- While the scope of the syringe guidance focused on interconnectivity of devices and specifically identified needleless glass syringes prefilled with a drug or biological product, the recommendations included in this guidance were generally applied to all prefilled syringes and manufacturers learned of the broadened scope of applicability of these expectations in pre-submission meetings or during the review for their application.
- Both guidances are written to support development, verification, and validation and do not include expectations regarding control strategies for the device constituent or combination product, but manufacturers began learning about these expectations during reviews of their applications beginning in 2015. See discussion on essential performance requirements in Section "Essential Performance Requirements and Control Strategies".
- Both guidances outline submission expectations, but do not provide clarity with regard to content and location expectations. See later discussion on submission expectations in Sect. 3.4.

Through the efforts of a cross-industry and regulator working group, in 2015 the Parenteral Drug Association (PDA) issued a technical report on *Prefilled Syringe User Requirements for Biotechnology Applications*.[73] This document consolidated the best practices and learnings from across industry related to the considerations and requirements for use of the 1 mL long glass prefilled syringe for biotechnology applications, including material selection and evaluation, syringe preparation and handling, human factors, drug product compatibility with the syringe materials, and mode of delivery. Throughout this document, there are practical considerations, including:

- Assessing compatibility between the syringe materials and the drug products for potential impacts due to compounds that may be present in the syringe materials, e.g., tungsten, silicone, adhesive
- Assessing for impacts to the protein due to shock, vibration, shear or other factors that can damage the molecular structure
- Interface considerations between the components of the prefilled syringe system

Human Factors Guidance

These early combination products were developed in parallel to the evolution and increasing importance of human factors in medical device design. The 2000 CDRH human factors guidance introduced the need to consider "use error" within the risk

[73] *Technical Report No. 73: Prefilled Syringe User Requirements for Biotechnology Applications.* Parenteral Drug Association, 2015. www.pda.org/bookstore

analysis and recommended human factors techniques to reduce the risk of use errors. Combination product often involve user interaction for correct administration, therefore human factors expectations have understandably evolved for combination products. Following the issuance of the 2011 draft human factors guidance, "Applying Human Factors and Usability Engineering to Medical Devices," it became a commonplace for combination product manufacturers to receive requests to submit human factors protocols to FDA to be reviewed prior to running the summative studies. While industry appreciated the opportunity to receive the FDA input prior to running the studies, the turnaround time for the review of these protocols became a bottleneck in the development process, and this was a driver for PDUFA VI commitment relating to establishing timelines (see Section "PDUFA VI Commitments").

In early 2016, FDA finalized the CDRH human factors guidance[74] and issued the draft version of the combination products human factors guidance.[75] The CDRH guidance is intended to be the overarching guidance, with the combination products guidance outlining details relevant to combination products. Included within the combination products guidance are several process considerations, namely:

- Considerations on whether to submit combination products human factors study data
- Considerations for design changes after human factors validation
- Human factors information to submit in a combination product investigational application
- Marketing application review of human factors studies and certain labeling

Additionally, the guidance contains a detailed chapter on the relationship of human factors and the major (or pivotal) clinical studies. In this chapter, the FDA outlined that the human factors validation study should be conducted on the final finished combination product before use in the major clinical study. This expectation was a shift for combination product manufacturers, who had historically performed the human factors validation for commercial in parallel with the use in the major clinical study. The impact of this new expectation was directly observed on the development timelines needed to incorporate the final finished combination product in the major clinical study, as this new expectation resulted in at least several additional months being added to the timelines, especially when time is needed for protocol submission and review by FDA prior to study execution, as described earlier.

Reference is made to Chap. 31, for an in-depth discussion of human factors.

[74] FDA, Applying Human Factors and Usability Engineering to Medical Devices, February 2016. https://www.fda.gov/downloads/medicaldevices/.../ucm259760.pdf

[75] See footnote 12.

Essential Performance Requirements and Control Strategies

Starting in 2015, industry began to receive requests from the FDA regarding Essential Performance Requirements (EPRs), but there was no published guidance regarding the definition of EPRs, the submission content expectations, and control strategies for EPRs. For example, Biogen received a request to "evaluate in-process control tests…ensure the consistency of the assembly…in context of essential performance of the combination product" for a prefilled pen.[76]

Following these initial requests, industry sought to understand what EPRs were, how to define them, and what the associated expectations were for the EPRs. Companies looked to existing guidance and international standards to develop further understand what an EPR was. As the requests originated from the USA, the starting point was FDA guidance. The Quality System Inspection Technique (QSIT) Guide[77] and the design control guidance[78] provided some insight, but not enough clarity. The QSIT Guide explains that design outputs which are essential for the proper functioning of the device must be identified. Typically a risk analysis tool such as FTA or FMEA is used to determine essential outputs. This guide recommends that the inspector utilize these essential outputs/essential requirements as the aspects of the design project to review during the inspection. The design control guidance outlines that "the essential quality aspects and the regulatory requirements, such as safety, performance, and dependability of a product (whether hardware, software, services, or processed materials) are established during the design and development phase," but this guidance doesn't provide clarity regarding how to define these aspects. Industry also looked to IEC 60601-1,[79] which defines essential performance as performance required to avoid unacceptable risk despite the absence of, or degradation of, a function or feature. Industry's conclusion was that essential performance related to both safety and effectiveness.

Over the next few years, companies continued to receive feedback from the FDA as part of pre-submission meetings and information requests regarding the FDA expectations. It was understood that FDA had developed a set of general feedback related to combination product development expectations and manufacturing and control strategy requirements, including EPRs, in lieu of authoring guidance. This general feedback is typically provided to sponsors as part of meeting feedback, submission feedback, or information requests. The content included in this general

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[76] Roan, S. Integration of Essential Performance into Combination Product Control Strategies (Presentation), October 16, 2018, 2016 PDA Drug Delivery Combination Products Workshop, Huntington Beach, CA

[77] FDA, Guide to Inspection of Quality Systems, August 1999. https://www.fda.gov/downloads/iceci/inspections/ucm142981.pdf

[78] FDA, Design Control Guidance for Medical Device Manufacturers, March 1997. https://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm070642.pdf

[79] IEC 60601-1:2005/AMD1:2012/COR1:2014, Medical electrical equipment — Part 1: General requirements for basic safety and essential performance

feedback has evolved over time, creating a "moving target" for industry. During 2018, FDA presented on EPR requirements in a few public meetings and explained their expectations in greater detail.[80] From this combination of interactions, industry was able to conclude that:

- EPRs were defined as the subset of design requirements necessary for your device constituent to safely and effectively achieve the combination product's intended use
- FDA expected that the lot release specifications would include the EPRs
- FDA considered EPRs to be similar to drug critical quality attributes (CQA)

The evolving nature of this general feedback has led to lack of clarity for sponsors with respect to what is required for combination product development and commercial manufacturing and control strategies. The impact to manufacturers can be significant, as the control strategies are often agreed during the later stages of a marketing submission review.

Combination product manufacturers are utilizing design control practices that are highly integrated with risk management, which drive the manufacturing and controls appropriate for each product. Requests for new or different testing approaches from those proposed by the sponsor can result in delays due to the need to develop, validate, and transfer additional test methods to the manufacturing and/or testing facilities late in the process, as well as pose challenges for sponsors in defining limits for the acceptance criteria, since there may not be a large number of batches tested.

FDA has been actively interacting with industry to listen to and understand the concerns, as well as sharing the goals behind FDA focusing on EPRs. FDA has explained that goal is for applications to include sufficient information to demonstrate that the device is safe and effective and that design controls are implemented as applicable. By focusing the FDA review on the essential "subset" of requirements, the review can focus on those aspects that are most important. Further guidance is expected on EPRs and how they can be applied across the product life cycle.

Delivery System Bridging

The 2013 draft guidance for rheumatoid arthritis products[81] included a section which outlined the expectation for use of the combination product in clinical trials and approaches for bridging changes to delivery systems. Similar to the expectation

[80] Stevens, A., Technical Considerations for Design and Quality Evaluation of Drug Delivery Devices (presentation), 2018 PDA Container Closure and Packaging Integrity Conference, June 13, 2018; Dorgan, C., Regulatory Considerations for Complex Container Closure Systems (presentation), 2018 PDA Container Closure and Packaging Integrity Conference, June 13, 2018

[81] FDA, Rheumatoid Arthritis: Developing Drug Products for Treatment, draft, May 2013. https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm354468.pdf

for use of the final finished combination product in major clinical trials from the combination product human factors guidance, in the rheumatoid arthritis guidance, FDA indicated the need for "a substantially complete clinical development program to support efficacy and safety of the entire combination product. We anticipate that the to-be-marketed drug-device combination product will be used in the pivotal studies supporting the efficacy and safety of the combination product for marketing approval." Additionally, the guidance outlines expectations for ongoing evaluation of device performance incorporated into the pivotal studies for the combination product.

The guidance provided an example framework when transitioning from a prefilled syringe to an autoinjector delivery system. This framework involves the following, at a minimum:

- Human factor studies to evaluate potential use-related risks of the modified combination product.
- Pharmacokinetic bridging study that demonstrates similar delivery of the drug product to the same biospace across a range of body weights.
- Real-life patient handling experience to assess device performance.
- Depending on the extent of the proposed changes, additional clinical data may be needed to support efficacy and safety, including immunogenicity.

While authored as a guidance specific to rheumatoid arthritis products, the concepts relating to bridging have been generally applied for changes to the delivery system across all therapies. The extension of the scope of this guidance to other indications was one of the drivers for the PDUFA VI commitment relating to a guidance for bridging (see Section "PDUFA VI Commitments").

Refer to Chap. 32, for further details on this topic.

3.4 Submission Expectations Related to Part 4

Declaration of Quality System

The cGMP companion guidance clarified an expectation for combination product manufacturers to declare the Part 4 quality system approach employed for each relevant facility in their premarket submissions. This is typically added to the list of manufacturers (e.g., 3.2.P.3.1).

Structure and Content Placement Expectations

Around the same time as the publication of Part 4, manufacturers started to receive feedback from the FDA regarding preferences for placement of device constituent information in the electronic common technical dossier (eCTD). These recommendations were

provided direct to sponsors as standard "Additional Comments" as part of Type B/C meetings[82] and included:

- All device information pertaining to manufacturing or assembly of the finished combination product and documents necessary to demonstrate compliance with applicable 21 CFR part 820 regulations should be located in Section 3.2.P.3., along with a reference to FDA Guidance, *Quality System Information for Certain Premarket Application Reviews.*[83]
- Not use Module 3.2.R for device constituent information.
- Reference files under 3.2.P.7 which are not currently listed as numerical items in ICH and FDA specifications and guidance.
- In 3.2.P.7 you could include a leaf titled something similar to the following, "Table of Contents for Drug-Device Autoinjector." This leaf/document could provide reference links to the other files in module 3.2.P.7. Obtaining concurrence from the Review Division on the proposed outline is recommended.
- Although it's not required, providing a "Information to Reviewers" or "Reviewers Guide" document in Module 1.2 cover letters can be helpful to provide a high-level overview (with reference links) of the submission's content and list where the information is located in the eCTD.

The initial requests from CDRH Office of Compliance to submit documents necessary to demonstrate compliance with 21 CFR 4, and the applicable 21 CFR 820 regulations in eCTD Section 3.2.P.3 included expectations to submit quality system documents, e.g., standard operating procedures (SOPs). Industry struggled with these requests, as for a NDA or BLA, it is not common for SOPs to be included in the regulatory submission, rather these documents are reviewed during facility inspections. Most concerning to manufacturers was that the SOPs were expected to be included in 3.2.P.3, Manufacturing, which is a section that describes the manufacturing details for the product and is subject to postapproval change supplements. As quality system SOPs are periodically reviewed and updated, manufacturers were concerned with the potential life cycle management impact of including these procedures in the eCTD and whether the manufacturer would need to submit postapproval supplements if these procedures changed. Manufacturers sought to gain clarity that these procedural updates would not trigger the need for postapproval supplements and, in some cases, received feedback that changes to the procedures could require supplements.[84]

[82] For example, refer to Administrative Documents and Correspondence for BLA 125522 Initial Review, page 104. Available at: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/125522Orig1s000AdminCorres.pdf .

[83] FDA, Quality System Information for Certain Premarket Application Reviews, Feb. 2003. http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm070897.htm

[84] For example, refer to Administrative Documents and Correspondence for BLA 761029 Initial Review, page 202. Available at: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2016/761029Orig1s000AdminCorres.pdf

Defining the most appropriate locations within the eCTD to place the device and combination product content to facilitate review, linkage to other sections and life cycle maintenance was an aspect where there was variability across industry. This variability made it more challenging for FDA device reviewers to locate the content that they needed in order to complete their reviews, resulting in information requests relating to providing the location of the specific data within the submission. Included within PDA TR73[85] are recommendations on the types of content to be included in regulatory submissions, as well as a tabulated descriptions of the elements related to prefilled syringes to consider for inclusion in module 3 sections of the BLA or NDA.

In October 2015, FDA issued the initial version of the eCTD Technical Conformance Guide[86] as a supplement to the eCTD guidance,[87] which enumerated many of the same recommendations that had previously been provided to sponsors as "Additional Comments." Following feedback received from industry, FDA made a significant revision in September 2016, providing more flexibility regarding the placement of content, along with expanded expectations for an informative reviewers guide to device content to be provided as an attachment to the cover letter to facilitate the device review.

The 2018 MDI/DPI draft guidance also outlined submission content and location expectations for combination products, including expectation that quality system information be contained in 3.2.P.3. Manufacture and a detailed listing of information to be included in 3.2.P.7 to support the CCS and the device constituent part.[88] These submission expectations outlined in the draft guidance were not aligned with the expectations from Industry, as exemplified in the comments submitted by the Combination Product Coalition (CPC) on June 18, 2018.[89] The CPC comments encouraged FDA to not be overly proscriptive in the placement of the content, and instead, allow manufacturers the flexibility to include supportive information in the pharmaceutical development or regional sections.

Master Files and Referenced Submissions

With partnerships or supplier relationships, the applicant may not have access to all necessary details to support registration and may need to rely on MAF/DMFs or other NDA/BLA/PMA/510(k) submissions. It is important to coordinate messaging between the referenced document and application under review to ensure that there

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[85] See footnote 73

[86] FDA, eCTD Technical Conformance Guide, available at https://www.fda.gov/downloads/Drugs/UCM465411.pdf

[87] FDA, Providing Regulatory Submissions in Electronic Format — Certain Human Pharmaceutical Product Applications and Related Submissions Using the eCTD Specifications, available at https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM333969.pdf

[88] See footnote 67.

[89] See footnote 69.

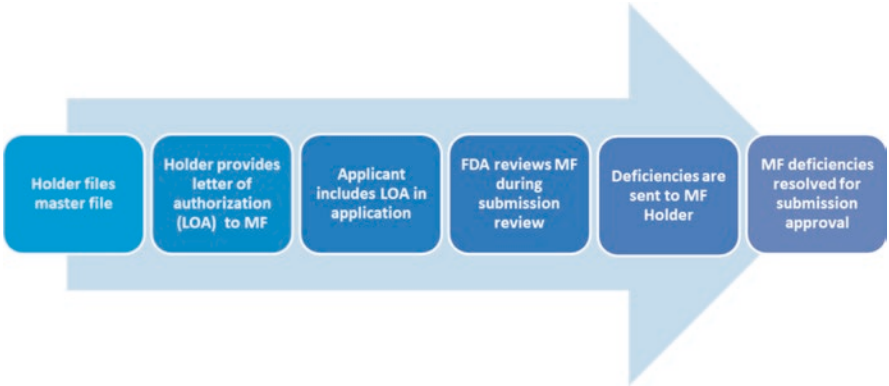

Fig. 1 US master file process

are no conflicts, as these can result in unnecessary questions and delays during the review.

Master files are a mechanism that material or component suppliers can use to share confidential information with health authorities without disclosing that information to the pharmaceutical company. In the USA, master files include Drug Master Files (DMF) and Master Access Files (MAF, or device master file). A "DMF is a submission to the Food and Drug Administration (FDA) that may be used to provide confidential detailed information about facilities, processes, or articles used in the manufacturing, processing, packaging, and storing of one or more human drugs."[90] The general master file process is outlined in Fig. 1.

It is important to note that a master file is neither approved nor rejected. Typically, a master file is not reviewed until referenced by an applicant in a submission. A master file permits the sponsor to incorporate the information by reference when submitting an application or an amendment or supplement to the application, or to authorize other individuals to rely on the information to support a submission to the FDA without having to disclose the information.[91]

As master files may be used to describe multiple materials, they can become very large filings. Therefore, it is best practice for the letter of authorization to the master file to identify the specific locations (section number, page number) for the referenced details to facilitate the review of the master file as it relates to the submission that it is supporting.

The material and component supply chain is often described using *n, n-x* nomenclature, where *n* is the pharmaceutical company, *n-1* is the supplier that provides the packaging or device components, and *n-2* are the suppliers of materials and sub-

[90] FDA, Guideline for Drug Master Files. September 1989. www.fda.gov/Drugs/DevelopmentApprovalProcess/FormsSubmissionRequirements/DrugMasterFilesDMFs/ucm073164.htm.

[91] Roan, SM, Use of Type III Drug Master Files in Product Registrations, *Regulatory Focus*, 2009, 14(12), p. 40

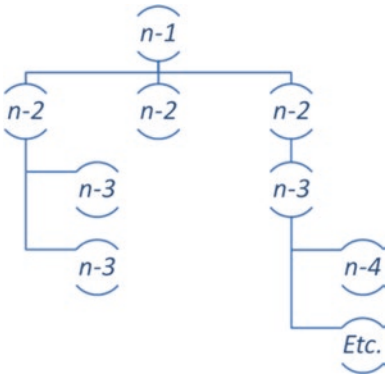

Fig. 2 Representative hierarchy of master files

components procured by the *n-1* supplier and so forth.[92] As the sponsor of a submission referencing the *n-1* master file to support an element of the submission, it is important to understand the hierarchy of *n-x* master files that may support the product. The review of the *n-1* master file may include review of master files referenced by the *n-1* master file, as shown in Fig. 2.

While master files are a useful regulatory tool, the preference from FDA reviewers is to ensure that the combination product submission is as complete as possible, supplemented by master file content. "Regarding the use of Master Files, these should only be for confidential proprietary information that is not otherwise known to the BLA holder. Also, if a master file is used only one file should be submitted for the information. Duplicate files should not be submitted in a DMF and MAF. To facilitate the intercenter reviews please provide master files in electronic format."[93] Additionally, it is important to understand the context of the information in the master file, as it relates to the intended use of the combination product.[94]

4 Life Cycle Management Considerations

4.1 Postapproval Modifications to Combination Products

Along with the issuance of the final rule for 21 CFR 4, the FDA issued a draft guidance regarding submissions for postapproval modifications to combination products,[95] which outlined FDA expectations for a manufacturer to holistically

[92] See footnote 91.

[93] See footnote 82 at page 99.

[94] McMichael, J., *Regulatory Challenges for Combination Products*, presented at PDA Combination Product Interest Group Meeting, 11 May 2017

[95] Guidance for Industry and FDA Staff: Submissions for Postapproval Modifications to a Combination Product Approved Under a BLA, NDA, or PMA, Draft Guidance, Jan 2013, https://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM336230.pdf

assess changes to combination products approved under a BLA, NDA, or PMA, including changes made to any of the individual constituents (i.e., drug/biologic constituent and device constituent). This guidance provided manufacturers with a framework upon which to assess the appropriate reporting category for changes to combination products. This framework provides that the sponsor should assess the change to the constituent part based on the submission type that would have been required if the constituent were a stand-alone product and then using the translation tables provided in the guidance, identify the corollary submission type based on the combination product original application type.

One significant challenge with this draft guidance is that the translation tables only include the medical device Premarket Approval (PMA) postapproval submission types. The majority of device constituents which are part of drug delivery system combination products would be classified as low or moderate risk if they were stand-alone devices, requiring 510(k) clearance and not a PMA. In order for manufacturers of drug delivery system combination products to utilize the guidance, the change assessment for the device constituent part involves applying criteria for a higher classification submission type, which essentially increases the reporting burden for the low or moderate risk products.

An example of this apparent up-classification is the Prior Approval Supplement (PAS) that was necessary to implement a minor design change to an autoinjector needle sleeve and associated changes to the instructions for use (IFU) to enhance the usability of the product.[96] Although assessment of the changes included in this supplement would have deemed that the changes were appropriately reported as changes being effected (CBE), FDA assigned change as a PAS based on the labeling changes.[97] The labeling (IFU) aspect of the change aligns with the criteria for a CBE outlined in the guidance on postapproval changes to specified biotechnology products,[98] as it adds or strengthens an instruction about dosage and administration that is intended to increase the safety of the use of the product in accordance with 21 CFR 601.12(f)(2) and aligns with the example change described in the guidance: "Clarification of the administration statement to ensure proper administration of the product."[99]

Industry has been advocating for a revised framework for postapproval change reporting which incorporates the appropriate reporting categories for low/moderate risk device constituents.[100] This framework is founded on the principle that since the

[96] See approval package for BLA 125289, supplement 103, available at http://www.accessdata.fda.gov/drugsatfda_docs/bla/2013/125289Orig1s103.pdf.

[97] Lipman, Jason, The Top Priority for PFS – Addressing Regulatory Compliance and Clinical Needs (presentation), SMi Prefilled Syringes East Coast, April 26, 2016

[98] Guidance for Industry: Changes to an Approved Application for Specified Biotechnology and Specified Synthetic Biological Products, July 1997, available at https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM124805.pdf

[99] See footnote 98 at page 8

[100] O'Connell, C. Determining NDA/BLA Submission Type for a Change in a Device Constituent Part: A New Approach Proposed by the CPC (presentation), Xavier Health Combination Products Summit, Sept. 2018.

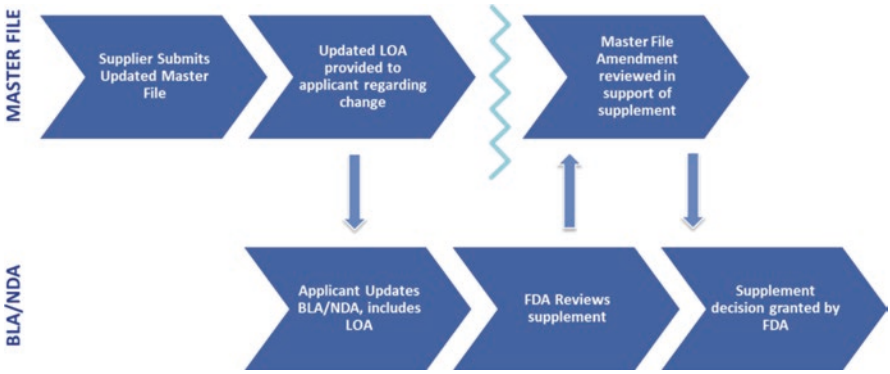

Fig. 3 Master file changes that impact applicant's BLA/NDA

requirements for the constituent parts are retained in the combination product, then device constituent parts should retain their risk-based classification for the purposes of postapproval change reporting, and a 510(k) analysis regarding assessing changes is appropriate. The expectation is that a revised version of the combination products postapproval guidance will be available in 2019.

With regard to changes to a master file that have impact to the BLA/NDA, there is added complexity, as two submissions need to be made to the FDA, and the review of the BLA/NDA supplement requires review of the master file. The process for master file changes that impact the applicant's BLA/NDA is outlined generally in Fig. 3.

4.2 Postmarket Safety Reporting (PMSR)

On December 20, 2016, the FDA published the "Postmarketing Safety Reporting for Combination Products" final rule.[101] The PMSR requires combination product applicants to comply with the reporting requirements applicable to the type of marketing application used to approve or clear their combination product. Additionally, combination product applicants must comply with a subset of six specified reports based on the other constituent parts (drug, device, or biological product). The rule also defines additional information sharing requirements for cross-labeled combination products with individual market authorizations held by different manufacturers. These requirements are highlighted in the Subpart B of 21 CFR 4 (§ 4.100-4.105).

While each constituent part of a combination product is governed by one of three differing sets of postmarket safety reporting regulations, and while each set of

[101] 81 FR 92603 (20 Dec 2016)

regulations have similar provisions, there are significant differences among these regulations which address the unique characteristics of the product type. FDA identified provisions specific to drugs, biologics, or devices that need to be supplemented to ensure appropriate PMSR for combination products.

The duties for both combination product and constituent part applicants are generally the same as for any other entity holding such an application. New requirements were also added for combination product and constituent part applicants.

The purpose of the PMSR rule is to build consistency and avoid duplicate reporting. Though conceptually possible, some reporting requirements for drugs, devices, and biologics are unique, and these requirements have created significant implementation challenges for industry related to electronic submissions. Given these challenges, in March 2018, FDA issued a compliance policy[102] for the rule to communicate that FDA intended to delay enforcement of certain requirements under the rule to ensure that combination product applicants have sufficient time to update reporting and recordkeeping systems, including information technology systems to comply with the requirements.

FDA issued the companion guidance[103] to the PMSR in March 2018, providing supplemental information regarding:

- Entities subject to the final rule and what reporting obligations apply to those entities
- Detailed discussion of specific combination product PMSR report types
- Guidance on where, how, and when to submit PMSR reports to FDA
- Hypothetical scenarios to illustrate how to comply with the PMSR requirements

5 Perspectives for the Future

Combination products regulation has made substantial improvements over the last several years, focusing on improving clarity of regulation, communication with sponsors, and intercenter coordination. However, as explained in this chapter, substantive policy challenges remain on the horizon. Further guidance is expected, specifically relating to bridging, EPRs, and postapproval changes.

One area to watch will be FDA's regulation of drug/biologic and software combinations. In late 2018, FDA suggested it was considering a framework whereby certain apps distributed by NDA and BLA holders would not be considered device constituents of combination products, but that their output would be treated as "promotional labeling," allowing for less burdensome regulation and oversight.

[102] Compliance Policy for Combination Product Postmarketing Safety Reporting Immediately in Effect Guidance for Industry and Food and Drug Administration Staff, March 2018, https://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM601461.pdf

[103] See footnote 31.

Wound care products which contain devices embedded with substances that might be viewed as drugs/biologics, and have traditionally been regulated as unclassified medical devices via the 510(k) process, are also an area of considerable interest to the Agency as it considers the future of these products and next generation products. A two-day public meeting in September 2016 highlighted significant differences in thinking between CDER and members of industry with regard to the direction FDA should take.

3D printing of therapeutics, nanotechnology that is a hybrid of mechanical and chemical function, the next generation of "smart" drug delivery systems, and larger volume delivery systems will likely pose a number of technical and regulatory challenges as well.

Finally, an issue that innovators, FDA, and Congress will need to continue addressing in coming years is the substantial cost and time required to take a product through CDER or CBER, which often underlies jurisdictional disputes with sponsors.

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Chapter 28
Evolving Regulatory Landscape for Drug-Device Combination Products: Europe and Other Major Market Perspectives

Amanda Matthews, Janine Jamieson, and Steve Dew

1 Introduction

As technology advances around us, our medicines are also evolving. We are seeing an upward trend to incorporate a broad range of emerging and often complex technologies in a way that facilitates patients self-administering their medication on a routine basis as well as physicians and patients more readily being able to monitor adherence. Combination products are a significant and growing area of the medicines being approved in the USA, and FDA regulatory requirements have rapidly evolved, but this is no longer a US-centric focus and more countries are reconsidering their regulatory requirements for development and registration of these products that incorporate both drug and medical device elements. However, with the growing interest in developing such products come challenges for regulators to ensure a consistent regulatory framework and assessment for such products, something that has been up to now quite variable across other major regions outside of the USA, including Europe.

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2 Inconsistency: It Starts with Terminology

The term combination product is widely used but commonly misused, as there has been no clear, official definition in Europe, unlike the USA, and only a small number of other major countries are now adopting the term as their regulations are starting to evolve. Until recently in Europe, the term "combination product" would refer to a fixed-dose combination product, combining two active ingredients. However, increasingly it is commonly understood that when used, this term is referring to products that have both a medicinal product and a medical device element, whether that be single-integral or separate entities that are co-packaged, although this is not as broad as FDAs definition under CFR Title 21 Part 3.2. (e).

Often, the principal mode of action (PMOA) of a product governs the regulatory pathway and route for approval, which is the perspective in Europe as well as other major countries or regions. As such, products are regulated as either a medical device or a medicinal product. Based on this defined PMOA, the lead authority for the review and approval is through either a notified body if it is a medical device or the governing Competent Authority or Medicines Agencies for single-integral drug-device products or those co-packaged and historically little cross-over between the two.

The inconsistency and interpretation of terminology goes beyond just the term combination product though and is broader across the complete development based on whether your approach has historically been from a pharmaceutical perspective or one of a medical device. For example, when speaking about specifications for a medicinal product, it is the release specification for routine testing of production batches. However, when considering a drug-device combination and specifications, it is looking at design inputs and requirements for the product and how those are related to a physical design and associated product specifications that control production and include drawings as well. These are often very different interpretations and development and regulatory teams must become bilingual in both medicinal product terminology as well as medical device language to understand the differences as well as similarities, to be effective and successful with product applications. Given that the device information is an application is reviewed by medicinal product quality assessors, it is important to present information in a language and format that is understood by the reviewers.

3 Challenges

Globally, medicines and medical device agencies are recognizing the increased convergence in medical technologies, blurring the traditional classification of being defined and governed as either one or the other. Increasingly, it is becoming more common that we see products crossing regulatory borderlines, and that single Agencies or Competent Authorities cannot cope alone with the full regulatory review of a product.

Competent Authorities or Medicines Agencies may have little or no specific medical device expertise, and the review of such products often lies with separate organizations such as a notified body (NB). This is certainly the current position in Europe and NBs are the organizations that have been designated by Member States to assess medical devices and conformance with defined standards. Consequently, given that a marketing application for drug-device products up to now falls to pharmaceutical assessors with little or no specific device-related experience or understanding, and limited or no collaboration with NBs for such products, this leads to variability in assessment requirements and ultimately the review.

With the regulatory framework being such that the product is either a medical device or a medicinal product, when a drug-device product is registered under a medicinal product application, the absence of, or variability in any defined requirements which truly relate to governing such combined, integral products for administration of medicinal products lead to significant divergence in approach. The medical device and medicinal product frameworks are very different with very little convergence from the overseeing regulatory bodies, and this leads to manufacturers and regulators working with these combined, integral products interpreting the limited guidance and requirements differently, resulting in inconsistency in approach when developing for the global market place but greater inconsistencies of information specifically relating to the device within the registration application. At this current time, there are limited EMA or other major market scientific guidelines for specific quality aspects of developing drug-device combination products. As a result, manufacturers are left to piece together the performance, verification, and safety requirements typically from either leveraging in part the medicinal product requirements, including pharmacopeia requirements, or utilizing the available normative standards such as International Standards Organization (ISO) requirements which are available for testing products such as aerosol drug delivery systems (i.e., ISO 20072) or needle-based injection systems (i.e., ISO 11608 series). However, availability and access of these standards within regulatory agencies and indeed for some manufactures may be limited due to associated cost and copyright issues and the application of requirements by Agencies has also been observed as inconsistent.

This absence of specific quality-related guidance is emphasized when manufacturers are then faced with managing the lifecycle of these products and find it challenging to appropriately assign and file the necessary post-approval changes within a suitably defined variations category, as the variation regulations are often medicinal product focused and do not adequately address device-related changes. A related challenge of lifecycle management is also the different speeds of post-approval change which is a direct reflection on the timeframe for product development of medicinal products versus medical devices. Medicinal products do not typically diverge significantly from the originally approved process and product, whereas it is more typical and expected that medical devices undergo continual iteration and product improvements.

Historically, the rationale for regulating either as medicines or as medical devices has been sound, but it is clear that it needs to become more integrated as technologies and possibilities for improved patient treatment evolve. As a result, we are also beginning to see Agencies also recognize and adapt. In response to the EU Medical Device Regulation requirements and publication of specific quality-related guidance, Guido Rasi, Executive Director of the European Medicines Agency (EMA), in recognition of this emerging landscape, publicly said, "*with the ever-increasing pace of innovation and the blurring of traditional boundaries between medicines and devices, it is inevitable for the Agency to assume new responsibilities in regulating complex medicines with a medical device component. The big challenge we face is to ensure we have the appropriate expertise and resources to adequately carry out these new tasks,*" which positions well what we can expect to see more of, as manufacturers embrace and advance different technologies to meet the needs of patients and healthcare.

4 Global Harmonization Opportunities

As mentioned in the previous chapter, the FDA have established the Office of Combination Products (OCP) which has focus on managing the regulatory process and formulating regulations and guidance documents for combination products. Europe and other major regions do not currently have an equivalent approach and historically fewer regulations and specific guidance have been available, which has led to uncertainties and a more variable approach to development and registration applications for such products across industry.

There are non-profit organizations past and present that are focused on global harmonization of regulation, guidance, and standards applicable to medical devices and as such were also often leveraged for combination products. The Global Harmonization Task Force (GHTF), founded in 1992, was a voluntary group of representatives from national medical device regulatory authorities (such as the U.S. Food and Drug Administration (FDA)) as well as members of the medical device industry, whose goal was the harmonization of medical device regulation across the world. The representatives from its five founding members (the European Union, the USA, Canada, Japan, and Australia) were divided into three geographical areas: Europe, Asia-Pacific, and North America, each of which actively regulates medical devices using their own unique regulatory framework.

In 2012, the GHTF was disbanded, but its mission has been taken over by the International Medical Device Regulators Forum (IMDRF), a successor organization composed of officials from only regulatory agencies from around the world, but with no industry participation.

4.1 International Medical Device Regulators Forum (IMDRF)

IMDRF is building on the strong foundational work of the GHTF with a mission to accelerate international medical device regulatory harmonization and convergence. Founded in early 2011, this organization is still operational at time of print, and its member countries are broader than the previous GHTF, including Brazil, China, Russia, Singapore, and South Korea with the World Health Organization (WHO) also as official observers along with the Asian Harmonization Working Party (AHWP), Pan American Health Organization (PAHO), and APEC LSIF Regulatory Harmonization Steering Committee as IMDRF Regional Harmonization Initiatives.

4.2 International Coalition of Medicines Regulatory Authorities (ICMRA)

The International Coalition of Medicines Regulatory Authorities (ICMRA) is a voluntary, executive-level, strategic coordinating, advocacy and leadership entity of regulatory authorities working together to address current and emerging regulatory challenges, identifying areas of synergy and leveraging existing initiatives and resources. It is a recent initiative that developed from a global summit meeting of heads of regulatory agencies marking 100 years of the FDA, in 2006. At the time of print, there are approximately 22 full members from across the world, 5 associate members, and WHO as an observer. MHRA currently chairs the organization.

In 2017, at their meeting in Tokyo, ICMRA identified Innovation as a key strategic priority, recognizing that a new paradigm was required to address the merging of different regulatory regimes and previously unregulated areas of manufacture. Although the name refers to medicines and the organization is linked to the formal WHO-led ICDRA (International Conference of Drug Regulatory Authorities), ICMRA is a more informal organization with topics merging with medical devices being considered, such as 3D printing, software, apps and digital therapeutics, as well as ATMPs.

4.3 Importance of Innovation in Medical Product Regulation

The ICMRA recognizes that one of the key challenges facing regulators today is the rapidly deepening complexity of medical products, with increasingly transformative innovations that do not fit neatly into the current regulatory framework and that, in order to apply appropriate regulatory scrutiny while not stifling innovation, regulators need to be open and adapt as necessary. The strategic priority of innovation

focuses on the research and development of novel and/or disruptive medical products, techniques, and technologies and how to apply risk-based approach in inspections, etc. Since many of the challenges posed by such innovations are common throughout the global regulatory community, ICMRA aims to provide strategic leadership and direction to avoid duplication of work by multiple regulators and to harmonize regulatory procedures.

4.4 EMA Regulatory Science Strategy to 2025

At a European level, again recognizing the need for collaboration to handle the innovative products identified during horizon scanning activities, the EMA held a workshop in October 2018, with the aim to learn from a wide range of different stakeholders about the challenges for the future and how to work together to solve them. Participants included patient representatives, healthcare professionals, academic infrastructures, health technology assessment (HTA) bodies, payer organizations, trade associations, and regulators. Following the workshop, a six-month public consultation on European Medicines Agency (EMA) human regulatory science to 2025 was launched in December 2018.[1]

Strategic goals and core recommendations for Human medicines include:

Catalyzing the integration of science and technology in medicines development through

- Supporting developments in precision medicine, biomarkers, and ‘omics
- Supporting translation of advanced therapy medicinal products (ATMPs) into patient treatments
- Promoting and investing in the PRIME scheme
- Facilitating the implementation of novel manufacturing technologies
- Creating an integrated evaluation pathway for the assessment of medical devices, in vitro diagnostics, and borderline products
- Developing an understanding of, and regulatory response to, nanotechnology and new materials in pharmaceuticals
- Diversifying and integrating the provision of regulatory advice along the development continuum.

5 Focus on the Current European Regulatory Framework

Medical devices can only be put on the European Market if they satisfy a set of criteria called “essential requirements,” as set out in Annex I of the Council Directive 93/42/EEC, Medical Device Directive (MDD). All medical devices must comply,

[1] EMA Regulatory Science to 2025.

where applicable, with these requirements to affix a CE mark to the medical device. In addition, manufacturers of medical devices are required to implement a quality management system appropriate for the specific classification of medical device, and most companies choose to align with requirements of ISO 13485.

In Europe, Council Directive 2001/83/EC, the Medicinal Product Directive (MPD)[2] requires evidence of CE marking, when applicable, if the product incorporates a medical device. In addition, the MDD stipulates in Article 1(3)[3] that "If, however, such a device is placed on the market in such a way that the device and the medicinal product form a single integral product which is intended exclusively for use in the given combination and which is not reusable, that single product shall be governed by Directive 2001/83/EC. The relevant essential requirements of Annex I to this Directive shall apply as far as safety and performance-related device features are concerned."

So, for products whereby the medical device is a separate entity but co-packaged or intended to be used with a medicinal product, evidence of CE marking would be available. The expectation of the applicant would be to demonstrate compatibility of the medical device and medicinal product and that the medical device performance is appropriate to deliver the required dose within the registration application, along with filing the CE certificate in eCTD Module 3.2.R (Regional) section of the dossier.

However, up to now, approaches taken by manufacturers developing single-integral drug-device products have been variable. While the relevant essential requirements of Annex I of the MDD are typically met as far as safety and performance-related aspects are concerned, it is known that evidence or substantial detail provided by manufacturers within a registration application differs between companies and is often not incorporated at all.

Moreover, development of products considering these requirements by manufacturers has historically been inconsistent and not always applied, for example, prefilled syringes have previously been considered as a primary container and met the applicable container closure requirements only, with little or no consideration of the MDD and Annex I at all. Similarly, it was not uncommon for manufacturers to position autoinjectors as functional secondary packaging and, while meeting the applicable Annex I requirements, would incorporate minimal evidence and information pertaining the autoinjector itself in 3.2.P.7 Container Closure of the application.

Although this may have been the historical approach taken by some, it was clear from the concept paper released by the European Medicines Agency (EMA) in November 2016,[4] from combined Quality and Biologics working parties, that such products were not considered complex container closure or packaging systems and as such guidance was needed on quality requirements for medicinal products incor-

[2] Council Directive 2001/83/EC, the Medicinal Product Directive (MPD).

[3] Council Directive 93/42/EEC, Medical Device Directive (MDD).

[4] EMA Concept paper: EMA/CHMP/QWP/BWP/661488/2016; Concept paper on developing a guideline on Quality requirements of medicinal products containing a device component for delivery or use of the medicinal product.

porating, or used with, medical devices. And recently, the EMA has openly stated on their web portal that prefilled syringes[5] are considered as integral products and will therefore be subject to certain medical device requirements for the device element. The EMA defined their intent to release a quality guideline to address the inconsistencies and often incomplete market authorization dossiers and, as a direct consequence, ensure that development of such products does not compromise the safety and efficacy of the medicinal product or result in adverse drug reactions (ADRs) or medication errors and ensure that the drug-device product is fit for its intended purpose. The intent of this quality guideline is to consider the quality aspects in relation to safety and performance of the medical device, whether it is an integral component of the medicinal product or a stand-alone device, and at time of authoring, a final quality guideline is still pending.

In addition, the current ICH and eCTD guidelines do not adequately cover single-integral drug-device products and what specific information is required for registration dossiers, or how they should be managed.

We are seeing a growing trend for medical devices supplied as integral to a medicinal product to have far greater complexity and functionality than simple container closures, which should be appropriately addressed in the registration dossier. With the introduction of more novel products that incorporate digital and technological advancements including electronics, manufacturers need to demonstrate product quality and safety, along with robust processes and controls.

In parallel, having an appropriate framework that ensures an acceptable review of such products is also of the utmost importance, to guarantee product quality and importantly patient safety while making available innovative treatment options to patients.

5.1 European Medical Device Directive and Times of Change

On April 5, 2017, the European Commission formally published in the Official Journal of the European Union the legislative Regulation (EU) 2017/745 (MDR) on medical devices,[6] amending Directive 2001/83/EC and repealing Council Directive 93/42/EEC (MDD).

The predecessor of the MDR, the MDD, defined the Essential Requirements (ERs) and introduced harmonized standards, helping to demonstrate conformity to the Essential Requirements (Annex I). These directives, which were introduced in early 1992, did work to a point and helped create a single market for medical devices across Europe.

However, the directives had some inherent weakness and the evolving changes in technology and science have demanded that the legislation be revised to better accommodate this going forward. Under the previous MDD, it was apparent that national member states could develop their own interpretation of the directive and

[5] EMA Human Regulatory and Medical Devices web portal.

[6] Official Journal of the European Union the legislative Regulation (EU) 2017/745.

this was not always consistent across all national authorities. While Directive 2007/47/EC[7] was introduced to address some of these shortfalls, this amendment did not address all the concerns and the system was still perceived to have failed to adequately protect the patient. The highly publicized Poly Implant Prosthesis (PIP) scandal in France which began to surface in 2009 was a key contributing incident which highlighted further weaknesses in the system whereby non-medical grade materials were used in the manufacture of the breast implants and incidents of device malfunction were observed which unfortunately led to some patient harm and a major safety recall was initiated.

Because of this scandal among other incidents, the European Commission embarked on a full regulatory overhaul of the medical device regulations. The goal was ensuring high levels of patient safety but, more importantly, implementing controls for both manufacturer and the notified bodies overseeing these companies, to ensure high-quality products continue to reach patients and ultimately restoring public confidence by ensuring such incidents did not happen again. While some of the new requirements were an extension of scope to specifically cover products that had previously been less regulated, i.e., cosmetic/aesthetic devices, many of the enhancements now incorporated apply across the wider range of medical devices under the regulation and significantly improve:

- Requirements for compliance from manufacturers are more stringent, to ensure patient safety and improved risk management of devices.
- Creation of a national registry where manufacturers have to register themselves and the devices they place on the EU market in a central database (Eudamed); the goal of the registry is to guarantee traceability through unique device identification (UDI).
- Greater transparency required along the entire supply chain of devices that obliges each entity in the supply chain to check compliance of the previous supplier and prevent inadequate quality materials, components, and processes being introduced.
- Ongoing conformity assessment of the device through its lifecycle is required, with specific requirements relating to postmarket surveillance including postmarket clinical follow-up. This also includes establishment of requirements where manufacturers are required to report serious events and corrective actions to reduce the risk of recurrence.
- Specific requirements for high-risk medical devices (Class III) including special procedures relating to an independent evaluation of clinical data with notified bodies preparing a Clinical Evaluation Assessment Report which is shared with the European Commission and the report is reviewed by an expert panel who issues a separate scientific opinion.
- Increased rights and responsibilities for notified bodies and their monitoring of all medical devices, as well as increased scrutiny of themselves by the Competent Authorities to ensure they are maintaining standards.

[7] Amendment to MDD, Directive 2007/47/EC.

While the above summary is primarily targeted to stand-alone medical devices, the goal of enhancing quality and scrutiny in support of patient safety directly correlates to integral drug-device products. Following formal publication, there is a transitional period, but from May 26, 2020, the requirements of the MDR will be fully enforceable and with this a requirement for drug-device products to meet a new requirement specifically laid out in Article 117.

Article 117 defines "the marketing authorization dossier shall include, where available, the results of the assessment of the conformity of the device part with the relevant general safety and performance requirements set out in Annex I to that Regulation contained in either the manufacturers EU declaration of conformity or the relevant certificate issued by a notified body allowing the manufacturer to affix a CE marking to the medical device.

If the dossier does not include the results of the conformity assessment referred to in the first subparagraph, and where for the conformity assessment of the device, if used separately, the involvement of a notified body is required in accordance with Regulation (EU) 2017/745, the authority shall require the applicant to provide an opinion on the conformity of the device part with the relevant general safety and performance requirements set out in Annex I to the Regulation issued by a notified body designated in accordance with that Regulation for the type of device in question."

With this new legislative requirement comes some convergence in regulatory framework of the different jurisdictions. While the EMA previously declared in their 2016 concept paper[4] that their future medicinal product quality guideline would cover the requirements with respect to quality as it relates to safety and performance of the medical device, the European Commission has taken this further with the requirement of the MDR and acknowledged that participation of notified bodies in the review of the medical device element is now necessary when integral to the medicinal product and not eligible to be CE marked, to ensure consistent and comprehensive review and approval for such products, which has not necessarily been the case up to now.

Therefore, for manufacturers of medicinal products that previously thought they were not working in the space of medical devices, they will be required to work with a notified body to gain approval for such medicinal products, which will bring with it new and probably unforeseen challenges based on a different approach to historical practices.

5.2 Notified Body Opinion

While the legislation of the Regulation itself is clear and defines a need for a third-party assessment of the medical device element, the process of how manufacturers will achieve this and specific requirements of the assessment are still not defined and at time of publication. It is likely that the new interactions and processes will continue to evolve while experience of the new procedures is gained, as recognized

in the EMA Q&A published in February 2019, which states that it is a living document that will be updated continuously. Although the quality guidance being developed by EMA and a draft expected mid-2019 is anticipated to address what is required within the MAA itself, the specific scope and requirements of the notified body opinion are not yet defined beyond knowing that the device is required to comply and demonstrate conformance with Annex I, no longer known as the ERs but the General Safety and Performance Requirements (GSPRs). The reality is such that these requirements associated with Competent Authority or notified body role and their respective review will probably continue to evolve as both parties and manufacturers start to interact and manage new product registrations and product lifecycle changes.

To ensure compliance with the GSPRs, it would be prudent for device manufacturers to consider treating the device constituent of the integrated product akin to a CE marked medical device, whereby the appropriate device design and development principles should be applied, and evidence should be compiled and maintained in the design and development file. This file would then serve as the evidence for meeting the applicable parts of the MDR Annex I and relevant GSPRs for your specific drug-device presentation. Construct and format of this file for drug-device combinations are something also not yet defined, but given the structure and content for CE marking a medical device are well articulated within Annex II of the MDR, it may be advisable to follow a similar structure for integral drug-device products.

In comparing the Essential Requirements under the MDD to the GSPRs of the MDR, it is clear the requirements have extended in scope because we move from 13 ERs to 23 GSPRs. Overall, text and requirements are expanded, but the general scope and topics are in the main consistent with the previous Directives. It is clear though there is an increased emphasis in a number of areas and more explicit requirements, which in many cases align with the harmonized and normative standards and industry guidance.

Some areas in Annex I that are considered to have impact on manufacturers based on their increased emphasis are summarized as follows:

- Risk management, There is much consideration to the risk profile and benefit to end users consistently throughout the safety and performance requirements, with an emphasis on ensuring risk profile is as low as possible and demonstrate as such.
- Further to current biocompatibility requirements, there is specific attention to material safety with respect to substances of concern, i.e., phthalates, as well as controlling components of carcinogenic, mutagenic, and toxic to reproduction or endocrine-disrupting substances.
- There are enhanced requirements for materials of biological origin and evidence of their safety, including traceability of origin, viral contamination and transmissible agents.
- Clauses with respect to software and electronic systems including emphasis on cybersecurity are new.

- There is growing emphasis to demonstrate usability and that a manufacturer has reduced the risks related to use errors. There are specific labeling and IFU requirements for devices to be used by laypersons, with the emphasis to ensure that risks are as low as possible.
- More prescriptive with respect to labeling and instructions for use, recognizing advancement in technology, i.e., nonpaper formats for IFUs as well as machine-readable bar codes and reduced-function devices (RFDs).

In addition to certain quality requirements some of which are discussed above, there are still undefined aspects which industry associations have tried to raise the profile for[8,9] to have the EC, Competent Authorities, and notified bodies also consider when developing implementation guidance and facilitate manufacturers in meeting the new requirements. This includes the quality management system requirements and whether application or even certification is required to device harmonized standards, i.e., ISO 13485, or whether medicinal product GMPs can be leveraged, akin to streamlined approach defined in the 21 CFR Part 4.4 cGMP[10] requirements in the USA incorporating specific design control requirements for the device.

Regulators are equally looking to bridge the gap between the new innovation, the new technology, and the current GMP guidance. And considering its interpretation, give assurance and be confident that the product and process are robust and that the essence of the requirements themselves is still being met. Rather than trying to force fit innovation into a legacy regulatory process or changing the regulatory framework to facilitate these innovations as they evolve, they are suggesting also being open to alternative approaches which might give greater assurance of consistency while meeting the outcome that is desired.

To this point, the Medicines Health Regulatory Agency (MHRA) has at different meetings and symposiums of late explored how current GMPs can be applied to new technologies and innovative ways of working, and if risk-based GMPs and appropriate control strategies could potentially be implemented, as regulators do not want regulations and guidance to be a barrier to innovation. For example, historical practice has commonly seen centralized manufacturing and distribution of products. However, we are now starting to see more products that require some final steps to occur at a local location or site, often immediately before administration to patient. This can be more than just a dilution or reconstitution for administration, e.g., adjusting the dose or removal of a cryoprotectant. This activity in many cases is part of the product manufacture itself, but it would be impractical if it means that to meet the GMP requirements, a Qualified Person is required at each of these local sites.

[8] EBE reflection paper on "Medicinal product incorporating a drug delivery device component: An Industry Perspective on the EU marketing application technical requirements, regulatory review process and post-approval device-related change assessment".

[9] EBE-EFPIA Reflection paper An Industry Perspective on Article 117 of the EU Medical Devices Regulation and the Impact on how Medicines are Assessed.

[10] 21 CFR Part 4.4 cGMP requirements.

Regulators are encouraging manufacturers to reach out to them with alternative approaches, acknowledging that new interpretations and new ways of working are required to support such situations, maintaining a system which fully meets the requirements around regulatory supervision but also makes products accessible to patients.

Other pertinent topics which industry is currently faced with understanding for managing drug-device combinations include but not limited to:

- Labeling requirements and whether Annex I requirements apply or does medicinal product legislation and requirements take precedence.
- Postmarket surveillance requirements specifically of the device element, given these products are regulated as medicinal products;
- Notified body assessment of clinical data versus the Competent Authority assessing clinical data within the MAA;
- Lifecycle management and what variations and the type of change that could require a further notified body assessment to support a change after the MAA is approved, versus what can be managed within a manufacturer's QMS.

It is hoped that pending and future guidance from different stakeholders will help define an acceptable path forward and facilitate future approvals.

6 Outside the USA and Europe

In parallel to the USA and Europe, other markets are trying to establish frameworks for the rapidly developing combination products sector. In general, most of these markets have adopted the same definitions (formal and informal) of a combination product as found in Europe, i.e., a product that integrally combines a medical device with a medicine or biologic, but have not always gone as far as the USA in defining different types of combinations such as companion, cross-labeled products, systems or kits.

6.1 Country and Specific Regional Overview

Based on the PMOA, often the drug-device product is governed under medicinal product legislation with some requirement to meet specific medical device requirements. While more countries are recognizing combination products within their regulations, very few have separate coordinating bodies for their review or oversight and requirements. Applications for combinations with drug PMOA are often exclusively reviewed in the medicinal product assessment.

The Asian Harmonized Working Party published a paper back in 2016 on the practices for combination products across different major regions. Figure 1, taken directly from that paper, highlights that there are broad areas of similarity in regulation

	Formal Definition in Regulation	Formal Status Determination Mechanism	Separate Co-ordination body	Evaluation Process	Fees	Manufacturing Controls	Labelling	Postmarket Reporting	Clinical Trials	Clinical Data Requirements	Planned Changes
USA	Y	Y	Y	P	P	P,C	C,X	P	P	P	
EC	N	Y	N	C	C	C	C,X	P	P	P	R
AUS	N	Y	N	C	S	P,C	P,X	P	P	P	
JPN	N	N	N	P	P	P	C,X	P	P	P	R+
CAN	Y	Y	N	P	P	P,C	P,C	P	P	P	
CHN	Y	Y	Y	P	Y	C	C	Y	Y	Y	R
SGP	Y	Y	N	P	S	P	P	P	P	P	G
IDN	Y	Y	N	P	P	P	U	U	P	P	
KOR	Y	Y	N	C	P	P,C	P,X	P	P	P	
HKG	N	N	N	P	P	P	P	P	P	P	
TWN	Y*	Y*	N	P	P	C	C,X	P	P	C	G
THA	N	Y	N	P	N	U	P	P	P	P	
MYS	N	Y	N	D	D	P	U	P	C	P	R,G
KSA	Y	Y	Y	P	P	P	P	P	P	P	R

Key

Y: Yes
N: No

P: Regulations or practise applicable to PMOA applied
C: Regulations for all components applied
L: Review coordinated by Lead agency
D: Regulations under development

S: Special Fees for combination products
X: Cross labelling requirements for co-dependent products
R: Changes to Regulation

G: Changes to Guidance
U: Undefined – no regulation or guidance established

Notes

* Guidance in preparation
+ Since conduct of the survey, Japan has issued a notification *Handling of Marketing Application for Combination Products* effective November 25, 2014, which states the scope and requirements for combination products

Fig. 1 Summary of regulation of combination products in international jurisdictions. (Ref: Guidance on Regulatory Practices for Combination Products Work Group 1 AHWP/WG1/F001:2016)

of combination products especially with respect to primary mode of action being used to establish which regulatory pathway the combination product will be assessed, with most of markets being like Europe and not having a distinct and separate pathway for combinations, choosing to have only the drug or the device routes of approval available. Most markets have so far opted for not creating a separate independent regulatory path for combinations as is current practice in the USA.

As a result, the lead agency responsible for the review of the combination product will rely on the resources of other appropriate agencies to provide an assessment of the "secondary" mode of action device. Some agencies have more formal arrangements for coordination of these activities, but this is in its infancy and is not always well defined. For example, as revised requirements emerge in Malaysia, they seem to be adopting some of these practices, in that they now require a separate ancillary medical device dossier for medium to high-risk medical devices, which seems to be based in many parts on the European Device Regulation and requirements under

Annex I, General Safety and Performance Requirements. However, they seem to be putting into practice these coordination practices such that if the device constituent of a combination product has regulatory clearance in a major region such as Canada, EU, Australia, or Japan, then this approval can be leveraged, and it would negate the need to provide substantial evidence relating to design verification and validation of the device itself. This in turn could expedite the review and approval for products and placing them on the market as well as reducing the burden of lifecycle maintenance long term.

6.2 Industry Perspective and Seeking Global Harmonization

While some guidance is available, it is recognized that it is fragmented across countries and organizations and may not be aligned across regions. As such, for combination products, this ultimately leads to the same problems previously mentioned, in that the content and format of regulatory submission files vary considerably and inevitably lead to wide variation in the level of detail required by different agencies. It is therefore hoped that the continued work of organizations such as the Asian Harmonization Working Party (AHWP), IMDRF, and ICMRA will help to define and align the content of device and combination product submissions globally as this space evolves.

Industry is keen to ensure combination products currently on the market and those in development meet these changing and evolving requirements to ensure patients continue to have access to important therapies. This presents challenges in terms of consistency of approach when developing combination products for a global registration. To address these challenges, the industry has been trying to work more closely together and in collaboration with some regulatory agencies, legislators, and other key stakeholders when possible.

Industry themselves are working toward gaining alignment on both advocacy topics and key areas outside of the US legislation, with a view to sharing common understanding of requirements and expectations as well as influencing through other external groups to gain alignment on approaches wherever possible. The Combination Products Coalition (CPC) is a cross-industry group of leading companies from the drug, device, and biologics industries, and it has a specific focus group on international registration requirements for combination products, as well as other focus topics including marketing submission topics, human factors, and digital health. In Europe, a cross-industry group led by the European Biopharmaceutical Enterprises (EBE) Biomanufacturing focus group, which is a specialized focus group of European Federation of Pharmaceutical Industries and Associations (EFPIA), has also been active in communicating with regulators on expected challenges of the new device legislation, offering up potential solutions for discussion and consideration in preparation of guidance.

6.3 Role of the Manufacturer Role in Regulation of Combination Products

Up until now, we have focused on the development of regulations and the regulators with respect to the converging worlds of pharmaceuticals and medical devices. However, one must remember that the industry has been slow to accept that the pharmaceutical and device worlds are converging with respect to how they are managed internally from a regulatory perspective.

As referred to earlier, it is not unheard of for medicinal product manufacturers to assume the device part is nothing more than a "container closure system," "accessory," or "packaging" and not consider medical device regulation, guidelines, and normative standard requirements appropriately.

Therefore, if a medicinal product manufacturer previously thought they "don't do devices," they may have to rethink quickly as this could prove to be a very costly philosophy when regulators are clearly thinking differently. For example, and mentioned previously, EMA has explicitly stated that prefilled syringes are considered as a "single-integral" product.

This inevitably requires a shift in mindset to embed device thinking into medicinal product development teams to ensure the device element is managed appropriately and supports future MAA submissions.

Official guidance from regulators is likely going to be too high level and too generic to provide specific guidance for a specific presentation. However, ensuring that the device element of the presentation is appropriately considered with respect to device requirements during the development cycle will help to avoid some of the frequently raised issues in dossiers for drug-device combinations and therefore reduce delays to an MAA submission.

Some of the more common issues found during MAA review are related to insufficient information about the device, drug/device compatibility, stability data, evidence of dosing accuracy, bridging data between the device used in trials and final presentation, human factors, and risk management plans that fail to consider device elements. All these issues should be considered as design requirements early in development and not left until an MAA is already under review.

Although the device component may not require a submission/registration itself, to move toward a common submission process and commitment to adopt international data standards, and provide greater alignment with drug submissions, some manufacturers are already migrating to the IMDRF-RPS structure put forward by the Regulated Product Submissions Table of Contents Working Group, 27 March 2018,[11] which is comparable to the electronic common technical document (eCTD), the international specification for the pharmaceutical industry to provide regulatory information to drug agencies developed by the International Council for Harmonization (ICH), as a baseline file for enabling core file for international submissions.

[11] Non-In Vitro Diagnostic Device Market Authorization Table of Contents (nIVD MA ToC), 27 March 2018.

It sounds burdensome, and much of the information may not actually need to be included within the actual MAA submission; however, adopting a mindset of "treat it as a medical device" may help prevent expensive redesign, development, and submission delays and validate the declarations of compliance to applicable requirements.

6.4 LifeCycle Management

There is a gap in global regulatory guidance that overarches drug-device products and specifically the registration requirements for any application and consequently the lifecycle and post-approval management, which has resulted in different interpretations and variability in device-related information that is filed.

With the introduction of drug-device combination products in the scope of draft International Council for Harmonization (ICH) Q12 *Technical and Regulatory Considerations for Pharmaceutical Product Lifecycle Management,* pharmaceutical companies consider that this could be one mechanism for regulatory convergence and consistency of device-related information within registration dossiers. It could in turn provide greater regulatory flexibility, allowing both marketing application holders and regulators to focus on the most critical changes that may occur in a product lifecycle to maintain product quality and ensure patient safety. Many of the concepts addressed by ICH Q12 are already embedded into design and development principles for drug-device products because of manufacturers' adherence with GMPs which has overlap with medical device standards such as ISO 13485, *Medical devices – Quality management systems – Requirements for regulatory purposes.*

Aligned with this, established conditions for device elements are assessed and defined as the characteristics of the medicinal product that are essential for its safe and proper use and defined by the control strategy, utilizing risk management tools in accordance with ICH Q9 or ISO 14971, depending on the manufacturer's quality system. Given the absence of reporting categories to suitably manage device changes when integral with the drug product, the same principles could be utilized to managing lifecycle changes and applying a risk-based approach to defining which device-related changes require reporting to Competent Authorities as a variation.

Moderate to high-risk changes (based on the intended use of the combined product, taking into account both device complexity and criticality of dosing accuracy) would be subject to review and approval as a variation, whereas low-risk changes would not necessarily require notification but managed within the manufacturer's quality system.

Although no current or future work item is defined, there is recognition that a specific focus on drug-device combination products and how aspects of device-related information as it correlates to patient safety and intended use with the given medicinal product (e.g., benefit/risk management and usability/human factors) would be of significant benefit. The aspiration to have such guidance would provide a mechanism of linking established concepts already in use through ICH Q12

effectively to drug-device products and the objective to ensure that pharmaceutical assessors across different global authorities are routinely receiving the necessary information to conduct an effective and efficient review while facilitating global consistency and harmonization utilizing the eCTD dossier structure including life-cycle management and post-approval change.

Chapter 29
Importance of Design Control and Risk Management Processes in Combination Product Development

Harold K. Yeager, Mary Roesner Brokovich, Beat U. Steffen, and Michael J. Roe

1 Introduction

A competent device quality system should implement a network of controls committed to the reduction, elimination, and prevention of quality nonconformities. The standard mantra from regulators and auditors goes something like "If you didn't document it, you did not do it." For combination products (refer to 21 CFR 4), rather than modifying current drug-centric quality processes (e.g., FDA GMP, ICH), establishing systems dedicated to the device constituent part (e.g., FDA QSR) is an important commitment in the pursuit of efficient and thoughtful drug delivery system development. The differences are substantial, not the least of which are the concepts of design controls and device risk management. Maintaining regulatory compliance, protecting patient safety, and maximizing customer satisfaction are problematic without that level of focus. The quality system should be expressed in terms of organizational goals, policies, processes, documentation, and the resources required to implement and maintain it. When properly implemented, the quality

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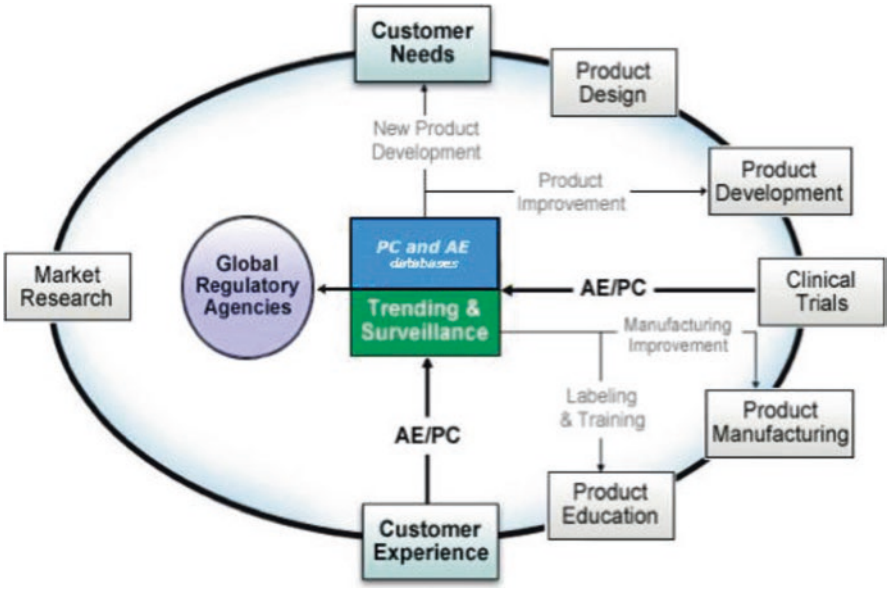

Fig. 1 Product lifecycle inputs and outputs. (*AE* adverse event, *PC* product complaint)

system will help the business reduce mistakes and increase efficiencies in terms of time and cost.

For purposes of this chapter, the notion of a device quality system should include the critical roles of both the regulatory and safety functions that may or may not be formally part of the quality organization. An overview of device risk management can be found in ISO 14971, and a discussion of design controls can be found in ISO 13485. Furthermore, the new EU Medical Device Regulations (MDR), which will fully replace the current EU Medical Device Directives (MDD), is an important document for sponsors of combination products to embrace and implement within their quality systems. Additionally, the MEDDEV 2.12/1 offers a solid overview of device vigilance requirements. These core documents are critical to managing a given product life cycle in terms of device inputs, outputs, safety, and compliance in general. A simple product life cycle map with inputs and outputs is shown in Fig. 1.

2 Design Controls

Design controls are a collection of procedures and behaviors driving a design and development process (refer to 21 CFR 820.30 as well as ISO 13485 for a complete overview). Design controls make systematic assessments of the design an integral part of development. As a result, deficiencies in design input requirements, and discrepancies between the proposed designs and requirements, are made evident

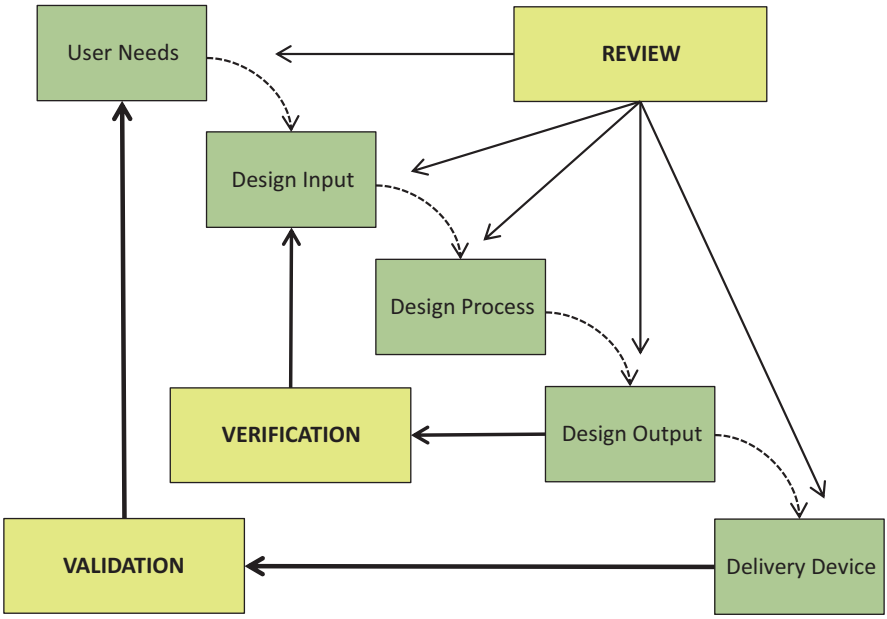

Fig. 2 Design process waterfall model

and corrected earlier in the development process. Design controls also increase the likelihood that the design transferred to production will translate into a device that is appropriate for its intended use as documented in the design history file (DHF). Consequently, the primary purpose is to arrive at a final drug delivery system that is appropriate for its intended use and endorsed through a robust design review process integrating the key stakeholders and related management across the organization. Controls and written procedures must be in place to ensure that specific design requirements are met. This process can be represented using the common waterfall design process in Fig. 2.

Design inputs can be both physical (e.g., device length, color, maximum dose setting), functional (e.g., end of dose confirmation, interface with needle), and performance based (e.g., dose accuracy, volumetric delivery rate), which must not be in conflict with one another. Design outputs include well-documented product specifications, manufacturing methods, engineering drawings, and training materials.

2.1 Design Verification and Validation

The ISO 11608 family of standards offers comprehensive guidance on performing design verification for needle-based drug delivery systems (refer to Chapter 30). Design verification is a structured methodology by which design outputs are com-

pared to the inputs relative to the product specification, as demonstrated through device testing (e.g., shipping/vibration, dose accuracy, human factors), inspection following environmental and mechanical challenges (e.g., cracking of parts, print legibility), evaluation of general design requirements (e.g., does the device allow visibility of the drug product), and analysis (e.g., statistical assessment of dose accuracy compliance). To that end, the ISO 11608 family (Part 1 in particular as the master document for all other parts) offers a set of general design requirements targeting basic functionality and, more importantly, safety considerations for the user interface. The ISO 11608 standards make these recommendations in a manner that is performance-based as opposed to design prescriptive such that innovation is not unnecessarily limited. As such, the "what" is prioritized over the "how." Additionally, the 11608 family mandates a series of environmental and mechanical challenges designed to assess product robustness when subjected to anticipated storage and use conditions.

Design validation (as compared to manufacturing process validation whereby objective evidence is used to establish that the process consistently produces the product conforming to previously established specifications) confirms that the user needs have been met based on the product's intended use through simulated use scenarios (i.e., summative human factors testing, which is a critical part of design validation). Acceptance criteria must be established ahead of testing, and all discrepancies identified (using objective evidence) must be addressed prior to commercialization. This includes the delivery system itself, its labeling, packaging, and training materials. Testing and analysis may also demonstrate the need for additional communications per risk minimization activities such as a letter to healthcare providers highlighting special concerns (e.g., awareness that concentrated insulins pose additional risk should a user attempt to withdrawal a dose from a prefilled pen using a standard U-100 syringe). That said, labeling and training are important, but they are not a substitute for good design and manufacturing processes.

All activities developed in support of design verification and design validation should be included in a master test plan (e.g., design verification and design validation master plan). The results of both the design reviews and verification/validation processes must be documented in the DHF which houses all records describing the design history of the final combination product.

2.2 Phase Appropriate Design and Testing

These assessments can be conducted at multiple points along the various development stages (e.g., early prototype testing, release of clinical trial materials, process validation in support of launch). As such, design verification methods can be phase-appropriate based on the maturity of the design and the immediate needs of the program. This allows the device organization to be more nimble in managing time, scope, and cost.

A case in point might be early-phase prototypes produced from aluminum single-cavity injection molding tools as compared to the commercial configuration pro-

duced from hardened steel multiple cavity tools. Here, a risk assessment for devices used in a pharmacokinetic/pharmacodynamic (PK/PD) study comparing a prefilled syringe (PFS) to a new auto-injector design might find it acceptable to not require a detailed evaluation of drop-testing or other more extreme handling conditions (i.e., a subset of the complete design verification protocol) given the controlled environment of a clinical setting and healthcare provider (HCP) oversight. This can provide valuable information in an expeditious manner with limited risk as long as the rationale for these "deviations" is sound and, of course, well documented.

If a device platform (refer to Chapter 26) is leveraged for another therapeutic in the same container design, efficiencies in completing release of the clinical trial materials (i.e., the drug delivery system) can be gained if prior successful design verification assessments can be reasonably transferred to the current product. However, if the new therapeutic is intended for different target populations or use cases, additional human factors/usability studies must be considered. As such, these attempts at efficiencies through deviations from master verification protocols must be carefully considered and fully documented as a function of the risk assessment and supporting rationale.

3 Risk Management

Risk management is a process that begins with identifying the intended use and a set of design input requirements (refer to ICH Q9 and ISO 14971 for complete overviews). The primary purpose of this effort is to address the needs of patients, HCPs, and the business stakeholders sponsoring or sanctioning the development effort.

Risk management plans serve to establish risk acceptability criteria and related activates conducted throughout the development and manufacturing process phases. Therefore, risk management plans must be created and executed for both the prelaunch and postlaunch environments. These plans should be reviewed and revised, as appropriate, at least annually.

3.1 Governance

The establishment of device governance structures is both tactically and strategically valuable. They should be cross functional with representation from development, safety, regulatory, labeling, manufacturing, legal, medical, quality, and other functional disciplines as appropriate. Governance should work closely with program teams and related functions to stay informed regarding evolving safety data such that risk-benefit profiles of development programs and marketed products remain acceptable. They should opine regarding new safety questions, signals, and risk assessments. Additionally, they can serve as gatekeepers by reviewing and approving key development phase milestones throughout product lifecycles and, in

so doing, provide management notification and awareness, which is critical in maintaining compliance with device regulations. Approval from the governance group for a given issue should be based on achieving a quorum among the governing members. If governance cannot reach a quorum, there should be escalation mechanisms in place for impacted business stakeholders to adjudicate the issue through leadership groups like a safety lead team and/or senior management.

3.2 Risk Management Plans

A general risk management plan is iterative and includes the following elements:

- Scope (combination product, accessories, etc.)
- Assignment of responsibilities and authorities
- Requirements for review of risk management activities
- Risk policy
- Risk analysis methods
  - Intended use
  - Hazard identification
  - Estimate of risk
- Risk evaluations
- Risk controls
- Evaluation of residual risk acceptability
- Risk management reports
- Verification activities
- Activities related to collection and review of relevant production and postproduction information

Risk analysis tools can be used to assess the drug constituent part and the device constituent part separately. Alternatively, there is benefit in attempting to characterize the overall combination product or system's residual risks as a whole. While this can be laborious and complex, it may prove very helpful in communicating product risks to regulators. Both approaches can employ the following tools:

- Preliminary hazard analysis (PHA)
- Failure modes and effects analysis (FMEA)
- Fault tree analysis
- Assurance cases
- Human factors/task analysis (see note)

Note: with regard to human factors/usability engineering and its relationship to risk management described in ISO 14971, ISO 62366-1 (Annex A) offers a useful graph highlighting the flow between these two processes.

The PHA is used early in new development programs to identify foreseeable hazards that directly result in harm where the harm can be assessed for severity (e.g., failure to deliver insulin resulting in nonserious or serious hyperglycemia).

These tools can be used to analyze the design, the manufacturing process, and the labeling associated with packaging and patient/customer use (including the instructions for use). In terms of the FMEA tool, there are three core evaluations:

- dFMEA (design): assessment of core design
- pFMEA (process): assessment of manufacturing
- aFMEA (application) or uFMEA (use): assessment of the use-related safety aspects (including but not limited to instructions for use (IFU))

In general, these methods are used to identify the hazards associated with a given design, design platform, or manufacturing process. Each identified hazardous situation is estimated and evaluated in terms probability of harm (typically a function of frequency of occurrence and detectability) and severity of harm (seriousness of the adverse event) using a numerical ranking system established in the risk management plan (risk policy) using the following ranking system as an example:

- Severity of harm: 1 to 5 where 1 might be "negligible" (e.g., mild discomfort) and 5 might be "severe" (e.g., potential for death)
- Probability of harm: 1 to 5 where 1 might be "improbable" and 5 might be "frequent"

While there are many approaches, one might identify and address risk by describing them in terms of three basic categories (some might argue that green is really an extension of yellow such that all risks should be reduced as far as possible compared to as far as reasonably practical):

- *Acceptable (*e.g.*, green)*: Identified risks, which are deemed to have a positive risk/benefit weighting relative to patient safety. In some instances, it may be required to justify specific green items as having reduced the risks as low as possible based on state of the art technology and/or information provided in the instructions for use (IFU).
- *Justification required (*e.g.*, yellow)*: Identified risks are considered undesirable. If they cannot be reduced as low as possible with state of the art technology and/or the IFU, justification should be brought before the established governance for discussion and documentation with respect to why they are acceptable.
- *Unacceptable (*e.g.*, red)*: Reds must be mitigated. If, however, the anticipated frequency of occurrence is extremely low (possible but not probable), these risks may be deemed acceptable and documented as such.

Refer to Fig. 3 for a tabular representation of how probability of harm and severity of harm could be used to assess risk acceptability using the aforementioned color-coding.

Here, red and yellow items may, depending on the review process and outcome, be deemed acceptable per risk/benefit weightings. Should an identified risk require mitigation, the implemented controls for the risk must be verified for effectiveness.

Risk Acceptability		Probability of Harm				
		1 Improbable	2 Rare	3 Occasional	4 Probable	5 Frequent
Severity of Harm	5 Severe					
	4 Major					
	3 Moderate					
	2 Minor					
	1 Negligible					

Fig. 3 Risk acceptability grid

Ultimately, overall risks are evaluated for acceptability. Again, a thoughtful governance structure can be extremely helpful in navigating these assessments and determinations. Once implemented and controlled satisfactorily, these risks must be monitored (e.g., analysis of complaint and adverse event data). Are there any new hazardous situations that were not foreseen in the risk analysis? Does the probability of occurrence correspond to the initial estimation or are certain hazardous situations occurring more/less frequently? The same process should be applied when a device development program involves clinical evaluations, market research, and other premarket evaluation opportunities.

Processes such as health hazard evaluations (HHE), risk minimization programs, collection of product complaints, and adverse events should be in place to allow for the evaluation and management of product risk. An HHE request typically comes from the manufacturing group in an effort to address a deviation or other "out of specification" conditions whereby a dedicated safety physician can assess whether that condition represents a public health safety concern.

4 Monitoring Processes

While regulations and logistics for monitoring investigational and marketed products may differ, the following capabilities are key components for effective risk management and compliance with regulatory requirements in either scenario (refer back to Fig. 1):

- Device vigilance process
  - Adverse event (AE) database

  - Expedited reporting capability
  - Surveillance and trending capability
- Product complaint process
  - Product complaint (PC) database
  - Product return and root cause investigation
  - Surveillance and trending capability
- Deviations tracking
- Audit findings and responses
- Health Hazard Evaluations (HHE)
- Counterfeiting
- Regulatory inquiries and responses
- Governance

Corrective and preventative action (CAPA) activities arising from product and process monitoring should be used to address:

- Design changes (including labeling and packaging)
- Manufacturing process changes
- Design verification and validation issues
- Effectiveness check outcomes

Figure 4 shows the relationship of the PC and AE monitoring processes and their importance to regulatory compliance, product development, and continuous improvement.

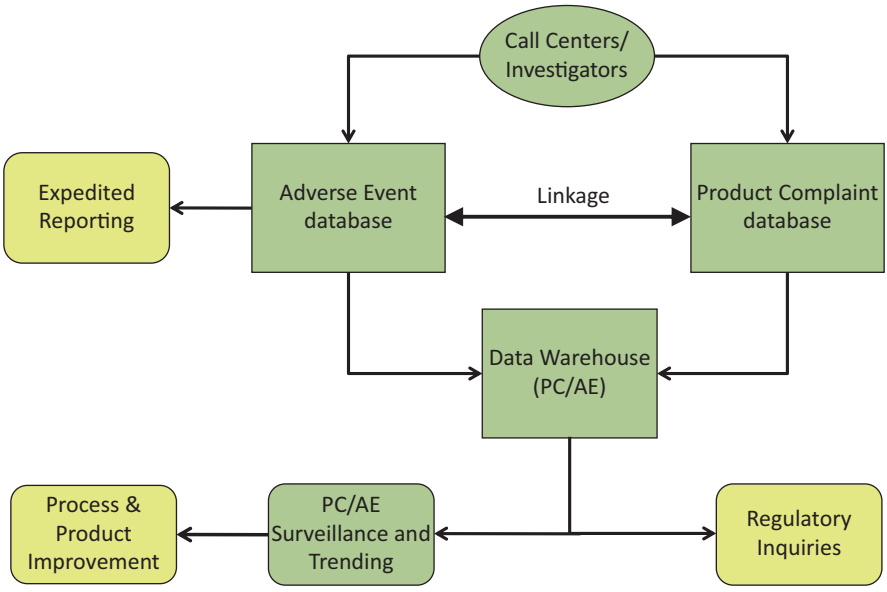

Fig. 4 PC/AE infrastructure model

Depending on the organization, complaint and adverse event group processes may operate out of separate parts of the organization and use multiple databases or they may be fully integrated. Pharmaceutical companies seem to have developed two separate databases, while device companies may have them more integrated (i.e., adverse events might be considered a special type of "medical complaint") from the start. Either way is acceptable as long as it is understood that most safety and quality issues that arise from consumer-based drug delivery systems are expressed in terms of adverse events associated with the medicinal product based on consequences largely due to over-delivery and under-delivery. As such, the adverse event data tends to be "symptomatic" of a potential issue whose root cause likely resides in the device's user interface, core design, IFU, or manufacturing process. Root cause data is likely captured in the quality/complaint group through investigation of returned product, house samples, lot/batch, and manufacturing process documentation. The robust linkage between AE and PC data is vital. Absent that link, context can be lost, making determination of root cause challenging. For example, high blood sugar might be linked to the manufacture of low-potency insulin, patient misuse, or dosing mechanism failure, the same event with three unique root causes. Only AE-PC linkage and formalized communication and cooperation between the safety and complaint groups can unravel the truth behind root cause.

Because these processes are required for both pre- and postlaunch activities, it is also worth considering how risk analysis language (e.g., FMEA descriptors) can benefit monitoring efforts by using similar or transferred FMEA language for the development of the product complaint descriptors. This can facilitate a common thread of risk language across entire product life cycles whereby theoretical frequencies of occurrence from development can easily be updated with complaint data from actual marketed products based on common language developed early on.

Product complaint processes should be constructed to facilitate the following:

- Collection of as much information as possible in a single complaint record.
- Description of complaints in terms of what the reporter said.
- Description of complaints in terms of what the engineers found.
- Mapping of complaint descriptors to regulatory expedited reporting codes (e.g., International Medical Device Regulators Forum (IMDRF) codes) to enable:
  - Expedited reporting
  - Trending and related surveillance methods
- Consideration that complaints of unreturned products are just as important as those with returned devices.
- Awareness of similar incidents based on investigation findings and other reported problems.
- Collection of product-specific use information (if possible) to provide context for complaint investigations.
- Awareness that when use-related issues are reported to a sponsor and a technical professional resolves the issue, the issue should still be captured as a complaint. In this way the use error can be tracked such that updates to the IFU and related training materials benefit all users.

Investigation conclusions should include some basic elements such that expedited reporting and surveillance are informed. Those key elements include:

- Whether the device was returned and evaluated
- Whether the device, upon testing, met its performance specification (e.g., dose accuracy and injection force)
- Complete listing of specific malfunctions and associated adverse events
- Complete listing of other defects that do constitute a malfunction
- Evidence of improper use and storage and associated adverse event
- Whether evidence of improper use and storage constitutes a malfunction such that the instructions for use are insufficient
- Planned or completed corrective and preventative actions CAPA) including dates of implementation

The complaint organization uses this information not only for their internal PC surveillance and improvement efforts but also to assist their safety colleagues in performing PC/AE surveillance, expedited reporting, and documentation with such requirements as Clinical Evaluation Reports (CER) mandated by the new EU Medical Device Regulations (MDR). The EU MDR will replace the EU's current Medical Device Directive (93/42/EEC), which may impact the current understanding of clinical evaluation reports currently described in MEDDEV 2.7/1.

4.1 Clinical Evaluation Reports

Clinical evaluation reports are a European requirement reflecting the collection and analysis of clinical data associated with a medical device or the device constituent part of a combination product for the assessment of whether there is sufficient clinical evidence to confirm compliance with essential requirements for safety and performance when the device is used in a manner consistent with the instructions for use. The evaluation should be appropriate to the device under evaluation, its specific properties, and its intended purpose. Additionally, the benefits and risks of the product should be quantitatively and qualitatively elucidated relative to the targeted population based on current knowledge and the state of the art. The CER is a component of the devices' technical documentation. Given the nature of the document relative to risk/benefit, the CER may have several authors from various disciplines, but the appropriate owner is likely the medical organization. Refer to the current version of MEDDEV 2.7/1 for a discussion of CER requirements (this guidance might change once the EU MDR is fully implemented).

4.2 Details for Device Vigilance System

Device vigilance is the process of collecting, evaluating, investigating, and assessing safety information from adverse events and product complaints for the purpose of trending, reporting, and risk management. Refer to the current version of

MEDDEV 2.12/1 for an overview of device vigilance systems. The device safety files, in addition to the information gathered in collaboration with the complaint group, are used to decide and document whether to report or not. The device vigilance group is charged with conducting surveillance activities, analyzing vigilance data for periodic safety reports, and communicating signals and improvement opportunities to their management as well as the appropriate governance group as noted above. This function, given the confluence of the drug and device expertise, should include safety physicians and engineers; both of whom should be members of the governance function.

Combination products are a challenge for existing regulatory and reporting frameworks, worldwide. However, progress is being made to address the differences in the drug and device constituent parts. In Japan, prefilled pens were once reported as a device but were later changed to be reporting as a drug. Growth hormone reusable pens, for some, have been regulated in the USA as a drug while most everywhere else in the world as a device. As such, reporting requirements can vary greatly across the world in terms of both reportability and timing.

While a clear lack of harmonization persists in terms of regulatory review and expedited reporting, companies must stay current with the evolving requirements and adapt to proactively stay in compliance. Moreover, it is the internal decisions and resultant monitoring processes associated with device surveillance that actually protect patient safety and the business. Flexibility and creativity are critical in creating a surveillance capability that recognizes these differences and similarities between, for example, a prefilled insulin pen and a reusable insulin pen. For the same serious hypoglycemic event (with a PC and/or IUS), a prefilled insulin pen (regulated as a drug) would not report in an expedited fashion because, per drug labeling, hypoglycemia is a known and listed insulin adverse event. A reusable pen associated with a similar PC/AE event, conversely (regulated as a device), would drive an expedited report independent of drug labeling given the more conservative device rules whose threshold is merely the possibility of serious harm (should the event recur with the regulatory assumption that it will).

A robust surveillance system should consolidate the richness of data available across both drug- and device-regulated products and embrace the more important similarities between prefilled and reusable devices. From the pure safety perspective, one should create a system that treats them equally for the purpose of analysis across national borders and patient demographics. That is a key philosophical point to grasp in creating and managing PC/AE processes to monitor device safety and quality; all drug delivery systems are devices, and they require harmonized consideration independent of how they are regulated. This will help “inoculate” a sponsor’s safety system from the inevitable changes to come in how combination products are regulated globally such that accommodation for these changes is more nimble and timely. What follows are several key elements of a robust device vigilance capability.

4.3 Case Management and Expedited Reporting

- Requirements for a *valid* device case are more conservative in that only two inputs are needed (drugs require patient and reporter as well).
  - The device
  - *Potential SAE* or any other adverse event
- Device regulations have additional expedited reporting requirements.
  - Results of investigation and corrective actions
  - Evidence of improper use and storage
- Expedited reporting thresholds are different (see Note below).
  - Prefilled pens are regulated as a drug. In general, drug reporting is based on seriousness and drug event expectancy per the drug label (e.g., severe hypoglycemia is a known and listed event when administering insulin).
  - Reusable devices are regulated as devices where reporting is based on:

    Potential for significant harm (independent of AE)
    SAE with malfunction or malfunction unknown
    SAE with user error (can vary by country)
    Hospitalization (e.g., required Intervention to remove a broken needle from the skin)

Note: it should be noted that the FDA Final Rule for combination product reporting does close the gap between drug and device reporting for the USA – as the USA will have additional reporting responsibilities based on the constituent part of the product (i.e., a product approved as drug or biologic containing a device constituent part will have the typical drug/biologic reporting requirements but will have some additional device reporting requirements (e.g., malfunction reporting, corrections, removals, and 5-day reports)).

Despite the differences in reporting thresholds, it is recommended that combination products with a device constituent part should be managed from both the drug and device perspectives. This creates global consistency for all data by choosing the lowest common denominator, independent of regulatory differences and rules.

4.4 Device Safety Surveillance

A basic device surveillance process is shown in the Fig. 5.

Primary functions of surveillance include:

- Present governance with periodic reviews of device-related serious adverse events and reportable malfunctions.

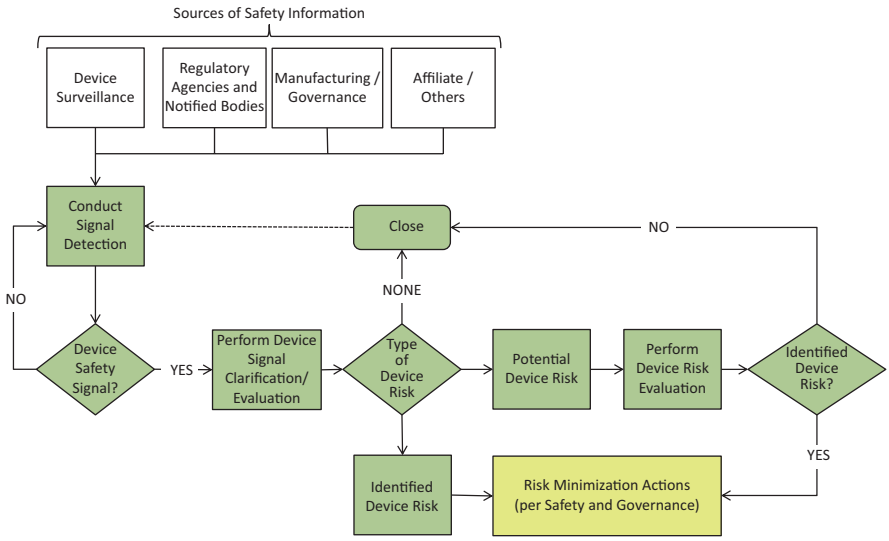

Fig. 5 General device surveillance model

- Conduct device safety trending of reportable events, events that are typically exempt from reporting and events that are usually not reportable.
- Use post-marketing data analyzed to update current risk analysis assumptions (e.g., FMEA assessments of severity or occurrence).
- Use statistically robust analysis tools (e.g., proportional reporting ratio (PRR), empirical Bayes geometric mean (EBGM)) and existing risk assessments to describe and present device signals as either:
  - Potential risks requiring further evaluation
  - Identified risks requiring remediation
- Use these tools to inform governance of process and product improvement opportunities.
- Support clinical evaluation reports (CER), which are performed during the conformity assessment and clinical development processes prior to market approval and continue throughout the product's life cycle as new information (internal and external) becomes available.

4.5 Other Issues Impacting Device Safety

Beyond the day-to-day activities of a device safety organization and its interaction with other elements of the risk management infrastructure, there will always be special design and manufacturing concerns as well as considerations unique to a

given demographic and disease state that requires additional device vigilance capabilities and proactive risk management solutions:

- Use of concentrated drug products in prefilled delivery devices
- Pediatric use of devices
- Device use by the visually impaired
- Electromechanical sophistication for device function and communication
- Connected devices generating concerns for patient privacy and cybersecurity:
  - Malicious hacking intended to harm (e.g., hacking a pump delivery rate)
  - Unauthorized access to cloud data
- Counterfeiting of devices producing lower quality, new failure modes, potentially life-threatening patient safety concerns, and damage to business reputations
- Health Hazard Evaluations (e.g., request from manufacturing for safety review of a specific lot of product)

5 Conclusion

Ultimately, there is much flexibility in constructing a robust quality system that enables development of novel combination products while maintaining compliance with an ever-changing set of global regulatory requirements. An understanding and a commitment to design controls and risk management are critical in producing innovative and safe drug delivery systems that satisfy the needs of the intended users while minimizing malfunctions, medication errors, and product complaints. Competent monitoring processes and a multidisciplinary governance structure providing oversight empower efficient and patient-centric product development while facilitating accountability and management awareness throughout.

Chapter 30
Standards for Injectable Delivery Devices: ISO 11608 Series and Others

Robert R. Nesbitt, Harold K. Yeager, Michael J. Roe, and Niels Hansen

1 Introduction

As may have been evident so far in this book, there are a number of international standards which impact medication delivery systems, in one aspect or another. This chapter provides an overview of the historical origins and evolution of the international standards which specifically address medication delivery systems.

ISO standards are intended to be globally relevant documents which can be used by affected industries and other stakeholders in countries around the world. An effective ISO standard can reduce global trade barriers by defining a common set of design or performance-based guidelines to ensure broader access to quality and safe products and is written to provide guidance without hindering technical progress or innovation.

The standards are intended to be globally harmonized through the broadest international participation as possible. Although ISO standards are voluntary, they are increasingly used in various regions as an efficient way to ensure a safe and effective product.

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The Technical Committees which create and update these standards are comprised of industry and regulatory experts. It is recognized that standards which a Technical Committee develops will have a business impact on firms with products covered by the standard; however the highest priority is placed on patient safety.

Figure 1 provides an overview of many of the standards that developers may need to refer to in supporting the design and development of their medication delivery system.

From a historical perspective, international (ISO and IEC) standards existed for medication product primary containers (e.g., dental cartridges, vials, and syringes) and components (e.g., stoppers, seals), but there were no standards addressing the design and verification of a medication delivery system. However, as medication delivery devices for patient self-administration began to appear in the market, starting with the NovoPen® insulin injection pen device, the lack of standards addressing safety and quality requirements for medications demanding tight dose accuracy (such as insulin) became apparent.

To address this gap, in the early 1990s, under the auspices of ISO Technical Committee 84 (or TC84) – the Technical Committee which is responsible for medication delivery devices ("Devices for the Administration of Medicinal Products and Catheters") – a number of pharmaceutical firms that manufactured and distributed insulin (including Novo Nordisk, Eli Lilly, and Hoechst, the predecessor of Sanofi), as well as a selection of medical device manufacturers (including Owen Mumford, Becton Dickinson, and Disetronic, the predecessor of Ypsomed), assembled to scope and develop an international (or ISO) standard containing requirements and test methods to govern this class of products. The standard that resulted from this effort, **ISO 11608**, has grown to include seven parts and provides broad coverage for injectable medication delivery systems. The following provides the history on the development of this standard and describes the focus of each of the seven parts of this foundational standard.

2 The ISO 11608 Family of Standards – Needle-Based Injection Systems

2.1 ISO 11608 Parts 1, 2 and 3

The standard would govern design and performance verification for multidose injection devices used with a medication container either integrated at the point of manufacture (i.e., prefilled) or provided separately and assembled by the user. The standard which resulted from this effort, **ISO 11608**, was initially focused on the technical limitations of the containers containing the medication. Initially published in 2000, the ISO 11608 series comprised three parts – **Part 1**: "Pen-injectors," **Part 2**: "Needles," and **Part 3**: "Finished Cartridges."

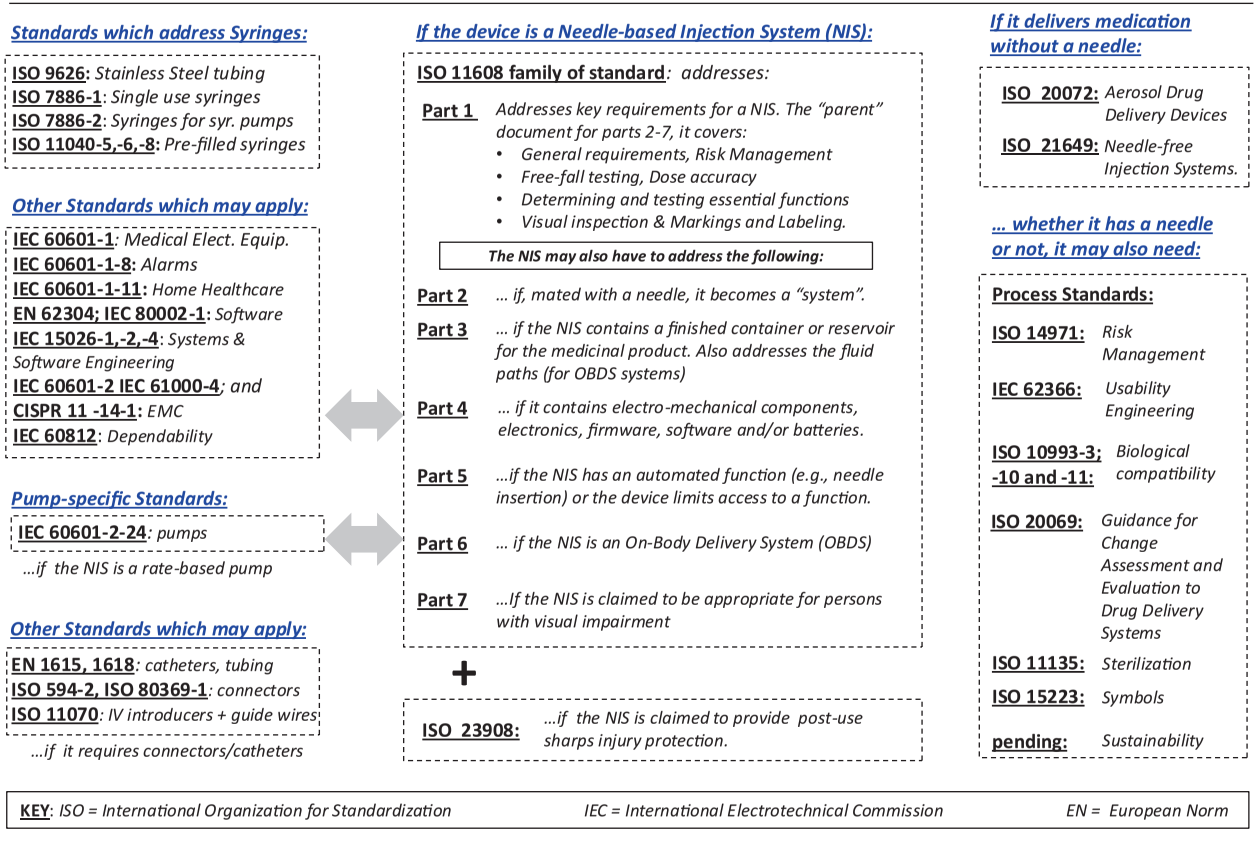

Fig. 1 Standards which impact medication delivery devices

The initial focus was design prescriptive by providing dimensions to ensure interchangeability between pens, cartridges, and pen needles. Although it initially addressed the unique and exacting requirements of insulin delivery, it quickly became the de facto standard for developers, notified bodies, and regulators for most all injectable medication delivery systems for self-administration.

ISO standards are intended to be globally relevant documents which can be used as broadly as possible by affected industries and other stakeholders in countries around the world. An effective ISO standard can reduce global trade barriers by defining a common set of guidelines to ensure broader access to quality and safe products and is written to provide guidance without hindering technical progress or innovation. The ISO standardization process encourages and is valued based on the broadest international participation as possible. Although ISO standards are voluntary, they are increasingly used in various regions as an efficient way to facilitate safe and effective products.

The first versions of ISO 11608 Parts 1, 2, and 3 contained specific dimensional, material, or construction requirements to ensure consistent global use of these products. Such an approach (i.e., design prescription) is used for products like pallets and global shipping containers, where consistently applied dimensions and materials were intended to ensure an interchangeable product. Based on this philosophy, the first version defined and specified a "Type A" device and needle, where specific dimensions were spelled out. This was intended to enable interchangeability across different manufacturer's device system components (i.e., needles), to enable patient convenience in obtaining other brands of replacement needles for their pen injector designed to use Type A components.

However well-intended, the "Type A" designation neither addressed the "system nature" of a medication delivery system (i.e., not every combination of component parts would assure safe and appropriate function) nor the business realities that it necessitated (e.g., manufacturers agreeing to redesign existing products or have knowledge via quality agreements that components were being changed). Therefore, in the update started in 2010, ultimately published in 2012, the standard eliminated the Type A designation and focused on ensuring safety by mandating compatibility testing for component combination labeled for use as part of the system. This represented a significant departure from the design prescription of interchangeability and a move in the direction of performance testing of the finished system. This highlights an important shift in the way in which TC84 standards have developed over time – they increasingly outline performance expectations ("what the system must do"), rather than outline dimensional or material (build) expectations ("how it should look").

Another change impacting ISO 11608 Parts 1, 2, and 3 between the 2000 initial version and the 2012 version was recognizing an increasingly broad definition of what a medication delivery injection system could look like. Although insulin injection devices were initially shaped like writing pens (and were therefore called "pen injectors"), the revision of ISO 11608 (which began in 2010) expanded the terminology used for the delivery system to allow for a broader range of form factors. Thus, the 2012 version referred to these delivery systems as "needle-based injection

systems" (abbreviated as "NIS"). In addition to addressing injection devices which are not "pen-shaped," it also addressed single fixed-dose devices, such as auto-injectors, and the widespread use of prefilled syringes (more on that later).

Finally, a significant area of change between the 2000 and the 2012 versions of ISO 11608 was the statistical basis for acceptance criteria to be used for design verification. The original 2000 version suggested pulling four samples for each dose setting (e.g., min, mid, max) from each of 15 devices to create a representative sample of 60 for each dose setting with which to conduct design verification. However, statisticians from Germany in their DIN international review noted that this approach essentially "underemphasized" inter-device variability, suggesting instead that recording one sample per each dose setting from each of 60 devices would more correctly balance inter- and intra-device variability. This change was incorporated in the 2012 and future versions.

Although the standards referred to in this chapter are global or international ("ISO") standards, various regions develop local versions of these standards based on local regulation. However, some local versions can be significantly different than the global versions on which they are based (an example of this is Japan where ISO 11608 Parts 1, 2, and 3 have been locally modified and published as JSA – JIS T 3226 – 1, 2, and 3). As such, these local versions may not match the global document on which they are based.

As happens with standards in a variety of technology areas, the scope of devices which ISO 11608 Parts 1, 2, and 3 address is broad and continuously evolving. As such, the series has also needed to evolve over time. The graphic below and the details to follow illustrate how ISO 11608 Parts 1, 2, and 3 have been expanded over time to reflect increasingly varied functionality in needle-based injection systems (Fig. 2).

2.2 ISO 11608 Part 4 (2006)

The first new part added to the ISO 11608 family was **ISO 11608 Part 4** ("Requirements and test methods for electronic and electromechanical pen-injectors"). This part, published in 2006, was developed to address requirements applicable for a growing number of needle-based systems which incorporated electronic dose displays and/or electromechanical drive systems. To address requirements not covered in Parts 1, 2, and 3, Part 4 included requirements and test methods applicable to devices with electronics and batteries, such as protection against fluid and dust ingress. Because of the content and in order to not replicate sections of the existing **IEC 60601-1** – ("Medical electrical equipment – Part 1: General requirements for safety, collateral standard, safety requirements for medical electrical systems"), this new Part 4 referred the reader numerous times to the IEC 60601-1 standard.

In the years since the publication of Part 4, TC84 has received feedback from users that the need to refer to IEC 60601-1 created confusion as to how much of

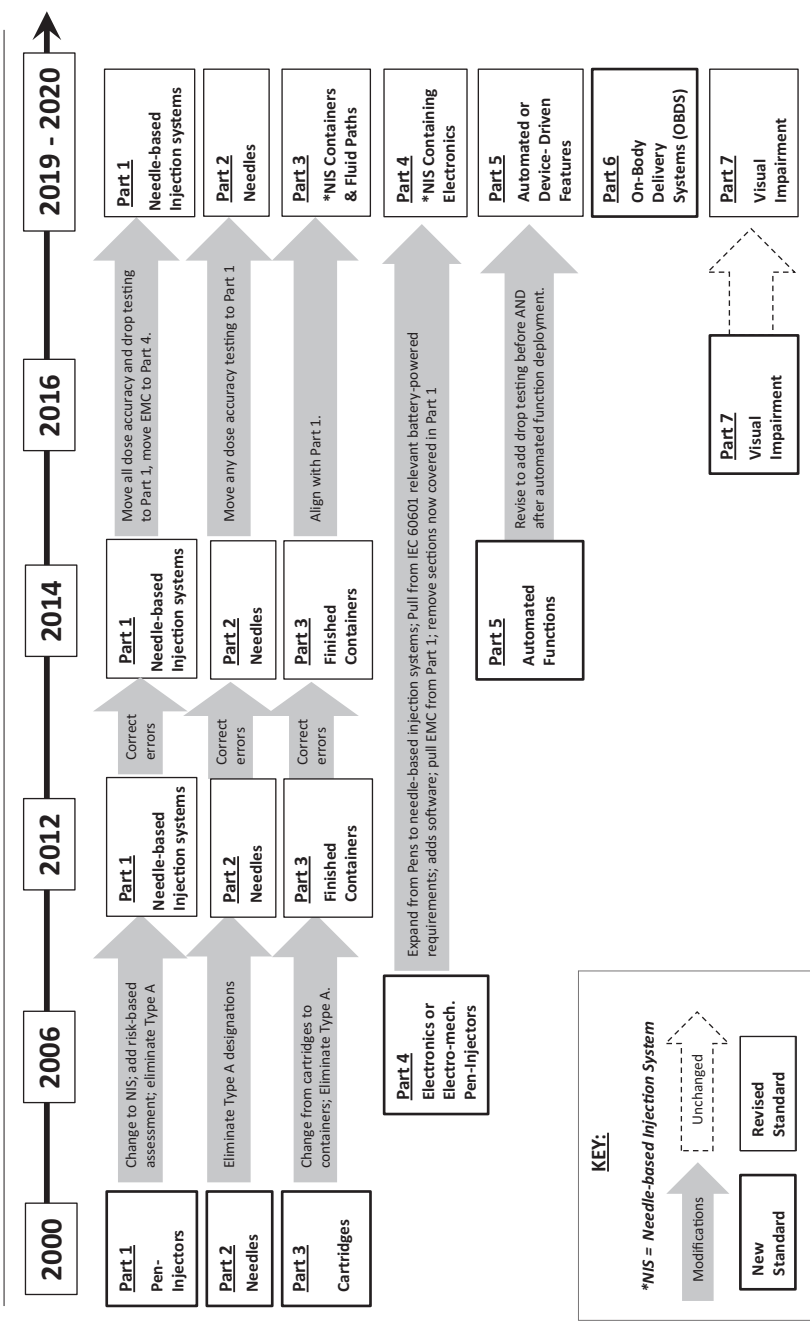

Fig. 2 How the ISO 11608 series has evolved over time

that document (intended to address large and mains-powered medical equipment compared to handheld and battery-powered devices) was applicable to the patient self-administered delivery systems described in ISO 11608. To address this confusion, the latest revision of Part 4 (to be published in 2019 or 2020) more clearly spells out (and includes in its requirements) exactly what is expected for performance of the electronics component of needle-based injection systems covered in ISO 11608.

2.3 ISO 11608 Part 5 (2012)

Whereas ISO 11608 Parts 1, 2, 3, and 4 were principally created to address cartridge-based systems, (whether multi- or single-dose), TC84 recognized that this did not include delivery systems which incorporated pre-filled syringes (governed by standards like ISO 11040-8, "*Prefilled syringes Part 8: Requirements and test methods for finished prefilled syringes*," which address dose accuracy) whose operation typically includes by some level of automatic needle insertion, delivery, retraction, or related functions. These systems have historically been termed "auto-injectors." To address these gaps, TC84 chartered a working group to develop an "auto-injector" standard.

The initial phase of any standard development process is scoping the effort – what content is to be included in the body of the document. One of the challenges in establishing that scope – which would include a pre-filled syringe – was that other existing (non-TC84) standards address syringe accuracy (as noted above). Therefore, such an "auto-injector" standard could not impose additional requirements on dose accuracy for a standalone pre-filled syringe (PFS) – but it would need to address the "system" performance (e.g., drop test) of the PFS with automated functions.

Further complicating the scope of such an "auto-injector" standard was that most variable-dose insulin injection devices (the impetus for ISO 11608) were generally manually actuated (e.g., thumb-driven) and ISO 11608 Parts 1, 2, 3, and 4 did not specifically address automated functions (e.g., "spring-driven" insertion of the needle or delivery of the medication contents).

The decision was made to change from creating an "auto-injector standard" to creating a new sub-part of the ISO 11608 series, which would address *any* automated function – whether dosing, needle insertion, needle removal, etc. The standard which resulted – **ISO 11608 Part 5** ("Needle-based injection systems for medical use – Requirements and test methods Part 5: Automated functions"), published in 2014 – addresses any automated feature of a medication delivery device covered by other parts of ISO 11608 (even pre-filled syringe-based systems). In the latest recent revision (targeted for publication in 2019 or 2020), the scope has been expanded to include features by which the device itself accomplishes a task which would otherwise be accomplished by the user. These are referred to as "device-driven features." For that reason, the revised version is called "Part 5: Automated or Device-driven features."

2.4 ISO 11608 Part 7 (2016)

Through the years, members of the TC84 committee recognized that the majority of medication delivery devices are labeled "not for use by visually impaired without assistance" – and even if they were, there were not international standards that addressed minimum performance requirements for, and test methods to confirm the effectiveness of, these devices in that demographic. However, injectable medication delivery devices are regularly used by persons with visual impairments (e.g., diabetes can adversely impact vision). Acknowledging this "gap," TC84 engaged a team to develop design requirements specific to those devices which are claimed to be appropriate for use by persons with visual impairment. The design intention in this standard was to provide guidance that would allow a device to be designed for *all* patients – not just for persons with visual impairments, which might otherwise result in a special, limited-use device that was cost-prohibitive and defeat the purpose of such a standard. The result of this effort is **ISO 11608 Part 7** ("Needle-based injection systems for medical use – Requirements and test methods – Part 7: Accessibility for persons with visual impairment"), published in 2016.

2.5 ISO 11608 Part 6 (Planned for 2020–2021)

As more parts of ISO 11608 were developed to address handheld, patient self-administered medication delivery devices, TC84 recognized that there was not yet a standard to address a new, emerging class of product – delivery systems intended to deliver a discrete volume of medicinal product by placing the device on or close to the body when delivery required more than 10–15 seconds, making handheld approaches ergonomically challenging.

Although such systems may resemble a "pump" and may informally be referred to as such, TC84 recognized that the key therapeutic drivers of these systems are different than those in true pumps. The foundation of an infusion pump is that *delivery rate* is the key to therapeutic efficacy and safety (e.g., insulin delivered in units/minute). Such systems are governed by international standards such as **IEC 60601-2-24** – ("Medical electrical equipment Part 2-24: Particular requirements for the basic safety and essential performance of infusion pumps and controllers").

As the therapeutic driver of this new class of body-worn medication delivery devices is volumetric accuracy (and not rate), neither the collective ISO 11608 series (Parts 1, 2, 3, 4, and 5), which addressed "handheld" delivery of a fixed volume, nor the IEC 60601-2-24, which addressed continuous "basal-bolus" delivery, would appropriately address the needs of "body-worn," fixed-volume medication delivery devices where rate impacts only tolerability or comfort.

To distinguish these body-worn medication delivery systems from pumps, TC84 created a new term to describe them – "on-body delivery systems" (or OBDS). And, to address the unique requirements of this class of delivery system, TC84 created

ISO 11608 Part 6 ("Needle-based injection systems for medical use – Requirements and test methods – Part 6: On-body delivery systems") which will be published in 2021.

As Fig. 1 illustrates, there are a wide variety of other international standards which address medication delivery systems outside of the ISO 11608 series of standards. An overview of some of those standards follows.

2.6 ISO 23908 – Post-Use Needle Stick Protection (2012)

The ISO 11608 standards addressed so far in this chapter are all needle-based injection systems. Recognizing the growing health hazards of post-use needle stick injuries and a limited number of standards which addressed the design of protection devices to prevent or minimize the risk of post-use needle sticks, TC84 created a standard to address this issue as well. Published in 2012, **ISO 23908** ("Sharps injury protection – Requirements and test methods – Sharps protection features for single-use hypodermic needles, introducers for catheters and needles used for blood sampling") provides specific design requirements and test methods to demonstrate that any system which claims to provide post-use needle-stick protection can be demonstrated to do exactly that which is claimed, sharps protection.

Although the discussion so far has focused on needle-based injection systems, there are also international standards which address medication delivery by use of technologies that do not utilize a needle. An overview of some of those standards follows.

3 Non-needle-Based Medication Delivery Systems

As the scope of TC84 includes standards that ensure safe and effective use of *all* "devices for the administration of medicinal products" (not just "needle-based"), TC84 has also developed standards which address systems which deliver the therapeutic without the use of a needle. To that end, TC84 has developed two such standards.

3.1 ISO 21649 – Needle-Free Delivery Systems (2006)

To address the diversity of systems that deliver medication product through the skin without the use of a needle, TC84 developed **ISO 21649** ("Needle-free Injectors for medical use – Requirements and test methods"). This standard, which outlined requirements and test methods for needle-free (or "jet-injector") delivery systems, was published in 2006.

Whereas the ISO 11608 series requires product performance to be verified through confirmation of volumetric delivery accuracy, needle-free system performance could not be guaranteed using similar methods alone. For example, without a requirement to ensure the desired volume penetrated the skin, accuracy alone might have resulted in wet injections. Therefore, ISO 21649 established performance-based requirements and the development of (in vitro) engineering surrogates which could be used to demonstrate effective delivery without the need for additional clinical studies once mapped to these surrogates (e.g., depth and dispersion characterization of delivered volume). By offering the freedom to define the desired "performance profile" and a means to demonstrate compliance, rather than simply outlining dimensional or "build" specifications, designers can iterate with new technology and designs, provided they demonstrate the final, verifiable, performance, and human factor results.

3.2 ISO 20072 – Aerosol Drug Delivery Devices (2009)

Just as TC84 addressed injections without a needle, it sought to similarly address medication delivered via the patients' respiratory tract rather than through the skin. Therefore, TC84 developed a standard to address requirements and test methods for aerosol drug delivery devices (commonly called "inhalers"). These systems are addressed in **ISO 20072** ("Aerosol drug delivery device design verification – Requirements and test methods"), published in 2009. This effort was initiated when major insulin manufacturers began to develop inhaled insulin delivery systems (i.e., systemic delivery via inhalation), which drove the urgency for creating a standard to develop performance requirement for such systems (e.g., dose accuracy).

4 Other Important ISO and IEC Standards Which a Medication Delivery System Developer Needs

Each of the standards referenced above encourages the reader to use a risk-based approach based on **ISO 14971** ("Medical Devices – Application of Risk Management to Medical Devices") to ensure that the design is appropriate for delivery of the intended therapeutic to the intended patient population and in the context of use that they are likely to experience. Although in the early 2000s, the ISO 11608 standards were created to address the unique requirements of insulin and hGH delivery; they have evolved over time to enable use with a variety of therapeutics, including ones with wider therapeutic windows (for which the risks of dose variability may be lower).

In addition, these standards are designed based on the assumption that the user will utilize a process which will confirm that the design meets the user needs. All of

these standards assume that the assessment of usability by the ultimate user of the system will be based on the application of **IEC 62366** ("Medical Devices – Application of Usability Engineering to Medical Devices").

Finally, TC84 recognized the need to address how best to evaluate changes to medication delivery systems that should be expected throughout their life cycles. Numerous things can drive a change in a delivery system, including changes in materials of construction, product adverse event or complaint data, production processes, or material suppliers. So, for any change that could impact the medication delivery system's safety or efficacy that occurs any time from initiation of pivotal or registration clinical study all the way through to the end of commercial supply, TC84 has developed **ISO 20069** ("Guidance for assessment and evaluation of changes to drug delivery systems") to assist companies in properly assessing and evaluating such changes.

5 Conclusion

As this chapter has illustrated, there are a number of relevant standards which address medication delivery devices. Although this chapter has focused primarily on those that fall under ISO Technical Committee (TC) 84 and the ISO 11608 family (for needle-based injection systems), there are other standards which may help inform the developer.

Finally, as a reminder, standards are not regulatory requirements, but guidance documents which are increasingly used by regulatory agencies as a basis for their review of medication delivery systems.

Chapter 31
Human Factors Regulations and Standards in Combination Product Development: IEC 62366 and FDA Guidance Documents

Melissa R. Lemke and Deborah Billings Broky

1 Introduction to Human Factors

FDA defines human factors engineering as "the application of knowledge about human behavior, abilities, limitations, and other characteristics of medical device users to the design of medical devices including mechanical and software driven user interfaces, systems, tasks, user documentation, and user training to enhance and demonstrate safe and effective use" [1], and IEC 62366-1 defines usability engineering similarly [2]. Human factors applied throughout combination product development can make the pre-market submission process more efficient and cost-effective while benefiting end users and sponsors with optimized safety and effectiveness. A thorough human factors process can help sponsors save time and budget, including at the end of the pre-market process when regulators are making decisions about the safety and effectiveness of a product for ultimate approval. Human factors can be invaluable to consider post-market as well for two main reasons: (1) human factors best practices can be used to address post-market issues that could arise over time (e.g., design deficiencies that lead to a corrective and preventive action (CAPA)); and (2) collecting post-market data around actual use of the product can provide helpful use-related design insights that can be used to develop an improved version of the product or subsequent new products.

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1.1 Scope of Application

Human factors can benefit development teams working to create any type of combination product, such as auto-injectors, prefilled syringes (and safety syringes), inhalant devices, implanted delivery systems, transdermal systems, etc. There are different human factors tools, methods, and strategies that can be used to inform user interface development decisions during initial concept development, generation of early prototypes, late-stage optimization phases, final validation testing, and post-market surveillance and product updates.

FDA's definition of combination products goes beyond "obvious" devices such as auto-injectors and inhalers that deliver integrated drug therapy to users (i.e., products that have two or more regulated components such as drug/device or drug/device/biologic that are physically or chemically combined and produced as a single entity, per 21 CFR 3.2(e)(1)). From a regulatory perspective, combination products also include co-packaged kits (per 21 CFR 3.2(e)(2)) and cross-labeled products that are not co-packaged but labeled to be used together in order to achieve the intended therapeutic effect (see Fig. 1 for definitions of these products according to 21 CFR). Note that while US FDA clearly defines what it considers a combination product, EU notified bodies recognize combination products somewhat differently (see chapters 27, 28 and 29 in this book for additional details). An example of a co-packaged kit is a new formulation of liquid medication in a bottle and the associated dose administration tool that is already available on the market (e.g., an oral syringe

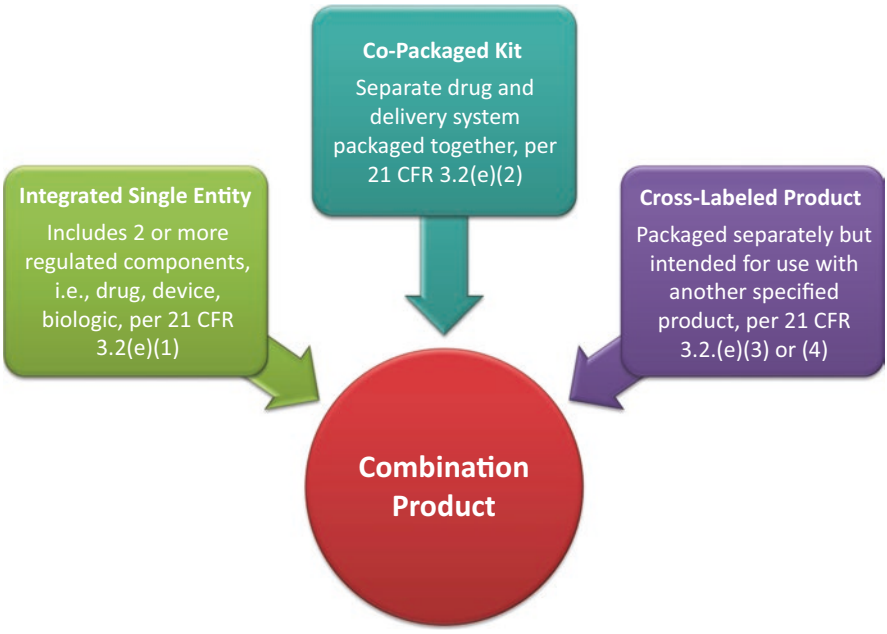

Fig. 1 According to 21 CFR 3.2(e), a combination product includes integrated single entities, co-packaged kits, and cross-labeled products, which are defined here

or medication cup) combined into the same patient packaging that is dispensed from a specialty pharmacy. An example of a cross-labeled product is an insulin pump or a pen injector that requires a separately packaged insulin cartridge and is intended to only be used with specific insulin, which is referenced by brand in the product labeling [3].

2 Key US and International Guidance

US and international human factors industry standards and guidance documents have become more harmonized in recent years. This consistency in guidance principles and recommendations makes global development and meeting different regulatory requirements more streamlined from a human factors perspective.

2.1 US Guidance

In the United States, there are several key guidance documents to consider, including the references outlined in Table 1. This list is not exhaustive, and additional references that may be applicable to combination product development can be found in the chapter's reference list. FDA continuously publishes new and updated guidance documents that may be relevant to different types of combination products, so it is important to regularly check for applicable guidance. The following websites are good resources to check periodically to ensure the most recent US FDA-recognized industry standards and FDA guidance documents are considered:

- *AAMI industry standards updates*: http://www.aami.org/newsviews/content.aspx?ItemNumber=2704
- *Recent FDA final medical guidance documents*: https://www.fda.gov/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm418448.htm
- *Recent FDA draft medical guidance documents*: https://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm407274.htm

CDER's Definition of Critical Tasks

All of the FDA guidance documents rely on the sponsor accurately identifying use-related critical tasks, which provides the framework for human factors validation testing to demonstrate that the combination of the product user interface is safe and effective for use by the intended users, uses, and environments. CDER defines critical tasks as, "user tasks that, if performed incorrectly or not performed at all, would or could cause *harm* to the patient or user, where harm is defined to include compromised medical care" [7]. Note that this definition is slightly different from CDRH's

Table 1 Key US guidance for incorporating human factors in the development of combination products (also included in reference/resource list)

Standard/guidance	Purpose
FDA CDRH's final guidance: *Applying Human Factors and Usability Engineering to Medical Devices* that was issued on February 3, 2016 [1]	***General guidance for all medical devices submitted to FDA***: Intended to guide industry on appropriate human factors and usability engineering processes to optimize device user interface and increase the likelihood that new medical devices will be validated as safe and effective for the intended users, uses, and use environments.
ANSI/AAMI HE75:2009/(R) 2013, *Human Factors Engineering – Design of Medical Devices* [4]	***General industry standard for all medical devices submitted to FDA:*** Intended as a reference for human factors principles, management of use error risk, design considerations, and human factors solutions.
FDA CDRH's draft guidance: *List of Highest Priority Devices for Human Factors Review* that was published on February 3, 2016 [5]	***General industry standard for all pre-market medical devices submitted to FDA (i.e., PMA, 510(k)):*** Indicates which device types should have human factors data included in the pre-market submission.
FDA CDER's draft guidance: *Comparative Analyses and Related Comparative Use Human Factors Studies for a Drug-Device Combination Product Submitted in an ANDA* that was published in January 2017 [6]	***Specific to abbreviated new drug applications (ANDAs):*** Intended to guide industry on comparative analyses to determine whether or not human factors data are required for a particular submission.
FDA CDER's draft guidance: *Human Factors Studies and Related Clinical Study Considerations in Combination Product Design and Development* that was published in February 2016 [7]	***Specific to combination products:*** Intended to guide industry on the principles of human factors studies and considerations for human factors methods during the development of combination products.
FDA CDER guidance: *Safety Considerations for Product Design to Minimize Medication Errors* that was published in April 2016 [8]	***Specific to prescription and nonprescription drugs and biologics regulated by CDER:*** Intended to guide industry on best practices that lead to improved drug product and container closure design.
FDA CDER *Guidance for Industry: Safety Considerations for Container Labels and Carton Labeling Design to Minimize Medication Errors* that was published on April 2013 [9]	***Specific for container and carton labeling for prescription drug and biologic products:*** Includes guidance on design of container and carton labeling that can help reduce medication errors.
FDA CDER draft guidance for industry: *Considerations in Demonstrating Interchangeability with a Reference Product* that was published in January 2017 [10]	***Specific for therapeutic protein products:*** Intended to guide industry in demonstrating that a proposed therapeutic protein product is interchangeable with a reference product for the purpose of submitting a marketing application or supplement under section 351(k) of the Public Health Service Act (PHS Act) (42 U.S.C. 262(k)).

(continued)

Table 1 (continued)

Standard/guidance	Purpose
FDA CDER draft guidance for industry and FDA staff: *Contents of a Complete Submission for Threshold Analyses and Human Factors Submissions to Drug and Biologic Applications* that was published in September 2018 [11]	***Specific for drug and biologics products:*** Guidance on conducting threshold analyses for human prescription drug products, including biologics, that are subject of an investigational new drug application (IND), a new drug application (NDA), a biologics license application (BLA), or an abbreviated new drug application (ANDA), as well as human nonprescription drug products that are subject of an IND, NDA, or ANDA.
FDA CDER's *Labeling for Biosimilar Products: Guidance for Industry* that was published in July 2018 [12]	***Specific for proposed biosimilar product labeling:*** Includes guidance and considerations for biosimilar product labeling content.
FDA CDRH's *Guidance for Industry and FDA Staff: Technical Considerations for Pen, Jet, and Related Injectors Intended for Use with Drugs and Biological Products* that was published in June 2013 [13]	***Specific for pen, jet, and related injectable therapies:*** Includes technical and use-related information to consider during product development and submissions, including human factors design considerations.

definition of a critical task (i.e., failure on tasks that may lead to *serious harm*). CDER's definition is broader by including "harm" instead of "serious harm" as a possible outcome for task failures, which often translates to a higher number of tasks that are categorized as critical for drug delivery products. For example, a task to remove the needle cap from a pen injector may "only" cause a missed dose or delay in therapy. This pen injector may be intended to administer a drug that is not life critical. However, removing the needle cap likely would be categorized as a critical task because the combination product is intended to be used by patients to successfully self-administer a medication (and without being able to remove the needle cap, the patient would experience compromised medical care). Even if a one-time event with a combination product does not lead to patient injury or hospitalization, a missed dose or incorrect dose of medication could still be concerning to the agency. Critical tasks should be categorized based on the risk profile of each specific product, keeping in mind that CDER views any task associated with harm, including compromised medical care, to the intended patient or user as a critical task. Task criticality should focus on severity of harm related to potential use errors, not on Risk Priority Number (RPN) or frequency/likelihood of occurrence [14]. See Fig. 2 for examples of critical tasks for combination products found in CDER draft guidance [7].

Additional detailed examples of critical tasks, possible task failures and use errors, and possible hazards, harms, and results from failures and use errors can be found in Appendix A of CDER's Guidance on Combination Products [7]. Note that these examples can inform sponsors about which tasks CDER typically expects to

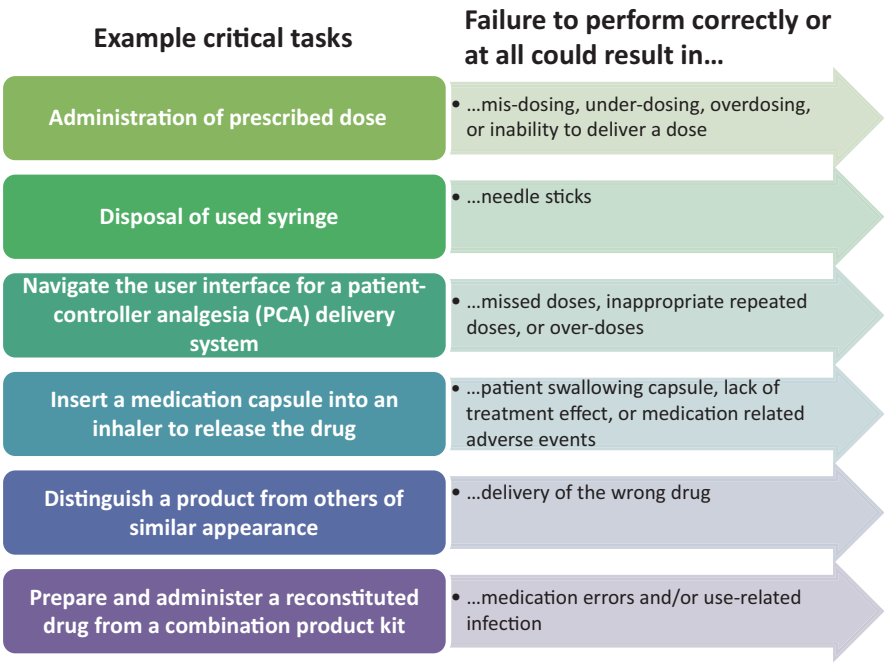

Fig. 2 Examples of critical tasks for combination products, outlined in CDER draft guidance [7]

be critical, but this does not represent an exhaustive list. Sponsors should identify all critical tasks relevant for their specific combination product, which may not necessarily be included in CDER's list of examples.

The Division of Medication Error Prevention and Analysis (DMEPA) within CDER will lead the review of human factors submissions for drug, therapeutic biologics, and combination products. The mission of DMEPA is "to increase the safe use of drug products by minimizing use error that is related to the naming, labeling, packaging, or design of drug products" [15]. To reduce risks related to failed or delayed submissions, FDA encourages sponsors to communicate regularly with the agency, including during the human factors process so that questions about strategies and validation protocols can be answered prior to conducting the validation study (instead of after a sponsor has completed an expensive and time-consuming human factors validation study that does not align with FDA's expectations). After a human factors submission, sponsors may receive requests for information from DMEPA, as well as feedback on Instructions for Use (IFU) and other product labeling that may include suggested revisions from DMEPA or the patient labeling group.

FDA reviewers have outlined some of the most common human factors and usability concerns uncovered during FDA reviews of human factors data in submissions, which provide valuable insight for sponsors [16] (See Fig. 3).

1. Human factors data are needed but not provided by the sponsor;
2. No human factors data were collected prior to the validation testing, leading to unexpected use-related problems during validation; sponsor attempts to justify the problems without adequately addressing them through the design process;
3. Sponsor does not effectively follow-up on residual risk analysis of performance failures due to design issues (e.g., blaming the users instead of implementing necessary design mitigations);
4. Tasks associated with use-related hazards are not identified or properly categorized, testing is not clearly related to critical tasks, relationship between testing and risk analysis is not clearly defined;
5. Potential use errors are omitted or not adequately described;
6. Subjective data from participants are not systematically collected and included in the root cause analysis;
7. Validation testing does not include participants representative of the intended user population;
8. Checklists and/or rating scales are the only data collected; and
9. Task selection and performance expectations are not based on comprehensive analysis of use-related risks.

Fig. 3 Nine common human factors submission concerns cited by FDA reviewers (Adapted from Ref. [16])

2.2 International Guidance

International guidance from notified bodies in the global market provide information for applying human factors outside of the United States; however, FDA recognizes many international standards and guidance documents to support US submissions as well, as described in Table 2. Note that US FDA guidance and international standards are aligned in many ways. For example, similar to US FDA guidance on identifying critical tasks, international standards also require identification and descriptions of hazard-related use scenarios, use errors, and all tasks (including sequences and severity of harm), which are integral for usability engineering processes and to support the determination of which tasks to include in a summative evaluation [2].

Similar to US guidance, international standards are periodically updated or replaced. The following websites can be used to subscribe to newsletters and notifications of new or updated standards:

- *IEC updates*: "Just published" updates: http://www.iec.ch/subscribe/
- *ISO newsletter*: https://www.iso.org/news_index.html

Table 2 Key international standards and guidance for incorporating human factors in the development of combination products (also included in reference/resource list)

Standard/guidance	Purpose
IEC 62366-1:2015—*Medical Devices Part 1: Application of Usability Engineering to Medical Devices* [2]	This process standard "strictly focuses on applying the usability engineering process to optimize medical device usability as it relates to safety." The standard notes that, "If the usability engineering process detailed in this International Standard has been complied with, then the usability of a medical device as it relates to safety is presumed to be acceptable, unless there is objective evidence to the contrary." *This is an FDA-recognized standard, applicable to all medical devices*: Identifies the process for a sponsor to analyze, specify, develop, and evaluate the usability and safety of a medical device that involves assessing and mitigating use-related risks.
IEC/TR 62366-2:2016—*Medical Devices Part 2: Guidance on the Application of Usability Engineering to Medical Devices* [17]	This technical report "contains background information and provides guidance that addresses specific areas that experience suggests can be helpful for those implementing a usability engineering (human factors engineering) process both as defined in IEC 62366-1:2015 and as supporting goals other than safety." *This is an FDA-recognized standard, applicable to all medical devices*: Provides additional details and context to the usability engineering (human factors engineering) process as described in IEC 62366-1:2015.
IEC 62366-1: 2015, *Annex C: Evaluation of a User Interface of Unknown Provenance (UOUP)* [18]	As part of the usability process standard IEC 62366-1, Annex C "was created in recognition of the fact that many manufacturers will be interested in applying the tools defined in this standard to user interfaces or parts of user interfaces that have already been commercialized prior to the publication of this edition of this standard. Such user interfaces or parts of user interfaces were not developed using the processes of IEC 62366-1 and as a result are of unknown provenance with respect to these processes." Annex C provides an alternate process specifically for user interfaces of unknown provenance. *This is an FDA-recognized standard specific to legacy medical device products that are being revised*: Annex C contains information for sponsors who are making changes to approved legacy products or user interfaces that already included human factors data as part of the original submission or did not undergo human factors testing because requirements were not in place at the time.
ISO 14971-1:2007 (R) 2016, *Medical Devices: Application of Risk Management to Medical Devices* [19]	This standard "specifies a process for a manufacturer to identify the hazards associated with medical devices, including in vitro diagnostic (IVD) medical devices, to estimate and evaluate the associated risks, to control these risks, and to monitor the effectiveness of the controls." *This is an FDA-recognized standard, applicable to all medical devices*: Provides details about risk management documentation, including information about developing use-related risk analyses (URRAs).

3 Expectations for Human Factors Data in Submissions

Requirements for inclusion of human factors data in combination product submissions have continued to expand over the past several years for both US and international health authorities, and there is a growing expectation that sponsors should conduct a human factors validation study (summative study) to demonstrate safe and effective use of the combination product as part of their submission. A human factors validation study differs fundamentally from a clinical study or trial in several ways, as described in Table 3.

When submitting human factors data as part of the product submission, sponsors should pay careful attention to guidance on the type of information and data analyses that are expected by the regulators. International standards for usability engineering processes specify requirements that largely align with FDA requirements, for example, sponsors are expected to complete comprehensive use-related risk analyses, conduct formative usability evaluations on product design(s), conduct summative validation testing, and conduct a residual risk assessment to determine whether additional design mitigations are necessary [2, 17].

4 Overview of Human Factors Process

Detailed documentation of the human factors process in the design history file (DHF) of a combination product is very important! A sponsor's documentation will provide evidence that the sponsor followed human factors best practices and guidelines to consider the needs and safety of the end users and implemented user interface design mitigations to reduce or eliminate use-related risks. While human factors documentation may not always be a requirement for a submission, it can be very useful if there are subsequent requests for data or justification for certain design elements.

The human factors process for a combination product usually culminates in a validation study once the product is finalized in order to demonstrate that the user interface can be safely and effectively used by intended users without serious use errors or problems [7]. However, human factors can (and should) be applied over the entire product development life cycle in order to optimize the user interface and design out potential problems before design elements are locked and making changes can be costly. Human factors formative evaluations conducted at key times during product development can provide crucial insight for the development team and can lead to an optimized interface.

The human factors process first involves defining intended use(s), users, and use environments for the product. A use-related risk analysis (URRA) should then be systematically developed to identify all use-related risks associated with the user interface of the combination product. FDA considers the user interface to include all components of the product that the user will interact with, including packaging,

Table 3 Differences between major clinical studies and human factors validation testing

	Major clinical study or trial	Human factors validation testing
Purpose	Intended to show evidence of safety and effectiveness of a product for a specific proposed indication (e.g., adequate and well-controlled studies and long-term extension studies [7]).	Intended to demonstrate that the user interface can be used by representative users without serious use issues under expected use conditions.
Timeline	Involves extensive planning prior to initiation, and the trial can extend over long periods of time.	FDA suggests that ideally a human factors validation study should occur before Phase 3 clinical trials [20]. However, in general, a human factors validation study must be completed and included as part of the overall product submission. The human factors validation involves careful planning but can typically be conducted in several months (including study planning, conduct, data analysis, and reporting).
Size	Larger scale involving high numbers of actual users in order to reach statistical significance for outcome data.	Relatively small number of participants who represent actual users (e.g., 15 users per unique user group is recommended by FDA for human factors validation testing).
Methodology	Seeks to demonstrate safety and efficacy of new drug and device products and therapeutic outcomes. Often includes specific user training, supervision, and scheduled follow-up for patients on how to use the product and to collect safety, efficacy, benefits and adverse effects of the drug.	Seeks to demonstrate simulated use of product under representative use conditions that should be sufficiently realistic so results are generalizable to actual use. Note that in some circumstances, actual use is required for a human factors validation study.[a] Participants should use the product independently, without assistance. If users will have access to labeling and/or training in actual use, the labeling and/or training is also made available during human factors validation testing. Users are not instructed to use labeling but may independently choose to use labeling if they wish.
Data emphasis	Gauges drug and device effectiveness and safety, does not generally focus on user-device interactions and associated use errors, and relies on *quantitative* analyses of safety and effectiveness of the product.	Both *quantitative* performance data and *qualitative* subjective feedback are collected, and FDA places emphasis on qualitative analyses. Data are analyzed to show that the design of the user interface has reduced or eliminated use-related hazards to acceptable residual risk levels such that the benefit of using the combination product outweighs the residual risk associated with using it.

[a]Actual use testing methodology is used when it is impossible or extremely difficult to test a product under simulated conditions (e.g., prosthetic limb). An actual use human factors validation study can be conducted as part of a clinical study. For more information, see Ref. [1]

labeling, instructional materials, physical devices, and training [1]. Likewise, IEC 62366 defines user interface as the "means by which the user and the medical device interact" which involves all components of the device that a user interacts with, including any accompanying documentation and medical device-specific training [2]. Thus, the sponsor should think carefully about how the intended user is expected to interact with all the elements of the product's user interface and how the product design eliminates or reduces risk to prevent adverse events, medication errors, and other serious issues. The main steps involved in the human factors process for combination products are outlined in the following sections.

4.1 Identify Users and Use Environments

Sponsors should first define who the intended users are, where users are expected to use the combination product (i.e., use environments), and what drug/device/biologic elements the users will interact with to use the product (i.e., user interface components). Intended users should be grouped into distinct categories if user characteristics are expected to impact use of the product. Sponsors should ask themselves: Are there any tasks, abilities, limitations, education, experience, or job roles that are unique to a specific group of users? For combination products, there are usually two distinct user groups including healthcare professionals (HCPs) who use products to administer medication to patients and/or train patients on use of a product and lay users (non-healthcare professionals) who use the product for self-administration or who administer the product to someone as a caregiver (e.g., family member [7]). In addition, within each HCP and lay user group, a sponsor may identify additional characteristics (or subgroups) that can impact use of the product. For example, if experience with injectable medications could impact lay user behavior with a new auto-injector, consider separating users into two groups: (1) injection-naïve lay users and (2) injection-experienced lay users. In some cases experienced users may even need to be further categorized into subgroups such as syringe injection-experienced and pen injection-experienced if this difference in previous experience is found to have an effect on the safe and effective use of the product. Note that the way the sponsor chooses to identify and categorize user groups is very dependent on the intended use of the product, use-related tasks, and user capabilities and limitations. Thus, it is always a good idea to systematically consider and document user characteristics to allow the sponsor to develop a strong rationale for user groups and subgroups that can be justified for human factors validation study-related decisions. Sponsors also should consider consulting with FDA on user group designations, because the agency often has expectations related to user groups.

Identifying intended use environments is another precursor to developing the use-related risk analysis (URRA). The sponsor should consider all environments where users may choose to use the combination product, for example, in clinical settings or nonclinical settings such as home environments or public places. Characteristics of the use environments such as noise, lighting, temperature, degree

of shared space (i.e., number of people in the use environment), and layout can impact how users interact with the product. If the combination product is intended to be used for emergency situations, it is important to consider aspects of environments that could impact use of the product, such as background noise, heavily or sparsely populated public areas, schools, patient inability to self-administer therapy, and bystander intervention to name a few. In these cases, bystanders may also be considered as intended users because in emergency situations those people may responsible for administering life-saving medication.

4.2 Identify User Interface Components

A combination product by definition is a combination of any of the following: drug, device, biologic. Because the parts of a combination product for the US market keep their separate regulatory status as drug, device, or biologic [7], it is important to thoroughly define all components of the user interface and describe how users are expected to interact with each part. This identification of user interface components becomes important for the use-related risk analysis (URRA), which systematically breaks down the user-device interactions and assigns risk categorizations to them. The physical drug, device, and biologic components are obvious parts of the user interface, but the sponsor should not overlook the user interface elements of product training and instructional materials (e.g., IFU, product packaging, product help line).

Drug/Device/Biologic Components

Identify the physical parts of the product that the user will interact with, including drug (e.g., oral capsule, liquid medication), device (e.g., software, drug delivery mechanisms), and/or biologic (e.g., drug product that is approved to treat rheumatoid arthritis). What are the user needs and requirements for each part in order to achieve the intended outcome? What must the user see, hear, feel, know, and do in order to safely and effectively interact with the combination product? Thinking critically about these questions can provide developers with a better understanding of how end users may ultimately interact with the product and the potential issues they could encounter. Applying good human factors design principles can help sponsors optimize design aspects of combination products early in the development life cycle (see Ref. [4] for a primer on recommended user-centered product design principles for usability).

Training

User training, if applicable to a product, is considered to be part of the user interface of a combination product. A sponsor may choose to include a trainer/demo device as a training tool that allows users to practice interacting with the tool before using the actual device. These trainer/demo devices may also be considered to be part of the product user interface. "Train the trainer" types of training, where sponsors may formally train HCPs who then train patients on use of the product, also may be considered part of the user interface. Sometimes the training is contained in the product packaging, for example, a QR code that links the user with online video instruction on how to use the product. Sponsors should ask themselves: Is user training necessary for the product? If so, how will the sponsor ensure that consistent training occurs for all users? What will the training documentation and management look like for the sponsor? If the product cannot be used without training, then there has to be a way that the sponsor will ensure that all users receive the intended training.

For combination products, it is often considered difficult or impossible to systematically and consistently reach the target audience or assume that everyone will be trained. Related to this difficulty, CDER typically expects that a human factors validation study will include untrained user groups [20]. Another potential benefit of evaluating a product with untrained users is to help assess how other UI elements support safe and effective use when users forget the training they are provided, which is highly likely for lay users, for infrequently performed tasks, and when significant time passes between training and actual use.

Sponsors should identify any formalized training that is a part of the product's user interface; formalized training includes any sponsor-managed training that is provided to all intended users of the product. Training decay (also referred to as learning decay) is another important consideration related to simulating training in human factors testing, which is the period of time between the training that a user receives and then interacts with a product or experiences a particular situation with a product. For human factors testing design, it is important to consider training decay in real-world situations and to implement representative decay in validation testing.

Instructional Materials (IFU and Packaging)

Sponsors need to consider the instructional materials that will be provided as part of the user interface. Instructional materials include the Instructions for Use (IFU) as well as any other labeling documents that the users will interact with, such as product packaging, quick reference guides (QRGs), pamphlets, etc. While not all users may utilize the instructional materials during actual use, it is important for a sponsor to optimize these materials to ensure that when users do in fact use or need the instructional materials that instructions do not lead to improper use or problems.

4.3 Develop and Leverage a Use-Related Risk Analysis (URRA)

A use-related risk analysis (URRA, e.g., failure mode and effects analysis (FMEA), fault tree analysis (FTA)) that is typically traceable to a detailed task analysis provides the framework for all human factors activities because it identifies the hazards, harms, potential outcomes, and severity of use-related risks associated with the combination product. The URRA helps the sponsor to identify where the most critical risks are in the user interface, which can then be reduced by implementing user interface improvements. The URRA is also the foundation of human factors testing because it allows the sponsor to categorize critical tasks that are required to be validated. When developing a URRA, consider known use-related problems with predicate or similar products, of which FDA CDER and notified bodies are typically well-aware and will expect to be addressed in risk documentation (e.g., premature removal of an auto-injector from the injection site before therapy is completely administered). A task analysis, which systematically identifies tasks and sub-tasks involved in device use and is often more detailed than a URRA, can be a valuable tool in URRA development and human factors evaluations because it enables sponsors to identify specific use-related steps along with potential use errors and associated outcomes from the perspective of the user or patient who is interacting with the device. The task analysis also typically provides the level of detail needed to adequately assess and validate use related safety and effectiveness by defining specific user performance requirements relevant to a product user interface. Refer to the international industry standard (which is recognized by FDA) for details about developing URRAs [19].

4.4 Apply Human Factors Guidance for Biosimilar Products for the US Market as Needed

FDA has identified additional human factors considerations that should be applied to a product when a sponsor has selected a biosimilar regulatory pathway for a combination product [12]. For example, FDA recommends that the biosimilar proposed product labeling be identical (or very similar) to the reference product labeling with the necessary modifications to accurately describe the proposed product. The sponsor should provide justification for any further changes to the labeling and determine if the labeling changes require human factors validation. The sponsor should also determine if human factors validation is needed for the product and other UI elements based on use-related risk. FDA recommends that a sponsor submits any human factors validation testing plan/protocol, in addition to the proposed labeling changes, to FDA for review prior to conducting a validation study. The FDA will complete a full review of the IFU and other labeling (e.g., patient information leaflet) during the submission review, along with any human factors data relating to

the labeling design (e.g., IFU formative evaluations). Specific labeling recommendations and example content can be found in Ref. [12].

5 Best Practices for Incorporating Human Factors Strategies

Once a sponsor has identified intended users, defined use environments, decided on a regulatory strategy, and developed a URRA (and corresponding task analysis, as necessary), the foundation has been laid for integrating human factors processes to support the combination product life cycle and optimization of the product user interface. The following four best practices are recommended for incorporating human factors strategies in combination product development, which are discussed in the following sections:

1. Start the human factors (HF) process early.
2. Leverage applicable prior work.
3. Keep the global market in mind.
4. Collect labeling-focused data.

5.1 Start the HF Process Early

From a cost/benefit perspective, the earlier that human factors processes are integrated into the design process, the better! A sponsor can avoid expensive and time-consuming late-stage revisions to product design by taking human factors into consideration at the beginning of development. Even if human factors processes are incorporated later in the development process (or toward the end), there is still value in assessing risks and working to optimize the user interface prior to conducting a human factors validation study. The following examples describe how human factors can be incorporated at different stages in the combination product life cycle.

Incorporate human factors when selecting device partners Sponsors seem to rarely consider (or consider too late) the potential human factors implications when selecting device partners, and many pay the price long afterward when they discover that the device cannot be safely used by their product's intended population, and device design cannot be changed. For example, a sponsor may select an inhalation delivery device that has been successfully used to administer therapy in younger adults as the delivery device for a new drug intended for elderly patients. Because the inhalation device has been used successfully and has no adverse events associated with it for the younger user population, the sponsor may think that the device is a perfect marriage for the new combination product. However, the intended elderly user population for the new product has different characteristics and physical limitations compared to young adult users, so the sponsor may discover too late that the same inhaler cannot safely and effectively be used by elderly patients. Consequently,

it is beneficial for sponsors to consider the use-related risk profile of the device and any human factors processes implemented on the device before entering such important partnerships. Sponsors may also want to consider the patent holder and determine if there is any flexibility to make changes to the device if human factors testing results are not favorable.

Incorporate human factors prior to clinical studies Often, sponsors are primarily focused on the conduct of clinical studies in which the combination product therapy efficacy is demonstrated for representative patients. While human factors considerations can often be an afterthought, the device user interface (i.e., the device itself, training, and instructional materials) can be evaluated in use-related human factors formative evaluations prior to starting clinical studies to ensure that there will be no unforeseen issues with users using the device that could negatively impact the clinical studies. By incorporating human factors prior to clinical studies, a sponsor may be able to avoid use-related issues that would require halting the study or restarting from the beginning (certain to be a costly effort). For example, if training is part of the clinical study, then the sponsor may optimize the training and/or training materials through human factors testing before conducting the clinical studies to prevent problems from occurring during studies that are due to poor or ineffective training.

Incorporate human factors in parallel to clinical studies Sometimes a sponsor decides to conduct human factors testing at the same time as clinical studies, which usually occurs when human factors actual use testing (as opposed to simulated use human factors testing) is necessary to demonstrate safe and effective use of a product. In some cases where clinical studies and human factors testing are conducted in parallel, human factors studies can involve actual use with real patients, which can provide the sponsor with unique insights into actual use of the device and continued use over time (when applicable). Human factors evaluations of the anticipated commercialized user interface (e.g., no training, revised IFU) in parallel to the clinical studies may also be beneficial from a cost and timeline perspective.

Human factors may need to be considered after clinical studies Ideally sponsors should conduct a human factors validation study before Phase 3 clinical trials [20], as this allows time for any necessary device modifications to optimize the user interface prior to completing the clinical investigation. Consider an example of a sponsor who conducts a human factors validation study after a Phase 3 clinical trial and uncovers human factors issues which make the product unsafe or ineffective for intended users. Now, the sponsor will need to determine how to mitigate the risks observed, which could entail significant revisions in some cases. If the updates require substantial changes to the product design, a sponsor's clinical results may become invalid because the final product design (after implementing modifications stemming from the human factors study results) will not match the product used for the clinical trials.

Even when human factors validation studies are completed prior to Phase 3 clinical studies, the sponsor may identify changes to the user interface based on clinical

study findings or changes related to differences between the clinical study and commercial user interfaces (e.g., training is provided in the clinical trial, but it is not intended for the marketed product). Even the most robust human factors testing cannot always predict and account for all possible issues that may arise during actual use. For example, Phase 3 results may identify the need for updates to the labeling or additional training requirements for the intended users. In these cases, the modifications to the user interface (e.g., labeling or training) should be identified, along with an analysis of how these modifications are expected to reduce or eliminate risk as well as if any new use-related risks are introduced as a result of the user interface modifications. Sponsors may need to run a supplemental validation study on modifications to demonstrate that the user interface updates are safe and effective and/or to ensure that any modifications associated with potential new risks do not adversely impact use-related safety and effectiveness.

Incorporate human factors assessments prior to human factors validation At a minimum, sponsors should include human factors processes prior to the human factors validation study because this will reduce a sponsor's risk of a validation study turning into an expensive formative evaluation due to unmitigated difficulties and use errors. Human factors formative evaluations allow the sponsor to observe the types of issues that may occur in a validation if those issues are not addressed. Formative evaluations are also useful to test implemented user interface design mitigations to ensure they are effective in preventing harm to users. Formative evaluations can also be used to assess validation study methodology (i.e., a pilot validation) so that use scenarios are clearly formulated for participants, instructions and questions are understood, and it is less likely for a use error to be attributed to study artifact. An important consideration for sponsors in planning human factors formative evaluations is the lead time necessary to acquire the correct study materials. The sponsor should recognize and plan for potential prototype limitations that do not mimic the real-world product because these types of factors will limit the generalizability of the study findings (e.g., placebo capsules that do not have the same perceptual cues as the actual medication capsule or asynchronization between the end of an injection and the audible click that is supposed to signify the end of an injection for the user). Limitations with the testing materials may mean that the use-related behavior observed during the human factors testing not directly correspond to how users in the real world would interact with the combination product, which presents a limitation in formative evaluations. According to FDA's human factors guidance [1], the product user interface in a human factors validation study should always represent the final design.

5.2 Leverage Applicable Prior Work

Many times sponsors will develop multiple combination products that have similar device designs and uses. In these cases, it is not always necessary to re-invent the wheel or repeat the same type of testing with the new product; rather, sponsors may be able to leverage human factors work and findings from their past human factors studies on similar UIs (for similar users and use environments) to inform design decisions on the new combination product. For example, consider a sponsor who conducted quite a bit of human factors testing and validation for a pen injector for use by adolescent users. Based on formative testing, the sponsor learned that participants were consistently confused by the dose increments in the dose window. Based on this information, the sponsor updated the display which effectively mitigated the issue. In the future, if the sponsor develops another pen injector for a similar user population, these findings can be integrated into the design to proactively optimize the design of the dose window. In addition, URRAs from similar devices can be leveraged where applicable. In this way, the sponsor can streamline the human factors strategy by conducting the necessary human factors testing on the unique design features of the new product without being excessive or burdensome. However, it is also important to consider if the new product has any unique use cases or tasks, labeling differences, branding differences, and other unique features of the user interface that may warrant additional testing with the new product.

5.3 Keep the Global Market in Mind

To a large degree, US and international human factors guidance and standards are harmonized. Sponsors who plan to submit their products for approval in both the US and global markets should plan for and consider the following:

- Mapping the similarities and overlap between US guidance (e.g., see Ref. [1]) and international standards (e.g., see Ref. [2]) enables sponsors to be more efficient in human factors documentation, testing, reporting, and submissions because the same data may be submitted for different intended global markets.
- For US submissions, human factors validation participants should reside in the United States, per FDA CDRH guidance [1], because typically the agency will not accept human factors validation data that were collected outside of the United States. However, formative evaluations outside the UnitedStates may be leveraged to inform the overall design process, as long as the differences in medical practice, types of users, and other factors are considered and accounted for when trying to generalize data as representative for other global regions.
- For US submissions, FDA also states that the user interface (e.g., device, labeling, training, etc.) should “correspond exactly to that which would be used for the device if marketed in the U.S.” This directly impacts submissions and human factors testing if training or other UI elements are intended to be different across different markets.

5.4 Collect Labeling-Focused Data

During human factors validation testing, participants cannot be directed to use any instructional materials to assist them in performing tasks, per FDA guidance [1]; instead, participants must independently choose to use those materials. What often happens in validation testing is that participants do not even pick up the instructional materials, which begs the question: How can a sponsor determine that the IFU (or other instructional materials) is safe and effective when participants may not reference it during a validation study? One option for confirming that the IFU is effective prior to the human factors validation study is to conduct formative testing focused on the IFU. In other words, conduct a formative evaluation but specifically direct the participants to reference the IFU as they complete tasks. In this way, the IFU can be optimized, and the sponsor can confidently say that the IFU, when used, is safe and effective.

6 Common Human Factors Challenges During Validation Testing

Validation testing is the culmination of the human factors evidence for a submission to ensure use-related safety and effectiveness for the combination product UI. FDA and others have discussed numerous challenges that exist for combination products during validation testing, including accounting for the possibility of negative transfer, relying on training and IFU as mitigations for design problems, providing data-driven design justifications, creating quick reference guides, determining how to confirm delivery of dose, considering auditory and visual cues in the actual use environment, and designing for user limitations. These are discussed in the following sections along with example situations to illustrate the challenges that developers face.

6.1 Account for Negative Transfer

Preconceived user expectations and behaviors can influence how users interact with a new product. These user expectations can originate from prior use of other medications, observing other people taking medications, or even from watching television shows or movies. Coming into a situation with expectations or prior knowledge can be positive (i.e., user expectations and behaviors can correctly transfer to the new product) or negative (i.e., those expectations and behaviors do not transfer to the new product). Examples of positive and negative transfer are described below:

1. *Positive transfer*: Patient A just injected medication using a new auto-injector. He sees the auto-injector cap on the table, but remembers that when he used a previous auto-injector, he was trained never to recap the auto-injector for safety reasons. Based on this previous knowledge, the patient throws the cap away and

properly disposes of the used auto-injector in a sharps container. This is an example of positive transfer—Patient A applied previously learned knowledge correctly to the new situation.

2. *Negative transfer*: Patient B has been prescribed a dry powder inhaler (DPI) to treat a new diagnosed illness, which looks similar to the pressurized metered-dose inhaler (pMDI) that her husband uses to treat his asthma. Patient B assumes she knows how to use her DPI and that it operates like her husband's pMDI. However this is not the case, and Patient B is in danger of mis-dosing her medication with her pMDI because she does not realize that pressing the inhaler button while simultaneously inhaling does not allow the medication to correctly load into the inhaler. This is an example of negative transfer—Patient B applied an incorrect expectation and associated performance based on how a different device operates, which caused her to experience a use error and incomplete medication dose with her inhaler.
3. *Negative transfer*: Patient C has been using a topical ointment daily for years to manage a skin-related disease that manifests on his hands. Recently, a new therapy came on the market that delivers a drug via injection to treat the patient's condition without the need for topical therapies. Patient C, who has never self-injected or injected others with any type of medication, is excited to try the new therapy but does not understand that the medication should be injected only in the thigh or abdomen. Instead, Patient C injects himself in the hand, where he applies the topical ointment. This is an example of negative transfer—an incorrect mental model of how the medicine works based on experience with prior therapies, which caused Patient C to incorrectly use the injection therapy.
4. *Negative transfer*: Patient D is an elderly patient that previously used a single-use auto-injector therapy to help manage his Type II Diabetes. Now his physician prescribed a new reusable pen injector that he must use twice per day. The patient assumes that he knows how to use the new pen device because it looks so similar to his old auto-injector, and he disposes of the new pen injector after using it one time. This is another example of negative transfer—Patient D assumed that because he had used an injection device in the past, they all operated the same way. When considering negative transfer like this, it is important for the sponsor to keep in mind that there could be many differences between similar looking injection devices that impact how a user interacts with a product, including single vs. multi-use, a priming requirement, dose dialing, pre-attached needle vs. the need to attach a needle, and units of measurement.

6.2 Cautiously Rely on Training as a Mitigation

Training may be an appropriate way to mitigate risks for combination products in some cases, but training is generally not the most effective mitigation and should not considered an "easy answer" for sponsors who identify use-related problems with their product. CDER recommends that sponsors assess the need for training by considering the following [7]:

- Implement modifications to the design of the device itself to remove any use-related risks that exist due to the design of the product can be the most effective means of reducing or eliminating use-related risk.
- Consider if use-related risks cannot be completely designed out of a product, training may be appropriate if the sponsor can implement and assure a consistent training program for all intended users before first use of the product.
- Include untrained participants in human factors validation testing if training is included as part of the user interface of a product, but the sponsor cannot guarantee that training will be consistent across users and/or if the training may not occur for all users. In general, CDER expects untrained participants to be included in the human factors validation testing unless there is strong evidence that all users will routinely and consistently be trained. So, if a sponsor cannot provide this evidence but still wants to include trained participants in the human factors validation testing, the sponsor should also include a group/subgroup of untrained participants to adequately represent those users who may not receive the training.

When developing training, a sponsor should consider design elements such as training duration (i.e., length of the training session, which could span hours or days), how often users will be trained or retrained, who will conduct the training, what the training objectives are, how to measure training outcomes, how to document training program requirements to ensure consistency in training, how to ensure all users receive training, and the realistic period of time between training and use of the product (included in a validation study as training decay period). This is not an exhaustive list of considerations, and sponsors should carefully consider any additional training factors that may be relevant for combination products on a case-by-case basis. For combination products especially, training can be difficult for sponsors to implement in a way that could be considered a consistent and effective risk mitigation. It is important to consider how the training program will be standardized so all users receive the same intended training and documented so the sponsor can ensure and track that all users receive the training.

Incorporating training in a human factors validation study should be considered on a case-by-case basis. Sometimes sponsors expect that all users will receive some type of training in clinics (e.g., from a user's doctor or nurse) prior to using the product, but this training is not a sponsor-developed or managed training program that can be expected to be consistent across all users nor provided to all users. In those cases, it may be beneficial for sponsors to include a trained user group/subgroup with representative training and an untrained user group/subgroup to represent users who may not receive the training before use. For some combination products, users may receive training with a trainer device, especially for products that are only used in emergency situations (e.g., epinephrine delivery device). Once trained, a user may not actually use the product for months (or even years) after receiving training. The implication for testing these types of products in a human factors validation study is that the knowledge users have learned during training has decayed so much by the time they actually use the product that it is similar to never receiving the training at all. In situations with long periods of training decay, including untrained participants in the human factors validation study may be most representative real-world use.

6.3 Cautiously Rely on the IFU as a Mitigation

As with training, sponsors should be cautious about relying on instructional materials such as the IFU as the only risk mitigation strategy for observed use-related problems with combination product use. Instructional materials fall into the category of information for safety, which is the least effective and least preferred risk control measure [19]. Often, multiple mitigation strategies are necessary to optimize the user interface of a product and effectively address use-related issues observed during human factors testing. Other times, modifications to the IFU or other labeling may be an inappropriate strategy altogether.

For example, pen injector "A" may be unintuitive to patients who are inexperienced with injectable products. As part of the product user interface, patients receive a 10-minute tutorial from a nurse before being sent home to use pen injector "A" to self-administer once weekly injections. During clinical testing, the sponsor received many reports of patients who received underdoses and overdoses because they were incorrectly dialing prescribed doses and were removing the injector from the injection site too early (resulting in a wet injection). Based on the reports, the sponsor revised the IFU to include large pictures that illustrated each step and emphasized the time users need to hold the injector on their skin to administer the therapy, assuming that these IFU modifications would eliminate the use related issues. The modifications to the IFU were effective in reducing the number of issues relating to wet injections. However, users continued to experience critical use errors related to dialing the dose. Further investigation by the sponsor revealed that the root cause for the continued issues was that the numbers and measurement increments printed on the dose dial were too small for the user population to clearly see, who were comprised of elderly users who frequently experienced vision problems. In this example for pen injector "A," a combination of design mitigations (i.e., IFU and device) was necessary to reduce the observed use-related problems with the injector and maximize the likelihood that the user interface is safe and effective for the intended uses, users, and use environments.

In some cases, modifying a product's IFU is an inappropriate and ineffective mitigation strategy due to the type of use-related issues identified. For example, a sponsor may observe in a formative evaluation on pen injector "B" for users with arthritis that many participants experienced difficulty unscrewing the pen cap or could not unscrew it at all. During debrief, these participants provided subjective feedback that they did not have the strength or dexterity to unscrew the cap or experienced a lot of pain when they could. Based on these findings, the sponsor redesigned the cap so that it included a grip that could be pulled off instead of unscrewed. Changes to the IFU would have been an ineffective strategy in this case, as no amount of labeling could compensate for the physical limitations of the intended users.

6.4 Provide Data-Driven Design Justifications

At the conclusion of every human factors validation study, sponsors should always analyze the validation testing data (objective performance data and subjective feedback), identify root causes of any use errors and problems, and perform a residual risk analysis to determine if residual risk is acceptable or if further design mitigations are necessary to maximize the likelihood that the device will be safe and effective for use by the intended users, for the intended uses, and for the intended use environments. Sometimes sponsors conclude that residual risk is acceptable only to have FDA disagree with the assessment and request modifications and supplemental validation testing. However, if sponsors have collected data during validation testing (or from prior human factors formative evaluations) that support the case for safe and effective use with no further modifications, they can present these data-driven justifications to FDA and other health authorities to assert that no further revisions to the user interface are required, often with great success.

6.5 Carefully Consider When to Use Quick Reference Guides

Quick reference guides are frequently developed when a combination product is complex and the steps to achieving the intended use are extensive and/or complicated. Sponsors should remember the purpose of a quick reference guide and also recognize potential risks in developing a quick reference guide. It is not intended to replace the IFU. Rather, it is a complement to the IFU and should serve as a cognitive reminder for users who may need to glance at it occasionally. However, one of the risks with a quick reference guide is that users may only choose to use the quick reference guide, which often means that important information is missed and lack of information may cause users to incorrectly use a product or experience use errors. Consider for example a sponsor whose new wearable delivery system has an IFU that is over 50 pages long, including text-heavy step-by-step instructions for each step, contraindications, warnings, etc. Findings from a formative evaluation revealed that although the patient participants received training before use (which was representative of the training patients would receive in the actual use environment), participants often chose not use the IFU during the initial setup steps where it is critical to hold the product with a particular grip to avoid touching the adhesive and inadvertently activating the needle. During the testing session debrief, participants often indicated that they would rarely use the IFU during the device setup steps because it seemed so easy during the training. Based on this information, the sponsor developed a quick reference guide to include on the inside of the product packaging to provide users just in time information that would guide safe and effective use of the product during the initial setup steps. After subsequent human factors testing, the sponsor concluded that the quick reference guide was effective in reducing the

incidents of unintentional needle activation but that participants never picked up the IFU and were not always aware of the additional critical information contained only within the IFU. Thus, sponsors should be aware that there may be unintended consequences with introducing quick reference guides, such as users relying solely on the guide.

6.6 Determine How to Enable Users to Confirm Delivery of Dose

Dose confirmation can be challenging to implement in a combination product. Slight variability in the mechanical components of the delivery device can impact the timing of the delivery dose. While sponsors may attach a visual or audible cue that alerts the user that a dose has been delivered (e.g., colored bar, audible click), the cues may not always align with the actual completion of dose delivery. For example, in one instance a sponsor conducted a formative evaluation on a new auto-injector device and observed that many participants in the study prematurely removed the auto-injector and did not administer a full dose. During the study session debrief, participants indicated that they thought the audible click that they heard after initiating the injection signified that the injection was complete when in reality the click was due to the mechanics of the auto-injector design and was unrelated to delivering the full dose. The sponsor ultimately revised the design of the auto-injector so that the audible click occurred after the injection was completed and also added a secondary visual cue (i.e., window that changed color once injection was complete) as another mitigation for this observed use error.

Key Messages

- Human factors engineering is an important part of combination product development, and regulatory bodies expect certain human factors standards to be met for successful pre-market submissions that demonstrate a product is safe and effective in the hands of end users.
- Sponsors should adhere to US and international regulatory guidance and standards for proper ways to implement human factors during product development and to identify regulator expectations early in the development process. US and international guidance documents are continuously being updated, and it is up to sponsors to stay abreast of current regulatory requirements for human factors.
- Strategic implementation of human factors methodologies include the following: (1) start the human factors process early, (2) leverage applicable prior work, (3) keep the global market in mind, and (4) collect labeling-centered data.

- Challenges that sponsors of combination products often encounter during human factors validation testing should be considered, including account for negative transfer, cautiously rely on training and/or labeling as mitigation strategies, provide data-driven design decisions, carefully consider when to use quick reference guides, and determine how to enable users to confirm delivery of a full dose.

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Chapter 32
A Science and Risk-Based Approach to Bridging Drug-Device Combination Products

John K. Towns

1 Introduction

1.1 What Is Bridging?

Bridging in the context of biopharmaceutical combination products is the connecting of two separate but related drug/device presentations by building the scientific argument that supports the conclusion that the two presentations are comparable with respect to quality, safety, and efficacy. For drug/device presentations, comparability bridging is a common and much needed exercise to demonstrate that the to-be-marketed combination product is comparable to prior combination product(s) tested in development. Comparability can be deduced from quality studies of device/combination product performance (benchtop testing) and human factors (HF) and may need to be supported by clinical studies. The extent of the studies required to demonstrate comparability will depend on:

- The phase of the development program where the drug attribute or device design change occurred
- The phase of the development program where the new device platform was introduced
- Impact of the delivery system to potentially alter or interact with attributes of the drug molecule or formulation and thus ultimately the delivery of the drug
- The availability of suitable nonclinical studies (e.g., analytical/functional testing) to detect potential product modifications caused by a delivery system design change

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The extent of bridging study data requirements that must be generated is risk-based and generally depends on the stage of development, how far removed (i.e., substantial design changes) the delivery device presentation is from the applicable prior presentation, and the extent of prior performance knowledge for the device platform. The same general assessment holds true for a biopharmaceutical formulation change. This assessment takes a risk-based approach comparing the original product formulation attributes to the new product and whether changes in, for example, excipient composition, viscosity, isotonicity, pH, and/or manufacturing process may adversely impact safety and/or bioavailability.

Ideally, the product tested in pivotal Phase 3 (Ph3) clinical trials is representative of the to-be-marketed product. This is best achieved by ensuring the device is introduced at the initiation of the pivotal Ph3 clinical trials, if possible, or at some point during the pivotal clinical trials (CTs) such as open-label extension arms. Along with eliminating the need for bridging studies, this inclusion in pivotal Ph3 demonstrates the sponsor's experience and confidence with the device. However, there are situations where the to-be-marketed device cannot be introduced into the pivotal clinical trial program, thus necessitating a scientific comparability bridge be built to link the clinical trial material to the commercial product.

A concern of FDA, expressed in End of Ph2 data package reviews, has been not including the to-be-marketed product presentation in the pivotal clinical trials. Specific concerns expressed by the clinical reviewers were potential structural damage to the biopharmaceutical as the molecule travelled through the device and into the subcutaneous biospace (e.g., shearing effect on monoclonal antibody) and the sensitivity of the analytical chemistry purity methods to detect such a change. Additional concerns were insufficient demonstration of device use in clinical studies that left lingering concerns of nonclinical bridging plans being sufficient to support product safety and efficacy. This uncertainty in what will constitute acceptable bridging studies has compelled sponsors in some cases to add a separate clinical efficacy trial into their study programs to mitigate any submission review risk that FDA deems the injector bridging data insufficient to demonstrate comparability to the to-be-marketed commercial presentation (e.g., see Cosentyx case study in chapter 34). The underlying concern seems to stem from the clinicians naturally leaning toward employing those studies they are most familiar with – using clinical trials to answer a given concern. However, in many cases, an alternate nonclinical bridging study plan would prove to be more informative, less costly, and a less time-consuming approach.

The decision of when best to introduce a new device platform (or a significant device design change) into the clinical trial program involves multiple factors, including prior device platform experience, patient use (e.g., self-administration in the home), and device availability (and continued supply) [1]. The timing of device introduction influences the design, conduct, and type of studies used to collect the technical information that plays a key factor in building the scientific comparability bridge. The decision strategy necessitates a risk-based approach that allows for sufficient experience and knowledge be gained in development to mitigate device concerns during regulatory review and ultimately to assure robustness once the product is out in the market.

1.2 Scope

The considerations and approaches in this chapter apply to biopharmaceutical products incorporated into a medical drug delivery system constituting the drug-device combination product. The principles explained apply to:

- A new device platform for delivery of the biopharmaceutical is introduced into the development program on the path to the to-be-marketed presentation (e.g., vial/syringe to prefilled syringe (PFS) to autoinjector (AI)).
- Design changes implemented within a given device platform (e.g., changes to the activation button dimensions for an AI implemented after human factors studies but prior to pivotal Ph3 CTs).
- Change(s) in biopharmaceutical attribute is implemented with established device delivery system. "Established" meaning device constituent part is already marketed in combination with the same or a new biopharmaceutical with similar solution attributes.
- New user group(s) is introduced (e.g., pediatric patient self-administration) with an established device delivery system.

Out of scope is bridging from a reference biological medicinal product to a similar biological medicinal product (i.e., "biosimilars"). This type of bridging exercise must take into consideration the added level of complexity of a new molecular entity that has been created and purified from a completely separate manufacturing process than the reference medicinal product.

1.3 Constituent Parts of the Combination Products

Biopharmaceutical injectable combination products are comprised of four constituent parts (Fig 1). Each of the four constituents should be considered separately and then the combined interactions when brought together into the to-be-marketed presentation. In the assessment for bridging, each constituent can contribute positively or negatively in impacting product tolerability, patient compliance, device functionality, and product usability, safety, and/or efficacy in the commercial setting.

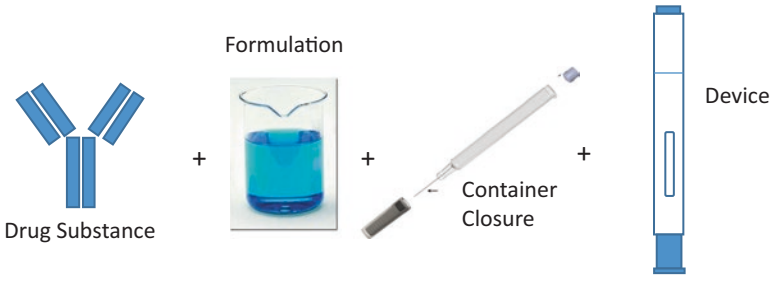

Fig. 1 Components of combination product

The constituent part details are:

- Drug substance (DS)
- Biopharmaceutical molecular entity that will include low levels of related product impurities and in-process materials carried over from the manufacturing process.
- Attributes of concern: molecular structure, product-related impurities, host cell proteins, drug substance stability, patient tolerability
- Formulation
- DS combined with excipients/stabilizers/buffers to meet drug concentration (dose strength), isotonicity, and shelf-life stability requirements.
- Attributes of concern: excipients, viscosity, concentration, volume, pH, osmolality, drug product stability, patient tolerability
- Container/closure
- Containment of the drug formulation with functional component attributes to aid drug delivery (e.g., siliconization of syringe barrel to aid plunger movement for more accurate delivery of drug).
- Attributes of concern: lubricants, extractables/leachables, particulates, breakloose/glide force, component(s) impact on dose accuracy and drug stability
- Device
- Device components combined with biopharmaceutical product primary container/closure that form the drug/device delivery system.
- Attributes of concern: device actuation, dose accuracy, needle insertion depth, glide force, speed of injection/delivery time, usability, reliability

1.4 The Bridging Exercise

Bridging takes a risk-based approach as illustrated in Fig. 2 that begins with (1) introduction of new device component into the biopharmaceutical development program, (2) changes in the device design within a platform, or (3) change in a biopharmaceutical attribute(s). The extent of bridging information required to support

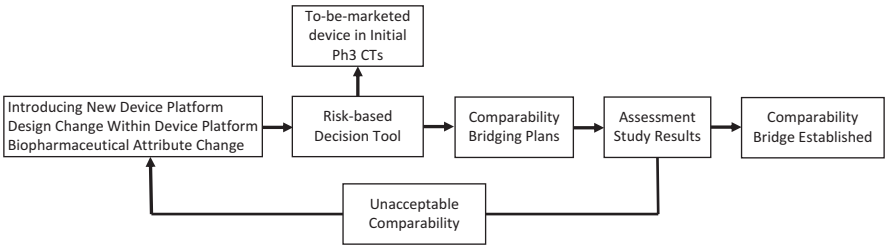

Fig. 2 Building the device comparability scientific bridge

the launch and commercialization of a new drug delivery system (or changes to an existing device platform/drug attribute) is then evaluated. The impact assessment requires the drug and device components be taken together to demonstrate confidence in the safety and efficacy of the to-be-marketed combination product. The decision tools take into consideration such factors as the device platform, drug and formulation attributes, patient user groups/scenarios, and established biomarkers.

Once a decision has been reached for the timing of device introduction into CTs, a stepwise additive testing strategy is undertaken for building the scientific comparability bridge (described in Sect. 6). This begins with fundamental lab bench testing of analytical and functional properties and progresses into design verification and product user interactions through human factors studies and into clinical studies. The comparability strategy that bridges to a new device/drug encompasses a multi-faceted testing scheme to confirm chemical compatibility, biocompatibility of materials, functional testing (design verification/engineering studies/modeling), human factors (HF), pharmacokinetics (PK)/pharmacodynamics (PD), real-life patient handling (RLPH), safety, and efficacy. Special focus is given to developing the appropriate comparability package that (1) demonstrates acceptable use and experience with the representative to-be-marketed combination product and (2) utilizes sponsor's prior experience and knowledge for the device platform within the intended user group(s). This information is used to establish the comparability bridge when either moving to a new device platform or for a change within a platform and/or biopharmaceutical product attribute.

1.5 Base Device Versus to-Be-Marketed Presentation

A base device is often used in the early phase clinical programs (e.g., subcutaneous bolus injections) to deliver the investigative drug dose. The base version may be a simple off-the-shelf device or a bare-bones version of what will become the commercial device. As the eventual commercial device component retains the basic design attributes and usability characteristics, this base device can be considered "representative" of the to-be-marketed device. The device may undergo minor design iterations implemented under continuous improvement with these revisions assessed to assure there is no adverse impact to safety and efficacy. An example of a minor design change iteration would be moving from a simple PFS used in early clinical trials to the commercial PFS with enhanced plunger rod, finger flange and needle cap, and/or a needle safety shield. Both iterations would require design verification and human factors studies to assure proper use of device (linking back to pivotal clinical trials), but as needle injection depth is controlled by the patient, these are considered "representative" devices, and no further clinical studies would be required.

For more extensive design iterations, a discussion with regulators may be prudent to confirm the necessary bridging studies are performed. For the FDA, where the Center for Drug Evaluation and Research (CDER) is the lead center due to the

primary mode of action being the drug, likely an inter-center consultation will be requested with the appropriate device review team (Center for Devices and Radiological Health (CDRH)). This consult would be to confirm no notable differences in the original base iteration of the device when compared to the versions used in later clinical trial studies and/or the to-be-marketed device.

2 General Principles

The goal of the bridging exercise is to ensure the quality, safety, and efficacy of the to-be-marketed combination product. This is attained through the collection and evaluation of relevant study data to determine no adverse impact in delivering the drug to the patient. This covers the situations when moving to a new drug delivery platform and post-device design change or change in a biopharmaceutical attribute.

The demonstration of comparability does not mean that the quality attributes of the delivery system are identical. The purpose is to demonstrate the new or revised delivery system can deliver the intact biopharmaceutical to equivalent specifications and equal or better reliability than the original delivery system and thus infer delivery to the appropriate area of the body and safety and efficacy is not adversely impacted in moving to the new/revised delivery system. The existing product knowledge needs to be sufficiently predictive to ensure that any differences in the delivery system would not impact safety or efficacy of the drug-device combination product.

A determination of comparability is based on a combination of analytical testing for compatibility of the drug product with the device system, functional testing/modeling including design verification, user interaction testing, and, in some cases, clinical trial data. If a sponsor can provide assurance of comparability through nonclinical studies (analytical, design verification, human factors) alone, clinical studies are not warranted. Other than those changes requiring clinical PK studies to address significant change in the delivery characteristics, clinical studies would only be required for novel delivery platforms, where there is little to no prior experience. Prior experience can help the sponsor to confidently determine that it is unlikely that there are unforeseen issues of safety or that would result in nondelivery of the drug (i.e., efficacy). However, where the relationship between specific drug delivery system and safety and efficacy has not been established, it may be necessary to include additional clinical experience of PK studies, RLPH experience, and/or safety and efficacy clinical trials. When needed, these can be conducted as standalone clinical trials or can be conducted during the open-label extension arms of pivotal clinical studies.

The scope of testing required to support a proposed change correlates with the potential to adversely impact patient safety and efficacy. Borrowing loosely from FDA guidance and international standards for drug manufacturing post-approval change assessments, combination products can follow the incremental escalation of

testing requirements from benchtop through to clinical studies (see Sect. 6). These studies take into consideration prior product constituent knowledge of the potential for adverse impact of the change to patient safety and/or efficacy. Nonclinical bridging data include drug/device compatibility analytical testing and functional benchtop characterization, biocompatibility testing, and HF studies.

The to-be-marketed product presentations may include multiple delivery systems due to device platform preferences (e.g., PFS, AI, bolus on-body injection) and product strengths (e.g., concentrations and volumes). When this is the case, the science-based approach may best be facilitated by establishing separate bridging "swim lanes" for each presentation, each with its own set of bridging requirements. For example, when a simple prefilled syringe is used in pivotal Ph3, the bridge to the commercial PFS may only require design verification and human factors studies. However, the bridge to an AI may require additional studies of PK relative bioavailability (RBA) to build the scientific comparability bridge to the new device platform.

Sponsors will increase the likelihood of gaining combination product regulatory approvals by demonstrating patient experience of the combination product and using the drug/device combination at every opportunity during development phase. By collecting patient experience during development, sponsors are able to demonstrate the patient's understanding of the delivery system and the steps taken to optimize device use.

Patient experience may capture learning on critical task steps that necessitate including focused instructions for use in patient labeling and/or targeted training for specific task(s) to ensure the proficient use of the combination product in a "real-world" setting.

To bridge to a different biopharmaceutical formulation, the data must assure the new formulation does not adversely affect the functional parameters of the delivery device (e.g., glide force, injection time) in the delivery of medication. Additionally, the bridge must assure no adverse injection site reactions occur that could compromise drug dosing. Many formulation changes (e.g., viscosity, excipients, pH) may only require analytical testing (including extractable/leachable testing), studies to assess both chemical and physical (e.g., aggregation, particulates) stability, functional testing, and/or HF studies to build the comparability bridge. For a major formulation change that could adversely impact drug disposition (e.g., a pH change to increase solution shelf-life stability), the comparability data would likely require a bioequivalence (BE) or PK comparability (RBA) study.

3 Considerations for Bridging

3.1 New Device Platforms

More extensive bridging strategies are needed when new delivery system platforms, especially those where the sponsor has limited experience, are introduced after the initiation of the pivotal Ph3 CTs. Important considerations when building the

scientific comparability bridge between the Ph3 and to-be-marketed delivery system include:

- *Product contact material change*: Differences in the product contact material (syringe barrel manufacturer, change in glass type, plunger change, needle, or needle adhesive) can potentially impact the resulting product quality. An assessment of product quality impact may be required as part of the analytical bridging package (real-time and accelerated stability studies, extractable and leachable assessment)
- *Differences in injection technique*: Injection technique may widely vary where the patient controls administration of the drug as compared to that controlled by a delivery system (PFS vs. AI).
- *Pressure or compression of injection site*: Consideration made for the variability between the two delivery systems at the injection site (abdomen, thigh, back of arm) such as pinched-up skin for a PFS vs. pushed down on skin by an AI.
- *Differences in the injection flow rate*: Injection flow rate/injection speed with manual devices is highly variable and depends on the capability and preference of the individual administering the dose. Consider the variability between the two injection systems, for example, the shift in injection speeds less than 10 seconds for an AI that may be within the injection time variability of the manual injection. If a significant change in injection time is expected, consider an assessment of the impact of flow rate on PK. Minor shifts in injection speed will likely not influence the PK of a drug product.
- (Note: Controlling overall injection time by reducing spring tension, thus slowing the speed of the push rod, is quite different than controlling the injection time by decreasing needle gauge. The milliliter per second delivered is the same in both scenarios, but changing the needle gauge may warrant confirmation that pushing the solution at a higher rate through a narrower needle opening has no impact on protein molecular structure.)
- *Differences in injection volume*: If significant change in the injection volume is expected, consider an assessment on the impact of this difference in volume on the PK of a biopharmaceutical. Minor shifts in injection volume as a result of component change (e.g., less than 5 mL injection volume) are not likely to influence or change the PK of a drug product [2].

3.2 Timing for Introducing Device Component into CTs

Sponsors need to make critical decisions on the appropriate timing for introduction of the device constituent part into the clinical trial study program. A continuing challenge for industry has been the use of the "to-be-marketed" delivery device in the start of pivotal Ph3 trials. Two key reasons for this are (i) investment in expensive delivery system development activities are often deferred until after clinical proof of concept is established (e.g., Ph2a) and (ii) finalization of the delivery system design for the specific therapeutic product may be delayed until key design inputs are known

(typically Ph2b). These early development unknowns may include specifics related to the dosage, dose volume, dosing frequency, and dosing (self-administered or given by a health-care professional). Based on the timing of these dosing decisions, the delivery system development and scale-up activities may need to run in parallel with the pivotal clinical trials, necessitating the need for bridging.

A predefined decision process greatly aids sponsors in determining the extent of clinical evaluation needed for a biopharmaceutical and device combination product [2]. Many biopharmaceutical companies have an internal position paper (e.g., a Comparability Bridging Manual) covering the points for consideration on clinical bridging strategies for injectable drug/device delivery systems. These decisions are based on the individual component attributes and then in combination that leads to the appropriate comparability bridging options. The most critical considerations for introducing the to-be-marketed device into the clinical trial may be simply logistics based (e.g., device component availability), while others are biopharmaceutical drug and/or formulation driven. Main themes to consider when devising a strategy for the acceptable introduction of the device into the CT program include:

1. Timing of new drug delivery platform (component) availability, including device assembly line access
2. Complexity of interactions between the molecule, formulation. and device components
3. Critical attributes of the delivery system to deliver the drug (Essential Performance Requirements)
4. Extent of changes in design for an existing product/system
5. Pharmacological attributes of the drug class and impact on study variability of comparability bridging studies

There is a growing expectation from internal product teams and global health authorities that a broad, in-depth data package be generated in support of the safe and efficacious use of the to-be-marketed combination product. A consolidated benefits/risks evaluation process assists in the decision for the appropriate timing for introducing the device constituent part into clinical trial program. The evaluation takes into account the demonstration of safety and effectiveness while considering any development program limitations of including the device in early development (ideally introduced by Ph2) and into the pivotal Ph3 studies.

In general, biopharmaceutical products for subcutaneous administration are most often delivered via a manual syringe (vial configuration) in Ph1 and Ph2 clinical studies. A PFS configuration may also be used in Ph2 and would typically be introduced at the start of Ph3. The AI or on-body delivery system (OBDS) for larger volumes may be introduced within the Ph3 study program or in separate bridging CT studies. For PFS, where the route of administration remains the same and administration (e.g., needle depth, injection speed) is controlled by the patient, there is minimal bridging required for introduction of a like-delivery system in Ph3. Consideration should be given to including a needle safety shield for the PFS if that is planned for the commercial presentation.

Given the challenges in bridging formulation, presentation, or drug product manufacturing changes, FDA has urged sponsors to meet with the respective review

division to ensure that such studies are adequately designed to meet the intended objective of bridging clinical data across cohorts. FDA may recommend additional clinical studies to bridge safety and efficacy data in support of a marketing application if drug product changes, such as formulation changes, production scale-up, manufacturing site changes, and manufacturing process changes during clinical development, are not adequately bridged. In the absence of such bridging information, it may not be scientifically valid to pool key clinical data, and this may significantly delay the marketing approval.

3.3 Holistic Approach for Biopharmaceutical with Multiple Programs

When embarking on a new biopharmaceutical drug program, consideration should be given to the multiple commercial presentations that may span across more than one device platform as well as line extensions that may be introduced during the life cycle of the molecule. Many biopharmaceuticals are targeted for multiple disease indications that may require different dose strengths or frequency of administration. These different strengths may or may not remain in the same volume and within the same delivery platform. For example, a single monoclonal antibody (mAb) molecule may cut across multiple disease states and dosing regimens leading to multiple delivery systems covering a variety of volumes and concentrations. The delivery systems may range from a small-volume pen injector for pediatric patients, all the way up to a large-volume on-body injector for indications requiring 100s of mg of a mAb in order to be most effective. These large-volume presentations may initially be evaluated in early CTs through multiple injections (e.g., 3 × 1 mL@166 mg/mL = 500 mg total) in a simple PFS and then move to fewer injection by AI (2 × 2 mL@125 mg/mL = 500 mg total) for more convenient dosing, followed by a larger-volume on-body injection (1 × 5 mL@100 mg/mL = 500 mg total). An accompanying small-volume single injection (e.g., 1 × 0.5 mL@100 mg/mL = 100 mg total) by PFS may also be introduced specifically for pediatric administration. The concentrations of the various drug solutions may vary depending on the phase of development and the solution viscosity constraints for a given delivery device. In the large-volume injector example, lowering the concentration from 166 to 100 mg/mL may facilitate delivering the full 5 mL within a reasonable timeframe (e.g., 10–15 minutes) due to the lower viscosity.

3.4 PK Study Criteria

The relevance of a bioequivalence (BE) study criteria being the correct measure has come into question when bridging to a different device constituent part (e.g., from a PFS to an AI) for subcutaneous (SC) administration [3]. Often in the development

program, the biopharmaceutical product (e.g., mAb) has well-understood physical, chemical, and pharmaceutical properties and known PK/PD (i.e., exposure-response) and safety profiles. Differences or lack of differences in formulation composition, device attributes, and human factors considerations may also be known. For mAbs, the absorption is typically slow from the subcutaneous biospace, with a concentration maximum being reached in 2–8 days postinjection. The mAbs typically have limited distribution and long elimination (half-life of IgG antibodies is typically around 25 days). In this scenario, the need for a PK study is not only dependent on the type of change to the biopharmaceutical formulation, or change in device design potentially impacting delivery, but also to the therapeutic index (window) and safety margin of the drug for its intended therapy.

The impact of drug delivery differences such as injection time (e.g., 5–10 seconds) between an AI and a PFS is unlikely to affect the rate and extent of exposure. The risk of antidrug antibody formation as a result of device changes is unlikely affected by whether the biopharmaceutical is administered by PFS or AI. If the same dose is delivered by the same route of administration over similar injection time resulting in a drug that is delivered meeting the same critical quality attributes (undamaged or unchanged), then PK comparability should not be required. If there are any potential differences, then these can usually be addressed through simple PK studies using relative bioavailability criteria [4]. In some cases, sponsors have been successful in gaining FDA agreement to conduct the pivotal clinical trial in a PFS presentation to address questions of safety and effectiveness for the same biologic in the AI presentation. No additional clinical safety and/or efficacy data was required. In others (see chapter 34), FDA feedback drove the conduct of separate PFS and AI Ph3 clinical trials. It is expected that the PDUFA VI agreement on draft guidance for bridging combination products will integrate a science and risk-based decision-making approach that appropriately addresses this lack of consistency.

3.5 Leveraging Prior Device Platform Experience

With the continued maturity of device platforms, there is the opportunity to leverage previous experience for a like-drug and/or like-user group to demonstrate comparability for the established device in combination with a new drug component. Many biopharmaceutical companies are fortunate to have mature and established device platforms on the market in combination with other biopharmaceutical products. This valuable commercial experience provides the opportunity to reduce bridging studies by leveraging prior knowledge, experience, and real-world evidence for a like-device when used in conjunction with a new drug component. Under the right conditions, data derived from real world sources should be used to support regulatory decisions on combination products. Applying prior device platform knowledge in support of iterations within a device platform is essential to remove non-value added studies and streamlining development timelines.

Typically, sponsors evaluate like-product attributes from prior products for the drug constituent (molecule type), formulation (composition, viscosity, stability), manufacturing process, primary container/closure, and device constituent part (design to deliver accurate dose to correct biospace). These drug and device attributes are then compared to historical biopharmaceutical product presentations with the quality target product profile (qTPP) setting the boundaries for each constituent part, including all aspects of the combination product, user groups (intended disease area, patient population, and dosing), and use cases (self-administration vs. in-clinic HCP, frequency). These key product attributes are compared to determine the relevance of the prior knowledge database (clinical, nonclinical, post-market product complaint data) to leverage previous study data in support of the new product.

The prospect of avoiding lengthy and costly clinical studies by appropriately leveraging existing clinical device platform data has been generally encouraged by health authorities. Development of guidance on the appropriate application of prior device experience would be of great value as sponsors would not be in the position of "starting over at ground zero" with a new clinical plan when a new drug component is combined with an established device platform.

Of special note is the successful leveraging of prior knowledge for human factors testing, where referencing prior experience with like-product attributes and user groups is included in summative protocols submitted to FDA for review prior to execution. FDA has provided feedback that has reduced the study requirements for the summative validation study and even eliminated further studies. Sponsors have conveyed that FDA had concluded that prior knowledge from a like-product with respect to the drug solution attributes, device presentation, and user groups was sufficient to demonstrate the product could be used safely and effectively.

3.6 Real-Life Patient Handling

Real-life patient handling experience has been interpreted by sponsors as requiring clinical data to be submitted that shows home-use experience with the product. "Sufficient experience" is a phrase sponsors have received in written FDA feedback in response to bridging studies for combination products. This experience by the patient or caregiver, in theory, would identify if there was a significant unforeseen product failure mode or reoccurring use error that results in failure to successfully deliver the biopharmaceutical. The implementation of this requirement has been understood to mean that a clinical investigation is required to assess the ability of the device to deliver a full dose of investigational drug when used by the intended user as a home-use device.

Since submission packages already include (1) a summary of in vitro test results, (2) a report of the results of the human factors (HF) studies (Formative and Validation summaries), and (3) a summary of drug administration in clinical trials, sponsors have surmised FDA is focused on any potential physical, environmental,

or unforeseen issues that would only be experienced by patients taking an actual injection at a time and place of their choosing. These studies have largely been conducted in the open-label extension portion of clinical studies with self-assessment questionnaires but have also included modified patient self-administered in-clinic studies with clinician-reported assessments [2]. These are relatively small studies with the primary endpoint as successful self-injection as evaluated by the proportion of successful injections of the total attempted doses, self-administered by subjects. The limited secondary and safety endpoints may include adverse events, adverse device effects, and device failure analysis. As the primary drug safety and efficacy data had already been generated with the initial injection device, this requirement was not interpreted to include any patient data or drug safety or efficacy information, only safety issues specific to drug delivery with the new device constituent.

Sponsors have found that RLPH studies have limited ability to provide actionable usability data due to the inability to observe and query users on use errors and possible patterns of misuse from the data collected. HF studies are specifically designed to reveal use errors and patterns of misuse that a RLPH study appears to be after. Sponsors continue to question the true value of these home-use studies and whether they address any patient experience or robustness questions. In addition, the use of such studies for assessing device robustness by collecting devices that functioned normally is of questionable value given the extensive reliability, stability, and verification testing sponsors conduct during development. The preferred approach would be to explore augmenting established bridging studies (e.g., PK study, HF testing) to answer any outstanding questions concerning patient handling rather than conducting a new clinical home-use study. FDA has been open to reviewing justifications for not requiring such studies based on leveraging experience from use of a similar device in prior clinical studies, on-market experience with the same or similar device (e.g., complaints), or justification for alternative in vitro studies to demonstrate patient experience that will adequately address FDA's concerns and any potential risk(s).

3.7 Changes in Device Design Within Platform

Design changes within a device platform (e.g., spring tension strength for AI to adjust injection time) during the product life cycle take a similar risk-based assessment framework that is used for the introduction of a new delivery device. For a post-approval device design change within a platform, the evaluation would follow according to the quality management system. The justification for comparability study requirements and regulatory reporting pathway would be commensurate with the potential to adversely impact device essential performance requirements and/or biopharmaceutical critical quality attributes. Risk assessment and design verification/validation (if applicable) should be performed to confirm that the device meets the intended user needs following the change.

For these device design changes that are introduced after the pivotal Ph3 CTs or post-product commercial launch, the comparability exercise must be comprehensive and thorough as this is representative of the to-be-marketed product. This is different than for changes introduced prior to pivotal Ph3 studies, where clinical studies can use the to-be-marketed combination product. The complexity of the change is evaluated in context of the established injection system(s) and the risk factors associated with the final device functionality and its impact to overall drug exposure, safety, and efficacy. The evaluation may also include review of literature data and/or existing clinical data generated internally or through public sources (e.g., FDA summary approvals) for similar change and delivery system product types. The outcomes of these comparability studies that are initially evaluating product quality attributes may lead, depending on the potential uncovering of moderate differences to the drug and formulation results, to additional nonclinical and clinical studies. For a simple device constituent change, functional testing only may be adequate. For those device constituent changes that may affect how the combination product is handled or the rate of dose administration, HF testing would be performed. For complex device constituent changes, for example, to novel drug flow-path contacting materials and/or that could adversely impact drug disposition to the biospace, a PK comparability study may be needed.

4 Global Considerations

International regulatory requirements are demanding increased attention from combination product developers and manufacturers. There are also a number of emerging markets that are increasing their regulatory expectations for medical devices and combination products. The bridging points to consider, study design approaches, and data requirements described in this chapter are largely based on regulator's expectations in the USA. Although written guidance from FDA has been limited, FDA has provided recommendations and expectations through conference presentations and approval summaries that form the framework for FDA's approach for bridging requirements [2]. The data packages based on FDA expectations have largely satisfied health authorities outside the USA in global market submissions in support of building the comparability bridge. There have been no relative ethnic sensitivity concerns raised from any global health authorities regarding the country of origin of the test subjects for bridging studies.

A relevant global standard is ISO/DIS 20069 [5] which provides illustrative guidance for consideration in the assessment and evaluation of changes to drug delivery systems throughout their life cycles. Drug delivery systems within the scope of the standard include needle-based injection systems for medical use, aerosol drug delivery device design verification (requirements and test methods), and needle-free injectors for medical use. The standard in particular focuses on changes to the drug delivery system from entry into pivotal or registration clinical studies through the end of commercial supply. The standard does not contain prescriptive technical requirements for assessing and evaluating drug delivery system changes.

One global consideration for bridging data packages is the need for batches placed on stability to be in the final to-be-marketed presentation. For purposes of assessing biopharmaceutical stability, putting the semifinished presentation on stability can be considered equivalent to the final consumer package. However, placing stability batches in full consumer packaging may be wise as an insurance policy against specific country regulators insisting stability studies be performed on the finished medicinal product. A sponsor may be unable to convince a particular country regulator that the device component had no impact on biopharmaceutical drug stability and eventually have to rerun the stability in the fully assembled to-be-marketed presentation. Specific country reviewers have cited ICH Q1A(R2) Guideline (section 2.2.4) [6] as unambiguously confirming the requirement to carry out stability testing with final (consumer) packaging and the Good Manufacturing Practice Rules: Part I – Basic Requirements for Medicinal Products, Chapter 6: Quality Control (6.28) [7] as stating the necessity of performing follow-up stability studies of finished medicinal products in consumer packaging.

The new European Union Medical Device Regulation (EU MDR) (Regulation (EU) 2017/745) issued in May 2017 [8] amends the directive on medicinal products for human use (MPD) (Directive 2001/83/EC) [9]. An important amendment to the medicine legislation is the marketing authorization application (MAA) for an integrated drug-device combination product; Article 117 requires the Marketing Authorization Holder to provide a Notified Body (NB) opinion for the device constituent for combination products [10]. The regulations position notified bodies (NBs) squarely into the review process for drug-device combination products [11]. An unresolved concern in the implementation of the new requirements is what information will be the focus of the NB opinion as opposed to the integral drug-device combination product review conducted by the Medicines Competent Authorities.

Of note is the EU guidance regarding bioequivalence study requirements. The 2000 EU guidance on investigation of bioavailability and bioequivalence [12] states for parenteral solutions the applicant is not required to submit a bioequivalence study if the product is to be administered as an aqueous intravenous solution containing the same active substance in the same concentration as the currently authorized product. In the case of other parenteral routes, e.g., intermuscular or subcutaneous, if the product is of the same type of solution (aqueous or oily) and contains the same concentration of the same active substance and the same or comparable excipients as the medicinal product currently approved, then bioequivalence testing is not required. This supports the justification of no BE study needed in the bridging from a PFS to an AI when all other parameters are held constant.

5 Device Component Quality Attributes

Considerations of the tests and acceptance criteria chosen to define the delivery system specifications and quality attributes are included in the comparability bridging exercise to establish evidence that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes. For devices,

quality attributes describe the functional requirements, formally documented as design input or product requirements in the quality system, used to evaluate the performance of a system.

Design control processes require an impact assessment be conducted for any design change implemented during development (or post-marketing) [13]. This involves updating the risk assessment to incorporate the proposed change to determine potential failure modes and their impact on device performance or usability. The scope of testing is determined by which product requirements and/or user needs may be affected by the change, and this analysis drives the verification and validation (as needed) testing plan required to implement the change. The impact of the change on the device/combination product to maintain robustness is minimally assessed through functional performance testing, modeling/simulation work, and potential human factors assessments. For functional testing, the devices have undergone various conditionings (e.g., accelerated aging) to simulate product life-cycle scenarios throughout the lifetime of the device constituent part and stability testing of the combination product as a whole.

5.1 Release Specifications

The criteria should confirm that the specifications for the new device are appropriate to ensure combination product quality. Batch release tests cover both the final product presentation and the primary container/closure (e.g., semifinished prefilled syringe, prefilled cartridge). Tests for consideration may include glide force, break-loose force, flow rate, injection time (both time required to deliver the biopharmaceutical and amount of time that the needle is in the body and retracted), dose accuracy, and needle insertion depth. It may be appropriate to conduct additional complimentary bench testing to demonstrate that the device functions as intended. Design verification testing to assess the mechanical specifications of the device may include force required for assembly, force required to actuate the injector, needle bond strength (i.e., force required to pull needle off the injector), and needle penetration force (i.e., force available for needle insertion and to engage retraction mechanism).

5.2 Essential Performance Requirements

The essential performance requirements (EPR) for a device are to ensure the acceptable delivery of the dose into the patient. The EPRs for an injectable delivery device are to measure and assure the device can deliver the correct drug dosage to the correct biospace within the body. For AI and on-body delivery systems, EPRs include dose accuracy, injection time, and needle insertion depth. For multidose pen injectors and PFS, where in each case the patient determines the injection timing and needle depth, EPRs would be glide force and dose accuracy. An EPR testing

can be conducted as a batch release test (e.g., dose accuracy) or an in-process control test (e.g., needle insertion depth). Further opportunities are being pursued to determine if EPRs could be assured through a validation control strategy for a given device assembly process. FDA's current expectations in marketing applications are a traceability matrix for the EPR with a reference to the location of the verification and/or validation testing documents.

5.3 Stability Testing

When conducting stability to establish the expiration dating for the to-be-marketed product, it is recommended for new device platforms that at least one of the batches be put on stability in the relevant fully assembled drug/device system. For established device platforms in combination with a new biopharmaceutical, where there is validation data and study experience demonstrating that the device component has no impact on container closure integrity or drug stability, putting batches up in the device is likely not warranted. The need for one of the stability batches to be in the final presentation is less a scientific need than an insurance policy to address concerns from global regulators that we have not submitted a complete marketing application package. Stability conditions and testing protocols include both long-term shelf life and in-use conditions. The in-use conditions and testing mimic the in-use period of a device (e.g., 28-day multidose pen, 3-day on-body infusion pump) and demonstrate the method of injection (rate, shear force, injection pressure) does not degrade or contribute to the denaturing of the drug/biological product and does not affect stability, safety, or effectiveness.

5.4 Device Reliability

Reliability testing is referring to the service life of a device component of the combination product. The components, subassemblies, and final device undergo a preconditioning regimen to simulate storage and real use conditions that could endure over its lifetime. It's important to note the difference between drug stability in setting product expiry dating and preconditioning of device samples in assessing device reliability. The product expiry dating is based largely on the stability profile (and acceptance criteria) of the drug component and secondarily the impact on the device component to deliver the drug. Reliability testing is an assessment of the device to perform as intended over the product lifetime, not to set expiry dating of the drug. The preconditioning protocols, testing, and acceptance criteria focus on the functional task of device ability to deliver an efficacious dose. Test methods and acceptance criteria can be different than those established for setting expiry dating and are aimed at confirming the device can deliver a minimum safe and efficacious dose at the end of product expiry dating.

There is a recommendation within the FDA RA draft guidance [14] to collect "a small number of devices (e.g., 100)" to establish device robustness. This device robustness recommendation is of questionable value given the extensive reliability, stability, engineering confidence, and verification testing sponsors conduct during development. An industry group representing combination products have shared their concern that such a requirement should not be included in future guidance on combination product bridging [2].

6 Bridging Studies

The complexity of each separate drug and device component, in addition to the interactions of the two together as the combination product, necessitates a comprehensive set of study tools be at hand to fully evaluate combination product quality, safety, and efficacy. The testing scheme includes drug product testing, device functional testing, container closure primary packaging, drug/device interactions, compatibility of constituent parts, human factors, and clinical studies. This long list of available tests and studies requires a significant level of scientific expertise in both generating (the sponsor) and assessing (the regulatory reviewer) the necessary submission data packages. Disciplines requiring coverage include analytical chemistry, formulation development, materials science, engineering, design, usability, pharmacology, and clinical efficacy and safety.

6.1 Stepwise Progression of Bridging Studies

The stepwise additive approach employed for the setting of testing plans and data package generation in support of the device bridging leading to the to-be-marketed product is described in Fig. 3. The testing plan begins with patient tolerance of the device component (usually with placebo solution), primary container and closure attributes, impact of the device on delivery of the drug (i.e., drug through the device), and device design verification. Following these quality assessments, the user interactions of the combination product are evaluated, refined through formative human factors (HF) studies, and validated through a summative HF study. The stepwise testing strategy escalation then moves to increasing levels of clinical study plans to assure that the drug will be delivered safely and efficaciously in the commercial setting.

The stepwise approach highlights the many advantages of applying the "right tool" in answering the question being asked when demonstrating comparability bridging. The right study tool in this case is the test or study that will provide the most insight and efficient design to answer the comparability question(s) being asked. While a clinical study might at first be thought of as the "go-to" study for

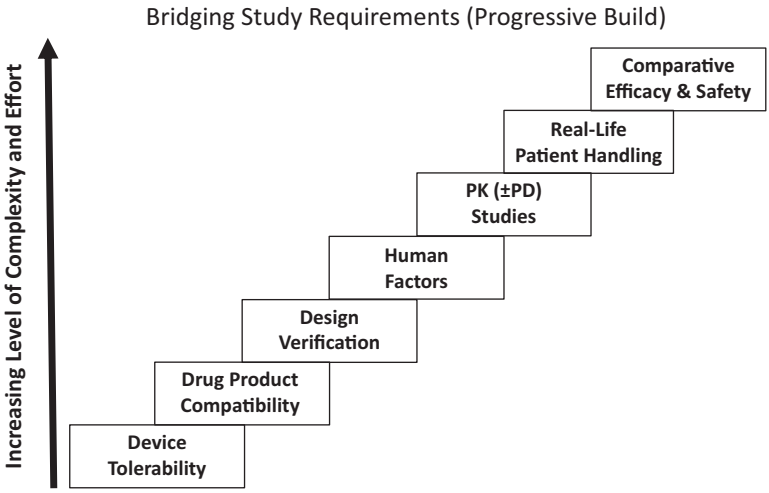

Fig. 3 Stepwise assessment of testing to support bridging

assessing combination product comparability, CTs may not necessarily be the "right tool" to answer the bridging question. In the case of patient-product use interaction concerns, a HF study would likely be the more revealing and cost-effective tool in answering a user interface question.

An impact analysis has proven a useful method in determining the "right tool" is employed for assessing the change to the combination product. This analysis focuses on the differences between the product used in pivotal trials and "to-be-marketed" combination product by making a side-by-side comparison of the potential differences in design, functionality, and user interactions. Then a risk assessment is performed for these differences to understand the potential impact on the existing dataset, considering the therapeutic window of the molecule, patient tolerability effects, existing experience with the delivery device and/or biopharmaceutical, and any HF data previously collected on the device. Targeted bridging studies can then be focused to winnow down to the necessary testing to answer the specific bridging question(s).

The stepwise bridging study categories as shown in Fig. 3 are described:

- *Device tolerability*: Evaluation of device when a tolerability study is needed for implementing new device platform technologies. Conducting a tolerability study early in program may help de-risk subsequent clinical studies. Device tolerability covers those aspects of delivery attributed to the device (leakage, raised subQ nodule, blister-like wheals) and not those attributed to the drug or formulation attributes (skin reactions). For injectable combination products, there is the added concern of specifically pain on injection that may occur immediately during delivery and then for some duration following the dose delivery. A minimal pain on injection experience is important to ensure product

adoption and continued patient compliance of their dosing regimen. This can also be a concern when patients are moved to different device platform (PFS to AI) for a given biopharmaceutical. It's generally accepted that some pain will occur, but not to the level that impacts product adoption or patient experience. The exercise is to quantitate the type of pain and the pain severity to ascertain what is tolerable pain. The pain visual analog scale (VAS) is a continuous scale intended to measure pain intensity, has been widely used in diverse adult populations, and ranges from "no pain" (score of 0) to "worst imaginable pain" (score of 100) [15].

- *Drug product compatibility*: Analytical testing of the drug product formulation to assure no adverse impact to (1) biopharmaceutical molecule or (2) increase in leachates/extractables from primary container/closure components when the biopharmaceutical solution is delivered through the device. For example, FDA has expressed concern of shearing of a mAb due to a perceived relatively fast injection times of large volume through AI (e.g., 2 mL in 5 seconds) and concerns of the sensitivity of the analytical methods to detect any molecular changes.
- *Design verification*: Ensuring the technical robustness/reliability of the device design. Design verification is an essential step in the development of any product as it ensures that the product as designed fulfils its intended purpose. The purpose of design verification is to confirm the design outputs meet the design inputs.
- *Human factors*: Usability assessments of device and instructions for use to demonstrate that the users can safely and effectively use the product in a commercial setting [16–19]. Aspects include the ability of users:
  - To read, understand, and follow instructions
  - To adequately set up the injector
  - To perform the injection or self-injection correctly
  - To dispose of sharps and other disposable materials safely and properly
- More on HF testing is described in Sect. 6.2.
- *PK*: For a drug product that exhibits PK variability that is consistent with typical variability (e.g., approximately in the range of 30–45% for a mAb), a clinical BE study (or comparability study (relative BA study)) can be powered to detect differences. For drug product that exhibits high PK variability or nonlinear PK, the risks may increase for building the comparability bridge between device components.
  - The clinical doses within the linear PK range are expected to pose a low risk to bridging outcomes. However, if the drug product exhibits nonlinear PK, the risk to bridging outcomes may increase.
  - An established device (already existing commercially and well characterized) that has shown successful bioequivalence with other drug products is expected to pose a low risk to bridging outcomes. Historical data may be leveraged for the PK study design.
  - Site of injection is not expected to impact PK; however, differences in PK profiles between injection sites (thigh, abdomen, arm) due to differences in absorption cannot be completely ruled out. For better study control, a health-

care provider typically performs the injections, and the injections should be restricted to a single specific injection site in bioequivalence or PK comparability studies.
  - For drug products with a wide therapeutic window (e.g., no maximum tolerated dose (MTD) or a drug product with at least 10x safety factor based on preclinical data), changes to exposure caused by differences in injection attributes have less impact on safety. However, if the therapeutic window of a drug product is narrow, changes in a device that increases drug exposure will potentially increase safety risks.
  - To reduce variability not related to differences between investigational products (drug product vs. device), PK studies should normally be performed in healthy volunteers, unless the drug carries safety concerns that make this unethical.

- *Real-life patient handling*: RLPH studies represent experience gained with users with the device in actual-use scenarios. Patient sample size is usually 50–100 with the information captured as rate of successful self-injections through a self-reported questionnaire. This experience can be obtained through actual home use as an open-label extension of the pivotal clinical trials that allows for self-reporting of successful injection in situation where normal distractions and use scenarios will naturally play out during the drug delivery process. As noted previously, the value of these studies continues to be debated between industry and regulatory authorities.
- *Comparative safety/efficacy trial*: Introduction of a novel device platform in combination with delivery of a new drug product may require a clinical bridging study to assess safety and efficacy per the commercial label claim.

6.2 A Word on Human Factors Testing

Special emphasis should be given to the important role Human Factors plays in building the comparability bridge for biopharmaceutical combination products. HF testing sits at the intersection of the progressive stepwise testing arsenal that begins with the benchtop analytical and functional testing prior to HF and then moves through to the more intensive studies requiring clinical trials (Fig. 3). In building the scientific bridge for a combination product, HF studies may be satisfactory in answering any remaining comparability questions, and no further CT bridging studies are necessary.

As clinical trial studies are more time-consuming and expensive, it is important to be absolutely certain that clinical studies are the appropriate tool to answer the question. Less expensive and more expeditious HF studies may be a more viable and appropriate study tool, certainly when answering user interaction question(s). HF studies are better suited for evaluating the product user interface and factors impacting the risk of medication errors. CTs generally do not provide the same insight on user interaction.

A similar argument can be made for the limited value of RLPH studies in the assessment of user interactions as compared to HF studies. The RLPH studies are not intended to evaluate HF. RLPH studies, which have largely been conducted as clinical home-use studies employing self-reported questionnaires, have limited ability to provide actionable user interaction data. Due to the nature of the home-use data collected, there is a general inability to observe and query users on possible use errors and limited ability to catch "close calls" or identify possible patterns of misuse. The design, execution, and information drawn from such home-use study self-reporting are much less comprehensive than for HF studies ("actual-use" or "simulated-use" designs) [16]. The limited value of RLPH includes inadequate test design and use limits and the inability to approximate worst-case scenarios, such as users receiving minimal or no training.

With respect to HF study design, the objective and purpose of running an actual-use HF study must be answered in context of what additional information is being gleaned that would not be addressed through a well-designed simulated-use HF study. The value must be addressed to justify the added time and effort compared to simulated-use studies. Actual-use studies may not be required to answer the question if no use-related risks are identified that would require such a study. Sponsors have been requested by FDA to supplement the comparability data package with an "actual-use study" to demonstrate that users could follow the instructions and the product quality is assured. However, simulated-use human factors testing may be a superior method in many scenarios for assessing potential use errors as compared to actual-use studies.

A recent development in meeting user interaction comparability bridging through HF study requirements has been the acceptance of an alternate HF data package. This package combines a formative HF study for the new combination product plus data/information leveraging prior knowledge for the device platform. This combined data package has been accepted in lieu of performing a summative validation study. Human factors is traditionally performed through a series of increasingly informative formative studies followed by a summative validation study. FDA has accepted a combined formative HF study plus prior experience with the device platform as demonstration of product use with justification for why a summative HF study was not needed. Leveraging of prior HF experience is of great utility and value, where predictions for a specific presentation can be gleaned from the prior knowledge gained from similar product presentations, user groups, and use environments.

7 Conclusions and Future Guidance

A well-designed combination product bridging program is essential for establishing effective and informative comparability assessments where a different device constituent is employed for the same biopharmaceutical and where the same device is employed across different biopharmaceuticals. The multiple study tools available

and numerous ways these studies can be combined into a comparability data package can lead to inconsistent application and setting of acceptance criteria if not appropriately applied. The significant variability in sponsor's bridging designs highlights the need for regulatory guidance in assuring the right bridging study is used to answer the question being asked. This will lead to establishing standard templates that ensure consistent submission data packages.

A science-driven risk-based approach is required to assure the most informative and efficient study plans are adopted in the bridging to the to-be-marketed combination product presentation(s). The extent of bridging study requirements is based on the risks associated with the identified gaps and level of change between the two combination products. Based on the risk assessment, there may be sufficient confidence that the risk of unforeseen problems or failures with the commercial combination product is low. This may focus on studies on analytical drug compatibility, functional device performance testing, HF testing, and prior experience with PK or other clinical studies that had previously been completed.

As delivery device platforms mature, the opportunity to leverage prior experience and incorporate real-world evidence becomes increasingly important in the demonstration of comparability for combination products. Regulators and sponsors have each obtained a tremendous amount of experience over the last several years for established device platforms (e.g., PFS, AI) to the point that prior knowledge could be provided that would negate the need to collect additional clinical data.

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Chapter 33
Design and Development Considerations for Autoinjector Delivery Systems: Technology Developer and Industry Perspectives

Lin Laurusonis, Ian Cleathero, and Hans Jorgen Jensen

1 Introduction

The options for delivery of biologic products to patients are heavily influenced by the very nature of those products; oral delivery is generally ruled out because the biologic product will be digested in the enteral tract and hence lose effectiveness. Therefore, the majority of biologics are delivered by injection of a liquid formulation. Many of the conditions treated by biologics are chronic, requiring regular dosage of the product, and therefore there is a clear desire to encourage self-administration by patients or administration by caregivers who are not necessarily healthcare professionals skilled in administering injections. In the development and commercialization of biologic products, these factors have fostered the development of delivery systems that allow patients and caregivers to administer injections reliably and safely without needing special skills or advanced training and without significantly disrupting the patient's daily routine. The majority of such devices are generally pre-filled with the formulation to be delivered, are single-use, disposable and deliver the formulation subcutaneously. Perhaps the simplest embodiment is a pre-filled syringe, where a single dose is supplied in a syringe, often with a pre-attached needle, and the user performs an injection much as a healthcare professional would do. However, in order to overcome the challenges of self-injection, such as the skill required, anxiousness about needles and self-injection, prevention of sharps injury and hence certainty of outcome, a pre-filled syringe (or indeed another form of primary container) may be assembled into a device that automates a number of the

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steps required for a successful injection. Such devices are generally known as “autoinjectors” (AIs). These products are known as “combination products” in the USA (being a combination of “biologic” and “device”), and whilst this term may not be used in a regulatory context throughout the world, for all practical purposes it is useful to consider autoinjectors in the same way.

A combination product is a product composed of any combination of a drug and a device; a biological product and a device; a drug and a biological product; or a drug, a device and a biological product. For the purposes of this chapter, we will focus on the combination of a drug and the device. The key challenge in developing such products is the coming together of diverse perspectives: (1) drug product development and device development, (2) pharmaceutical scientists and device engineers, and (3) pharmaceutical development (ICH/GMPs) processes vs. design control-driven processes. The challenges are exacerbated by the fact that the device development is typically not initiated until clinical proof-of-concept and a better understanding of the dose is defined; this typically happens at the end of Phase II or concurrent to Phase III. This situation can result in expensive and time-consuming iterations, if device requirements of the combination product are not clearly captured.

In this chapter we will describe some best practices and approaches to drive patient-centric and timely development of the combination product. We describe considerations, options, and process (including technology and partner selection) when developing a combination product consisting of a liquid biologic formulation and an autoinjector device, with the aim of providing guidance to the reader for developing such a product. Here we are concerned with development of the device element and assume that the biologic formulation in its primary container is an input; its physical and chemical properties are already defined, and the route of administration, dosage, frequency of administration, patient population and so on are assumed to be known. Development of a device for the delivery of a small-molecule (non-biologic) drug product would generally follow very similar lines, and therefore the guidance could be equally applicable to such products. Taking this into account, and for simplicity, the liquid formulation to be delivered will be referred to as “drug product” or “DP”.

2 Autoinjector Definition

Typically, an AI will contain a pre-filled syringe with an integral needle, which will contain the drug product, and will house a mechanism that enables insertion of the needle to a pre-defined depth in the patient’s skin, delivery of a pre-determined dose of the DP, retraction of the needle, protection of the needle to prevent needlestick injury, and disposal. However, there are many variations upon each of these factors, so definition of “autoinjector” becomes an extremely challenging notion. This challenge was recognized during the development of ISO 11608-5:2012, Needle-based injection systems for medical use — Requirements and test methods, Part 5:

Automated functions. The technical committee responsible for this work embarked on a path intended to develop a new international standard for autoinjectors, but it was quickly realized that the term "autoinjector" could cover many different types of device with different features and different levels of automation. As discussed briefly in Chapter 30, it was therefore impossible to limit the scope of the standard to one particular class of devices without excluding others, which would then be left without guidance for developers. In order to address this, it was agreed to dispense with the term "autoinjector" to define the scope of the standard, and instead to focus on individual features that may or may not be automated in a particular device, which approach led to the creation of the aforementioned Part 5 of the ISO 11608 standard series concerned with "automated functions". In order to claim compliance with this part of ISO 11608, a manufacturer must demonstrate that a device complies only where a particular feature or features are automated.

For the purposes of this chapter, we shall consider an autoinjector generally to be a device with the typical features and functions listed in Table 1.

The following additional features, which may be automated if included, are defined in ISO 11608-5:

- Drug product preparation (e.g. reconstitution)
- Air removal
- Priming
- Dose setting
- Disabling the device after use
- Needle hiding (note this is different to needle shielding, described above; it refers to obscuration rather than sharps protection)
- Needle removal

It is difficult to limit the features that could be incorporated in an autoinjector, and indeed to do so may stifle innovation. Limiting the features and level of automation for the purposes of this chapter is done only to avoid complexity.

Usage steps for a typical autoinjector as defined in Table 1 are given below:

- User removes cap from AI, which also removes needle cover from syringe, and removes any other cap or safety catch.
- After disinfecting the chosen injection site, the user applies AI to the injection site and actuates or "fires" the AI. Actuation may be accomplished automatically (by pressing a button or by pushing the AI against the skin) or by pushing on the AI to insert the needle to a pre-determined depth at which injection will be initiated automatically.
- AI delivers DP automatically over a duration specified within the product label and instructions for use.
- Needle is retracted either automatically by AI or manually by user lifting AI from injection site, with needle shield extending and locking to prevent access to needle.
- User disposes of device

Table 1 Typical autoinjector features/functions and usage steps

Feature/ function	Typical example	Examples of alternatives and additional functions
DP container	Pre-filled syringe (PFS)	Cartridge, user-filled syringe
Needle	Integral with DP container	Fitted by user
DP container closure	Rubber needle shield (with or without rigid shield)	Rubber tip seal (PFS without needle); rubber septum (cartridge)
Inspection of DP	Viewed through window in device	For user-filled, inspected in syringe or vial before loading
Preparation for injection	User pulls off device cap, which pulls off needle shield	Fit needle to PFS and load to device; set needle depth and/or dose if selectable; remove dedicated safety cap or clip
Needle insertion	User pushes against injection site prior to one of the following: (a) Needle inserted manually to injection depth by user's hand force (b) Needle inserted to injection depth automatically by device power source (c) User pushes button and needle inserted to injection depth automatically by device power source	Conventional manual needle insertion (as with syringe and needle)
Injection of medication	Automatic injection initiated when needle reaches required depth	User initiates automatic injection by pressing button; manual injection by pushing plunger
Needle withdrawal	(a) User withdraws manually when end of injection indicated (by audible click and/or visual means such as color appearing in window) (b) Needle retracts automatically into device at end of injection	User withdraws manually without indication of end of dose (time-based, typically 10–15 seconds); needle retracts automatically, initiated by user starting to lift device from injection site
Needle protection post-use	(a) Needle shield extends and locks during needle withdrawal (b) After automatic needle retraction, needle is locked inside device	No protection; manual needle shield extension
Power source	Coil spring or springs	Compressed or liquefied gas; gas spring; electrical drive; combustion

3 Autoinjector Examples

Table 2 shows a selection of autoinjector products that have been commercialized in various global markets with a comparison of some features. Note that the EpiPen as shown here differs from the other devices and the description above in that it contains a cartridge rather than a PFS and the needle emerges from its protective sheath as part of the injection process, with the safety cap being at the opposite end. It also

Table 2 Comparison of feature sets of several commercially available autoinjectors

Product		Route of administration	Volume delivered	Container	Needle insertion	Needle retraction	User steps to actuate
EpiPen®		Intramuscular	0.3 mL (contains 3 mL)	Cartridge	Automatic	Manual	2
Humira®		Subcutaneous	0.8 mL	1 mL PFS	Automatic	Manual	4
Benepali®		Subcutaneous	0.98 mL	1 mL PFS	Manual	Manual	2
Simponi®		Subcutaneous	0.5 mL/1 mL	1 mL PFS	Automatic	Automatic	3
Cimzia®		Subcutaneous	1 mL	1 mL PFS	Automatic	Automatic	2
Enbrel®		Subcutaneous	0.98 mL	1 mL PFS	Automatic	Manual	3
Actemra®		Subcutaneous	0.9 mL	1 mL PFS	Automatic	Manual	3
Cosentyx®		Subcutaneous	1 mL	1 mL PFS	Automatic	Manual	2
Trulicity®/Taltz® / Emgality®		Subcutaneous	0.4–1 mL	1 mL PFS	Automatic	Automatic	3

delivers a small-molecule (non-biologic) drug product but is included as it is probably the best known autoinjector on the market and has been in use for many years, albeit in slightly different versions, and is therefore a useful and interesting comparator for some of the newer products.

4 Design Considerations: How Do You Know What to Design?

There are two distinct parts to developing an autoinjector combination product; one is the drug product in its primary container (hereafter referred to simply as the drug product), and the other is the device. Whilst this may seem obvious, it is important that the distinction is recognized by those responsible for each and that the different disciplines have an appreciation for the processes employed and challenges faced by the other. In order to embark upon the development of an autoinjector device for a particular application, it is clearly important to understand the parameters within which it must operate, and these form the basis for "design inputs". As we are concerned here with development of the device element of the product, the drug product is considered to be an input and therefore its properties may already be defined. In fact, this is often the case because investment in device development may be deferred until after clinical proof-of-concept at which point the properties of the drug product (concentration, viscosity, injection volume ranges) are known and cannot be changed without significant delay to the project timeline. This is one advantage offered by AI platform designs (see discussion below) because the same design, with minimal customization, can be used with more than one asset, and for a given asset, the timeline for development can be streamlined.

As far as the drug product is concerned, design inputs will therefore include the following elements:

- Volume of DP to be injected – in other words, the dose
- Depth of injection required (subcutaneous or intramuscular)
- Viscosity of DP, including the viscosity/temperature relationship and shear rate dependence
- Primary packaging of DP (pre-filled syringe/vial, etc.)
- Needle dimensions
- Injection time required (minimum and maximum)

During development of a new drug product, there is usually uncertainty about many of these factors until completion of at least phase 2 clinical trials. It is therefore highly unusual for investment to be made into a delivery system at an earlier stage without certainty of technical requirements and commercialization according to clinical proof of concept. This leads to a common strategy of filing drug product marketing applications in a vial or pre-filled syringe presentation and following up with a combination product filing at a later stage, possibly after market launch. Considerations around such strategies are discussed in the next chapter of this book.

In addition to the drug product requirements, the needs of the patient and users must be considered. It is important to note that whilst the patient may be a user (in other words self-injecting), the needs of other users, such as healthcare professionals and caregivers, must also be considered. Below are some examples of such needs and how they will apply to design inputs:

- *Patient/user demographics:* Factors such as age and cognitive ability are important, as with any product, but an autoinjector which is intended to treat a medical condition must consider the effects of that condition on both physical and mental abilities to use the device. Therefore, the device must be designed in order to make its use as easy and reliable as possible for those users. An example of this is the "AutoClicks" product which delivers Cimzia for the treatment of autoimmune diseases such as rheumatoid arthritis; it has a large body with a soft surface to help those users with compromised dexterity to grip and operate it. Consideration of user capabilities is complicated by the fact that there may be more than one indication for a product, with different conditions having different typical challenges and furthermore by typical comorbidities such as poor visual acuity frequently being typical with people suffering from diabetes.
- *Delivery regimen:* Drug product delivery frequency varies significantly from product to product, and that frequency will in itself influence the circumstances in which the delivery is administered. For example, if a patient requires infrequent injections (every other week or longer), then it is likely that they will plan or schedule the injection and set aside time in their own home for it whenever possible such that the environment will be relatively controlled, predictable, and comfortable. However, if delivery must be undertaken once or more per day, it is likely that the patient will need to operate the device in different environments depending on their day-to-day routine, making it necessary for the device designer to consider more diverse usage conditions. On the other hand, frequent use is highly likely to make the user much more familiar with the operation of the device, whereas infrequent use may lead to challenges of unfamiliarity – quite simply forgetting how to use the device between doses.

5 Developing an Autoinjector Combination Product: Pharma/Device Partnership

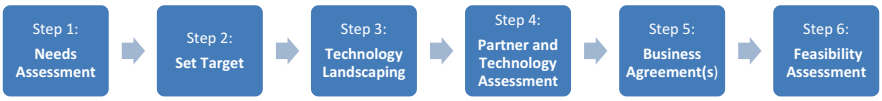
Many pharmaceutical companies rely upon partnering with device companies to access drug delivery technologies. While the delivery system technology, its stage of development, and extent of customization required are key factors in the "technology" decision, the assessment of the partners' capabilities including business and operations-related criteria by key internal stakeholders needs to be integrated to form a balanced and long-term view of the potential relationship. A pharmaceutical company must select not only the best/most appropriate device technology but also a partner that the pharmaceutical company can work with throughout the life cycle of the combination product. Working with a device supplier on a device program

adds complexity and risks to a combination product development program as the pharma company becomes dependent on the capabilities of the partner. Factors such as availability of resources from the supplier to support the customization of the device, experience in development of critical device data and documents for regulatory submissions, project management capabilities, openness and willingness to share risks and design concerns and issues, "cultural fit" between the organizations, and management commitments are highly important factors in any collaboration. These "collaboration factors" along with a thorough technical evaluation are critical elements to test in a feasibility study with a prospective partner prior to entering into a major development program.

In what follows, we discuss the key steps of a device technology landscaping and partner selection process using autoinjectors as the example. Executing such a process ultimately drives organizational alignment in the final technology and partner recommendation.

6 Technology Landscaping and Partner Selection

Selecting the right technology and the right partner is of utmost importance. Equally important is to ensure that the broader organization understands what goals and objectives the product development team has as well as insuring their buy-in to the product development team's recommendations for technology and partner selection. A technology landscaping and partner selection process can be used to select the right technology and facilitate cross-functional agreement on a partner recommendation. It consists of the following steps:



The first step of the process starts with obtaining a general understanding of an unmet need, business imperative, and/or specific technology needed to satisfy predefined product or performance requirements. The fundamental question being asked at this time is as follows: "What is the targeted area you are exploring?" The individual(s) participating in the landscaping effort and/or partner selection process should familiarize themselves with the products and companies involved within the targeted area to get a general understanding of the magnitude of the research that will be involved. In some cases, there may be a small number of options to consider, while in others the number of potential options may be significant.

In the case of autoinjectors, there are many drug delivery device companies with multiple technologies and/or product offerings. Additionally, there are consulting firms who have developed autoinjectors from a concept and a blank sheet of paper.

All of the technology options exist at various stages of maturity and need to be evaluated individually to determine which technology can achieve the top-/highest-

level requirements defined within the target product profile (TPP) and understand the potential risk level that each option carries. The TPP is a document which is intended to define the highest, top-level requirements and features of the combination product. Once the method and route of administration are determined, a second, more detailed device target product profile (dTPP) with additional device-specific detail can be created (Step 2 of the process). The dTPP would contain a refined level of requirements and key technical criteria which must be achieved. Information such as the injection volume, viscosity, required functionality, and specific features (if known) is described. This level of detail extends well beyond the TPP of the drug product, and ultimately the dTPP becomes a source document for product requirements or design inputs. These requirements contained within the dTPP are then used to perform a technology landscape assessment.

7 Team Formation

At this point, a cross-functional team responsible and accountable for executing the technology and partner selection process should be formed. Lessons learned from previous technology and partner recommendations suggested that the following functions should be considered:

Device R&D *(lead)*	Quality assurance	Legal/licensing
Purchasing	CMC-regulatory	Legal/intellectual property
Supply chain	Formulation development	Marketing
Manufacturing operations	Clinical development	
Equipment engineering	Clinical PK	

It is beneficial to identify “core” and “extended” partner assessment teams. The Core Team will actively manage engagement with technology companies and ensures the Extended Team is consulted and provides critical input to be considered and ratification of any critical decisions.

It should be noted that being inclusive and having cross-functional team participation even at the early stages of the process helps ensure high levels of collaboration and organizational alignment of the recommendation that the team will ultimately be making.

8 Landscaping and Partner Assessment

The technology landscape assessment (Step 3) consists of identifying any and all technologies which achieve all, most or some of the characteristics described in the dTPP. A few sources for this information are the Internet, databases available via a subscription, direct inquiry with technology owners, trade shows, etc. One benefit of this effort is for the team to start familiarizing themselves with the potential

options and features of existing devices. Additionally, the team will learn from the process by better understanding the current state of technology and any potential technology limitations. It is noted that device technology landscaping is an ongoing effort by the device R&D group for core delivery systems that support the pipeline; however, the value of completing steps 1 and 2 is they inform and direct the scope of the landscaping efforts.

All of the information highlighted above is entered into a technology and partner assessment matrix/spreadsheet (Step 4) which helps organize the information for future reference as well as provide a means for calculating relative ratings or scores for each of the device technologies and provides an objective, semiquantitative assessment to guide the debate to select the most appropriate partners for the particular product need. The following information should be gathered as part of the process:

Assessment criteria/questions: These are key assessment criteria and questions which ideally differentiate one technology and/or partner from another. They also include important business criteria which may influence the decision to engage a potential partner.

Categories: This is a label for a logical grouping of questions which are being asked. Categories of questions may include technical assessment, business, manufacturing, quality/regulatory, etc. Each category will be assigned an overall weight such that no one category over-influences the assessment simply by the number of questions that are being asked within a specific category. Table 3 pro-

Table 3 Technology and partner selection categories and example criteria to be assessed

Category	Assessment criteria/questions
Technology/design concept	Criteria defined based on the technology, design requirements, specifications, and user needs Evidence that product requirements can be met Robustness/reliability of design, evidence of design for manufacturability Container/closure and fill and finish process
Development experience	Device development experience/capability Depth and breadth of engineering staff Project management, Human Factors Engineering (HFE), industrial design Existing technology platform for customization
Manufacturing	Capability/capacity of manufacturing (Mfg) sites or preferred Mfg partners Supply chain sophistication, flexibility Supplier/vendor management and controls Post-launch support – sustaining engineering, complaint management
Quality/regulatory	Capability to serve as legal manufacturer/certifications Regulatory submission experience, Marketed products Perceived strength of quality system – via on-site quality audit Health authority/notified body audit results, ISO 13485 cert
Business	Estimated Cost of Goods Sold (COGS), feasibility/development timeline, and cost Intellectual Property (IP)/Freedom to Operate (FTO) – flexibility with joint ownership Size/scale of company and financial stability Intangibles - responsiveness, cultural fit, *transparency*

vides an example of the categories and technology-agnostic assessment criteria/questions associated with each.

Scoring guidance: It provides clear guidance on how specific responses from the potential partners should be rated. The scoring guidance includes a specific rating for the expected range of anticipated responses which will be collected.

Criteria/question and category weighting: Each criterion/question should be assigned an "importance" rating. Historically, a rating system of 1–5 has been used successfully and is recommended. Alternative rating scale could be used but should be discussed and rationalized within the Core and Extended Teams.

Exclusion criteria: These criteria are crucially important. Exclusion criteria are the ones that if not met, the partner is immediately disqualified from consideration regardless of their scoring. These represent the unconditional "must-have" items for the technology and/or partner. Scoring guidance should be provided for the exclusion criteria as well. The team should clearly define what is absolutely required to be considered as part of the assessment.

Once the categories, questions/criteria and scoring/weighting guidance have been defined by the cross-functional team, the needed information should be collected and entered into a partner assessment matrix. A Pugh matrix is one tool that can be used to objectively compare different options with disparate qualitative data inputs. Some information may require a Confidential Disclosure Agreement (CDA), and some may not. The team should populate the partner assessment matrix with as much available information as possible (from public sources and knowledge gained from past landscaping efforts) to start narrowing the potential technology and partner list. In the authors' experience, it takes time and effort to proactively identify the questions, assessment criteria and scoring and weighting guidance, but this pays dividends in the efficiency and ownership in reaching an aligned partner recommendation by the cross-functional team.

9 Business Agreements

Once the cross-functional team has agreed on the technology(s) and partner(s) to advance into feasibility, appropriate agreements (Step 5) need to be established (e.g., CDA, material transfer agreement, feasibility agreement, etc.). As the technology and partner assessment process is being executed, the technical team should continue to flesh out areas to be explored during feasibility. A key element of the feasibility agreement, beyond the legal aspects of the agreement, are the activities and deliverables which encompass the technology assessment itself. These items are described within a scope of work (SOW) document which is described in greater detail within the next section.

10 Feasibility Assessment

Drug delivery technologies typically require some level of customization to achieve the requirements specified in the dTPP. In many cases, program timelines and risk levels direct pharmaceutical companies to select a proven or low/manageable risk option taking into account some level of customization for the drug delivery device. Feasibility is the time to assess all of the information provided by the potential partners and confirm their claims relative to the criteria in Table 3. The SOW included in the contract is developed collaboratively between the pharma company and prospective device partner. The SOW should contain a description of the goals and objectives of the project, an equipment and/or tooling summary which will be used to produce device components during the feasibility study, engineering confidence testing and technical reports to be delivered, legacy technical documentation to review, audit or review of quality system documentation, and any other activities to be completed during feasibility. Also to be included are the cost and fee structure, a timeline including start date, major milestones, submissions (if applicable), any applicable working assumptions, as well as any exclusions which describe work considered outside of the scope of the study. From a technical perspective, it is critical that the deliverables and expectations are developed collaboratively between the pharma and device technology partner. Time spent working with your partner in developing and clarifying the deliverables will eliminate potential issues and concerns after the feasibility efforts have started. It also allows both partners to better understand one another and establish a common terminology, which may sound trivial, but the impact cannot be understated. When developing the deliverables, one should list the deliverable with a detailed description, explicitly state the purpose of the deliverable, identify which decisions, milestones, and payment gates it supports, and identify the acceptance criteria and timing for completion. Once the agreement is fully executed and feasibility commences, many "soft" pieces of information can be realized (e.g., how responsive is the partner, how open/collaborative is the partner, does the partner share the necessary values for success, how hard is it to work with the partner). If the technology proves to be appropriate but the feasibility experience was horrible, do you move forward into development? Probably not.

11 Platform Technology Approach

From a technology developer's perspective, to be selected as a supplier or partner for pharmaceutical companies is a challenging and difficult but critical undertaking. Historically, drug delivery device companies have traditionally been able to develop and market, for example, autoinjectors approved through a premarket notification route (510(k)) for the US market. Such devices were developed through little or even no interaction with pharmaceutical companies, and there were no agreements in place between the companies on either use of the device or drug products. Today's regulatory environment and human factors expectations require drug delivery

devices be tested with specific drug products and user populations. This means that almost all drug delivery devices will be treated as combination products and will require device and pharma companies to work together on achieving this. Pharma companies can have different approaches as to how to select suppliers/vendors for an autoinjector – this will vary from company to company including the maturity of the pharma company's device development function.

Irrespective of the process, a supplier has to demonstrate that it has capabilities that will fulfill the needs of the pharma company seeking a drug delivery device. Most pharma companies expect a potential partner to have at least one "platform" device (ideally a commercialized, proven technology) within its offerings for reasons stated in chapter 26 (e.g. faster development time and lower costs). For the sake of this chapter, a platform autoinjector will be defined as follows:

> a platform autoinjector is a device typically designed for an initial set of drug product specifications such as a specific syringe, fill volume, needle, drug viscosity and delivery time, which, within certain limits, can be customized to meet different specifications for different drug products

Platform devices can be of various maturities, ranging from early industrial design sketches/renderings showing the outer shape of an envisioned embodiment of a device to already approved and launched devices. Some suppliers will offer both mature and early device designs as a way to increase their range of offerings and increase the chances of meeting various customers' market needs for different devices. As detailed in other sections, different drugs, therapies and user groups often have needs for different features of a device to meet different user populations and user capabilities as well as differences in drug product-related parameters such as primary container, needle size, injection depth, drug viscosity and drug delivery time. When assessing supplier capabilities, the actual maturity of their offerings must therefore be carefully examined in order to obtain a true risk assessment of their device technology.

The term "within certain limits" used above in the platform definition is not a standardized term, and it is on purpose vaguely described, as it differs from device to device and from supplier to supplier, but may be an important element when selecting the right technology and device partner to work with.

In the following section, a more detailed description of the customization elements that should be included in any platform AI device assessment is made.

Syringe Historically, most autoinjectors have been using 1 ml, long glass syringes with staked needle (typically ½ inch, 27 gauge or a few devices, 29 gauge). As a high number of pipeline biologics require higher doses, BioPharma companies are looking for autoinjectors capable of delivering more than 1 ml; hence using a 2.25 ml syringe is becoming a need for the suppliers to address. For most autoinjector platforms developed for a 1 ml long syringe, this will require significant changes to a platform device, as the syringe is substantially larger in diameter (10.85 mm vs. 8.15 mm). The probability is high that the supplier has optimized parts holding the 1 ml syringe in place as well as kept the outer shape of the autoinjector small for better portability, storage, etc., hence requiring many parts of the autoinjector design

to be redesigned for a 2.25 ml syringe. Supporting the use of syringes from different suppliers (e.g., Becton-Dickinson, Gerresheimer, Ompi) and different types of flanges (cut, round, small round) may also require substantial design changes for a platform device, and also newer material syringes such as plastic syringes made of cyclic olefin polymers can drive the need for customization.

Insertion depth The needle insertion depth for an autoinjector is typically determined with input from the pharma company and the physical design of the autoinjector, whereas the needle insertion depth when using a PFS can be as deep as the length of the needle (typically ½ inch needle is most often used). The needle depth offered by the platform device must be carefully examined and may require a pharmacokinetic study or other assessment to demonstrate that needle penetration depth does not affect the bioavailability of the drug product, particularly if the autoinjector is introduced as a supplementary application to an already-approved drug product delivered in a PFS. Flexibility in the platform design for offering different needle depths should in this case be an element in the selection/decision process.

Needle shield type Most autoinjectors will have a "cap" added to the design that interacts with the PFS needle shield, such that when the cap is removed by the patient, the needle shield is also removed from the syringe. Different types of needle shield, ranging from soft to rigid needle shields, are available from a number of suppliers, and redesign of the cap/needle shield interface may be needed, if a needle shield different from the preferred or supported needle shield from the autoinjector supplier needs to be selected by the BioPharma company. A redesign of the rigid needle shield is typically viewed as a last resort option by pharma companies and is not preferred by syringe manufacturers due to a customization of a high-volume product line and associated costs and timelines for the customization.

Dose volume A challenging aspect for a number of autoinjector platforms is that most devices must be changed to accommodate various fill volumes. For a drug in development, the fill volume will only typically be decided in a phase IIb dosing study, and that may only be true for the first indications for a drug. Many biological drugs will be adding new indications following initial approval, and some of these new indications may require different dose volumes. Also for an autoinjector platform, having a device that can "easily" accommodate different fill volumes with minimal (or even no) customization can be highly important where the dose volume is not finally decided until late in the development process. This also holds true in, for example, dosing studies or other blinded studies with different dose volumes, where investigators (and patients) should not be able to distinguish between different presentations being given to patients.

Delivery time As all autoinjectors will be used to deliver a bolus injection, the injection should last a maximum of 10–15 seconds because it is generally considered to be reasonable to expect a user to hold the device in place for such a time. Most conventional autoinjectors over the last 20 years or so have been developed to deliver "water-like" viscosity drugs, but a number of new biological drugs and depot injec-

tions in pipeline typically have much higher viscosities. The alternative to higher viscosities will be to deliver a higher dose volume than can typically be administered with an autoinjector (as, for example, the on-body delivery system Pushtronix for Repatha, which delivers 3.5 ml in over 9 minutes). As the forces increase to deliver viscous drugs in conventional spring-based autoinjectors, risks associated with breaking the primary container or the components of the autoinjector increase as well. Therefore, suppliers are compelled to innovate solutions to address such issues for specialized "high viscosity" autoinjectors to be adopted by the industry.

Delivery time is derived primarily from the forces applied to the stopper inside the syringe, the needle gauge and length used, the volume to be delivered, the diameter of the syringe and the viscosity of the drug product. Forces to overcome the break-loose forces of the stopper as well as the gliding forces within the syringe also contribute to the forces that need to be applied to the stopper to deliver the drug product in the desired delivery time. As the break-loose forces of the stopper typically increase during long-term storage of the syringe, such parameters must also be taken into account.

Many factors affect injection pain including dose volume, injection depth, injection time, formulation characteristics, etc. Some data suggests that injection speed does not affect perceived pain, some data suggests that delivery of a more viscous drug product actually reduces perceived pain, and a study showed that even injection of larger volumes (>1.2 ml) does not provide unacceptable perceived pain when delivered as a bolus injection [1–3]. As a result, published data and conclusions may be confusing and/or confounding, and conducting an assessment of injection pain during clinical development may be beneficial.

As the final dose, and hence the volume of the drug to be delivered, may only be known at a relatively late stage, it is relevant to consider the importance for flexibility of a selected autoinjector platform. Conventional autoinjectors using compression springs are limited to changing the spring forces (which typically requires redesigning a number of components), electromechanical-driven syringe pumps (using a motor and gear train to push on the plunger rod) can easily be designed to support different force profiles but are significantly more expensive than spring-based devices, and finally some autoinjectors use gas as driving force and can simply use a different gas with a different pressure to adjust the delivery time once the other mentioned parameters are decided.

12 Trade-Offs of Platform Devices Versus Bespoke Device

So what are the main benefits of selecting a platform device vs. developing a bespoke device?

FTO An important factor is the so-called freedom to operate (FTO) assessment to determine whether a device and details of the device are already protected by third-party intellectual property. Confirming your design is not infringing on existing

intellectual property, or negotiating an access deal for existing intellectual property, can be a costly, time-consuming process and may impact a development project. Most suppliers with commercialized devices will already have determined that their solution has FTO, which may also prove true for platform suppliers who have developed the core technology and demonstrated the FTO over a period of time.

Development time As stated in Chapter 26, and depending on the maturity and fit of a platform device to the needs for a BioPharma company, development time will almost certainly be significantly shorter when using a platform device. It will of course depend on how "appropriate" the selected platform device is for your needs, but assuming these factors as described in the previous section, you will already have short-listed some platform devices at this point that may be customized for your needs. Development time can of course vary, and how much the development time impacts on your decision process is defined by the timeline requirements that need to be aligned to your drug program timelines.

Development and unit costs Development costs may in a number of cases be prohibitive to selecting to develop a bespoke device, as you may need a delivery device for an orphan drug with limited market size potential, for example, which simply cannot justify the high development costs of developing a bespoke device. Finding a suitable platform device may be the only option available in this case, and it may become more a question of ensuring the selected device will match the technical minimum requirements than getting the "optimum" device for your drug product. Unit costs may also not be as low as if you develop a bespoke device, and this factor should be assessed in the business case and be part of the selection process for the right platform device.

Device size and form factor One area that must not be underestimated or overstated is the critical need for conducting mandatory human factors (HF) engineering processes required for any drug delivery device/combination product. Depending on the studies and user groups for which a platform device may already have been investigated, and availability of using such HF study results, this task may be more or less complex. A larger device may not necessarily be a disadvantage, as it may provide a better "grip" than a smaller device for particular user groups. Recent approvals and introduction of larger delivery devices (such as the SelfDose™ injector from West Pharmaceuticals) seem to be very focused on usability and ease of use for groups such as elderly patients with dexterity issues instead of being small and slick design. For other patient groups and therapies, a small form factor allowing users to more discretely carry and use the device will be more important; hence suppliers of platform devices must be aware of such needs in their offerings.

Design risks Autoinjector devices are in many cases only considered and introduced later within development programs (typically later than Phase II(b) dosing studies) and, depending on needed customization of a platform device to suit the minimum device needs, may quickly become the critical path for a drug development program. If the delivery device is critical to the launch of a new drug product, minimizing the risks may be one of the most important factors for a BioPharma

company. Platform devices already marketed and existing in various customized versions should have less design risks compared to less mature designs that are still in development. Such platform devices should also have more reliability data available from design verifications and design updates driven by product complaints experienced during post market surveillance, all leading to lowered technical risks.

13 Future Challenges

The number of biologic products in development is growing rapidly, and given their generally well-tolerated safety profiles, there is a distinct trend toward increasing efficacy by increasing the dose through increased volume of injection, concentration, or both. In addition, efforts continue to minimize the burden on the patient and healthcare providers by increasing time between injections; this can be done by increasing the dosing volume vide supra, using sustained release formulations, or by engineering molecular properties into new biologics to increase their pharmacokinetic half-life. These factors lead to challenges for delivery systems of the future in terms of delivering higher volumes of potentially higher viscosity formulations with comparable or improved usability features.

- *Delivering higher volumes*. As has been pointed out, currently, most autoinjectors have a maximum capacity of 1 mL, although there are some that incorporate a 2.25 mL PFS now available. Whilst it is technically straightforward to design an autoinjector for even higher volumes, one important consideration is the combined effect of volume and delivery time on the discomfort experienced by the patient, and there is little publicly available data on this topic. Most devices claim a maximum of 10–15 seconds delivery time, but longer than this may lead to problems in holding the device to the injection site. There are therefore a growing number of "on-body delivery systems" (OBDS) being offered by device manufacturers. These devices are attached to the patient's body, frequently using an adhesive patch, and will deliver a dose over a sustained period of time, even up to several hours, thus removing the need for the user to hold the device and allowing the patient to carry on with normal activities whilst the OBDS is delivering therapy. With such devices the volume delivered can be increased dramatically, but the devices themselves have significant technical challenges to overcome, such as controlling the delivery rate appropriately, remaining attached to the patient for the appropriate length of time, and being convenient for the user (minimum number of user steps and minimal pre-use preparation). These challenges mean that current devices tend to be complicated and difficult to manufacture and are therefore expensive; as more products of this nature are developed, the complexity and cost of OBDSs could be driven lower. See Chapter 36 for a more in-depth discussion of OBDS systems.
- *Delivering high viscosity formulations.* High concentration formulations and sustained release technologies often mean much higher viscosities than were contemplated just a few years ago or were considered in the development of

many current autoinjector concepts. Simple fluid mechanics dictates that in order to deliver the same volume of liquid through the same needle in the same time, the pressure required inside the liquid container (syringe) is proportional to the viscosity. In other words, if the viscosity doubles, the pressure inside must double, and therefore the force applied to the plunger must more or less double (there will be an adjustment for friction, including deformation of the plunger). Some formulations already on the market have viscosities significantly higher than water, and the need for higher pressures means that higher forces must be applied to the plunger and be resisted by the drug product container. This is particularly challenging for glass syringes in spring-powered devices, where impact forces are greatly increased and fracture of containers becomes a significant risk. Advances in manufacturing techniques have improved the resistance of syringes to failure, but other emerging technologies such as polymeric containers, with much higher toughness, and alternative power sources such as liquefied gas, which lessens the impact loading of the container, mean that it is possible to contemplate delivery of much higher viscosity liquids. Further development of such technologies will improve their competitiveness and suitability for a wider range of applications. Alternatively, on-body systems provide an option here since it may be possible to dilute the protein concentration and deliver a higher volume with the caveats noted above.

- *Improved usability*. The drive toward a decreased frequency of dosing coupled with self-administration means that patients become less familiar with the device and therefore prone to errors when administering simply because it may be several months between doses. This leads to a real need for devices to be intuitive in their use, and far more so than is the case with, for example, insulin where patients may be dosing themselves four times per day. Whilst there is a far greater emphasis on usability testing and validation than even just a few years ago, the importance of continuing to develop devices (and the training materials) that are easy and intuitive to use cannot be underestimated. At the same time, developing devices that reduce patient trauma by minimizing discomfort and anxiety must be seen as a constant goal.

14 Future Opportunities: Connected Devices

A more holistic, patient-centered design approach for autoinjectors of the future will include factors beyond the delivery of the drug itself. There are opportunities to add functionality to autoinjectors by drawing on parallel developments in digital technologies.

The widespread adoption of smartphones and availability of communication technologies, sensors, and Internet of Things (IoT) devices enables a wide range of possibilities for the healthcare industry and can provide a number of benefits to patients, relatives, healthcare professionals, payers and BioPharma companies themselves.

The initial driver for adding connectivity to drug delivery devices was to improve adherence to prescribed dosing. It is generally recognized across therapies that adherence on average is said to be only 50%. Poor adherence affects not only patients due to reduced efficacy but also leads to increased healthcare costs. Most patients with a chronic disease visit their healthcare provider for follow-up visits typically every 1–6 months, and dialogue with the doctor is most often based on the patient's anecdotal information. Capturing and sharing patient and dosing-related data during therapy enabled by connectivity provide many theoretical benefits for the patient, caregivers and healthcare professionals. These benefits include automated recordkeeping for adherence tracking, remote monitoring, medication reminders, information related to device usage and performance, supply chain security and even document patient symptoms.

Some reusable autoinjector products are already on the market which employ on-board electronics/software technology such as RebiSmart and the BETACONNECT device.

As technologies evolve and mature and costs of electronics continue to drop, it is highly likely that single-use disposable devices will incorporate some form of connectivity, and when patients become more comfortable with information sharing, the true benefits of connectivity will be able to be realized.

Whilst injection devices, and in particular autoinjectors, are clearly a means of enabling the delivery of increasingly challenging formulations, they also offer many opportunities to assist the patient in the whole experience of injecting the drug product. It should also be noted that the device is usually what the patient sees as the product. As such, it is what shapes the patient's perception of the product, so making it as user-friendly, intuitive and functional as possible will encourage a positive attitude toward the product which is likely to reduce patient anxiety and assist in maintaining adherence and hence efficacy of the treatment.

15 Conclusion

Development of an autoinjector for delivering any drug product is by no means a trivial undertaking. There are many factors to consider in selecting the development approach and the design direction, and these must always be balanced with the commercial strategy for the product. It is advisable to ensure that the risks in the chosen development path are understood and that allowance is made for the realization of those risks during development. For example, if the drug product is itself groundbreaking and time to launch is critical to fulfil an unmet need for patients, then selection of a pre-existing, proven autoinjector technology with minimal need for customization may be the driving factor for device selection. This will generally minimize the risks associated with development of the autoinjector. On the other hand, if the goal is to improve the user experience for an existing product, for example by providing a device that addresses the needs of a particular group of patients, then it may be more appropriate to select an innovative technology but accept its

attendant risks. This approach would be typical of a life cycle management strategy, where the introduction of a new autoinjector (or other device) would be an incremental change to the overall offering of presentations, and so the consequences of failure are less than those of failing in a new product launch.

Once the overall strategy for autoinjector development is understood and decided, other factors such as selection of the right development partner should also consider the risks of different options. These risks are not only technical ones but also cultural – a small device technology provider may be extremely agile and innovative, but not familiar with the processes and rigor involved in combination product development that can cause longer lead times. At the same time, the pharmaceutical company may be uncomfortable with device development processes whose agility may be interpreted as lack of rigor.

There is a fundamental difference in the approach to development in the device world to that in the pharmaceutical world. In the former, a specification is first set, and then the device is designed to fulfil that specification, whereas in the latter the specification is developed largely through characterization as the drug product is developed. This difference does not necessarily lead to problems, but it is important for the teams, particularly at the interface of drug product and device, to understand each other's approach. In particular, understanding of the impact of change is one of the most important considerations; what may be a simple change in drug product development, such as selection of a different pre-filled syringe, can have a devastating impact on parallel autoinjector development, even to the extent of stopping design work in its tracks and starting again from scratch.

In short, the considerations for embarking upon development of a biologic/autoinjector combination product can be summarized as follows:

- Select an appropriate autoinjector technology based on
  - Drug product characteristics and requirements
  - Patient population
  - Product life cycle stage
  - Risk acceptability
- Select a suitable development partner based on
  - Technical capabilities
  - Technology availability
  - Track record
  - Cultural "fit"
- Understand the risks associated with the chosen technology and partner, and be prepared to modify choices to make the risk profile acceptable.
- Ensure that the drug product and device development teams understand and appreciate the dynamics of each other's processes.
- Allow time in development for iteration and risk mitigation; problems will arise and will need to be overcome.

Finally, whilst this chapter talks of caution, risk, potential pitfalls and difficulties in developing an autoinjector, the benefits to patients in doing so are clear: straightforward, reliable, safe, comfortable self-injection of remarkable drug products. The growing list of examples of such products is testament to this, and future advancements in autoinjector technology will continue to provide better care and reduced burden for patients.

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Chapter 34
A Case Study of Bridging from a Lyophilizate Formulation to an Autoinjector for Patient Self-Administration

Gerard J. Bruin, Marie Picci, and Kennneth Kulmatycki

In clinical practice, subcutaneous (SC) administration of biologics such as peptides, therapeutic proteins, and monoclonal antibodies is common with a large number of drug-device combination products available on the market. Devices for SC administration range from highly sophisticated pumps to prefilled syringes (PFS) and autoinjectors (AI). Clinical devices and formulations used during pivotal Phase 3 studies may differ from the final commercial device that will become available for patients after approval. In an ideal world, the device marketed needs to be intuitive to use, convenient and easy to operate for self-administration, and of course a preferred choice by patients and caregivers. The use of an AI for self-administration has become a common choice for patients requiring frequent and/or long-term SC administration, for example, of a monoclonal antibody. The first disposable AI for patient use was introduced in 2005. The use of an AI offers better quality of life, convenience, improved adherence to treatment, and more flexibility through less dependence on health-care professionals [1, 2]. Additional benefits of an AI include eliminating the need for patients or caregivers to measure injection volumes from a vial and minimizing the risk of needle stick injuries. Needle protection features such as safety shields and automatic needle retraction improve safety and reduce patient anxiety concerning handling of needles [2].

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During early clinical development, for instance, in first-in-human and proof-of-concept studies, monoclonal antibodies are administered either by intravenous (IV) or SC injection with still many unknowns regarding the drug development candidate. Final decisions about required dose level(s), dose volume, dosing frequency, weight-based or flat dosing, self-administration or administration by health-care professionals, and the final patient population(s) cannot be made earlier than at the end of Phase 2B dose-finding studies. In many cases, the timing of the Phase 2B results, complemented with the complexity of the targeted commercial device (e.g., AI) and its associated high development costs, and long development timelines limit the choice for the Phase 3 clinical study to a vial or PFS. Liquid formulations, such as liquid in vial (LIV) or PFS, are developed as the preferred choice and are becoming the most common standard practice for early clinical development (e.g., already beginning in Phase 2 clinical studies) due to the additional injection preparation steps required with a lyophilizate (LYO) formulation. However, if the drug molecule exhibits physical or chemical instability (e.g., antibody-drug conjugate) and/or the formulation is needed quickly, a LYO formulation may be developed and utilized well into development for an unprecedented target.

The incompatibility of clinical study timelines in Phase 3 and device development timelines is illustrated in Fig. 1. In the case of secukinumab, i.e., the therapeutic antibody that will be described in this chapter as a case study, the majority of the Phase 3 clinical studies was conducted with the LYO, and the proposed commercial

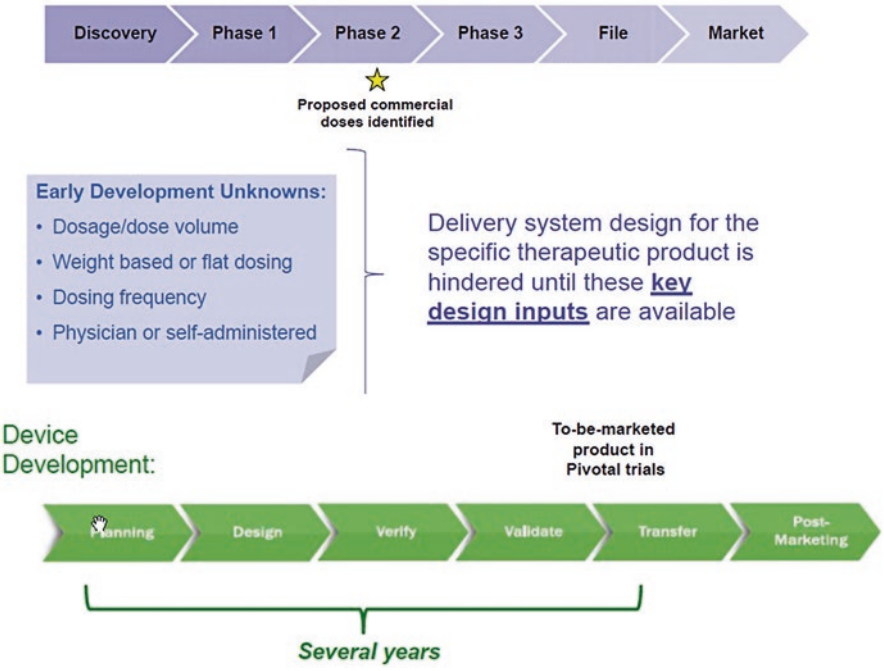

Fig. 1 From discovery to market and device development timelines

dose in the first indication (psoriasis) was defined approximately 6 months prior to submission only. Since a single commercial dose could not be defined after Phase 2, Phase 3 was conducted with two doses, i.e., 150 mg and 300 mg. It therefore poses the question of what type of bridging study would be required when switching from a LYO, LIV, or PFS to ultimately an AI in order to minimize foreseeable risks and ensure safety and performance of the marketed AI. Bridging strategies are needed to collect sufficient data that demonstrate good clinical response and to ensure safe and well-tolerated use of the to-be-marketed AI.

PK comparability bridging studies are often conducted with the aim to minimize clinical risks, such as loss of clinical response or safety. This could be the case when switching from a LYO formulation to a PFS and then to an AI. Under this scenario, the rate and extent of absorption of the antibody may be compared between the LYO form and PFS, or between PFS and AI after SC administration to healthy subjects. Moving from a LIV to a PFS can be regarded as less risky and could be justified based on analytical product comparability studies without conducting a clinical bridging study since the formulation changes are often minimal.

Monoclonal antibody PK properties such as slow absorption after SC administration with Tmax in the range of days, long half-lives in the range of weeks, and their often wide therapeutic index suggest that strict bioequivalence (BE) criteria may not be required [3, 4]. However, very different factors should be considered when deciding whether a PK bridging study is needed, such as formulation changes in case of switching from a LYO form to a solution with different excipients. Excipient levels may affect the rate and extent of absorption of the antibody after SC administration [5]. For example, changes in excipients in formulations may affect antibody degradation once injected into the subcutaneous tissue if the excipients interact differently with the antibody.

Although not necessarily a formulation change, increase or decrease in injection volume or concentration of the monoclonal antibody in the formulation may also need consideration in the evaluation of the need for a BE study. Thus, the decision whether to conduct a BE study should be justified based on concerns regarding the potential changes in rate and extent of antibody absorption. If not justified, then routinely conducting BE studies would not address any specific question and may not be required for the drug development program. It should be emphasized here that often the AI is assembled with the same PFS used in Phase 3. As an example for this situation, it was reported for the belimumab relative bioavailability study not initially powered for BE that PFS and AI were bioequivalent [6]. It therefore shows that the relevance of conducting a PK comparability study or a more strict BE study as a routine development process may be challenged in the case of a to-be-marketed AI containing the same PFS as used in Phase 3 or uses the same PFS and AI as for other commercial drug products. Those situations may be supported in many cases by the availability of extensive post-market surveillance data. In fact, other types of studies than PK comparability or BE studies may be more relevant, such as studies to address whether the intended patient population has the strength, mobility, and manual dexterity to perform self-injection with the AI correctly, for instance, for patients with peripheral neuropathies or rheumatoid arthritis. In those cases, a

human factors study may be more appropriate and meaningful. Therefore, it should always be carefully evaluated whether a BE study can really contribute to resolve the relevant development concerns for use of an AI [7].

The case study in this chapter shows how the monoclonal antibody secukinumab was developed for the initial indication of psoriasis and then later for two additional indications psoriatic arthritis (PsA) and ankylosing spondylitis (AS), with a formulation evolving from a LYO in vial to a PFS and AI for a SC route of administration.

1 From Intravenous Injection in First-in-Human to Autoinjector on the Market: A Long Journey

Secukinumab is a recombinant high-affinity fully human monoclonal antihuman interleukin-17A (IL-17A) antibody of the IgG1/kappa isotype, approved for the treatment of moderate to severe psoriasis, PsA, and AS [8].

Secukinumab binds to human IL-17A and neutralizes the bioactivity of this cytokine. IL-17A is the central cytokine of a subset of inflammatory T cells, the Th17 cells which, in several animal models, are pivotal for several autoimmune and inflammatory processes. IL-17A is also produced by memory effector CD4+ and CD8+ T lymphocytes. IL-17A is recognized as the cornerstone pro-inflammatory cytokine in autoimmune diseases such as psoriasis, PsA, and AS. Its neutralization can treat the underlying structural features of immune-mediated disease (inflammation, tissue destruction, and loss of function), as well as providing symptom relief [9–11].

The marketed therapeutic doses are available as a LYO formulation reconstituted with 1 mL sterile water for injection and a 1 mL liquid formulation either as a 150 mg/mL PFS or as a 150 mg/mL AI [12–14]. The highest marketed dose is 300 mg delivered as 2 × 1 mL injections.

The evaluation of formulation changes, and the road to the commercial AI for SC administration of secukinumab is described in the following case study. Every development program depends on many different factors, and the case study as summarized here should certainly not be regarded as the best, fastest, or most efficient road to approval of an AI device. It should be kept in mind that other scenarios would have been possible as well.

The pivotal Phase 3 program for secukinumab in moderate to severe psoriasis patients started with a LYO formulation. As depicted in Fig. 2, clinical bridging from the lyophilized formulation to a liquid formulation in PFS occurred by bridging pharmacokinetics in the following manner:(1) A PK comparability study in healthy subjects using BE criteria was conducted before the start of Phase 3 in psoriasis patients. (2) Between-study PK comparisons from sparse sampling schemes in psoriasis patient studies with multiple dosing scenarios with LYO and PFS. In addition, the clinical bridging was accompanied by a state-of-the-art analytical comparability package.

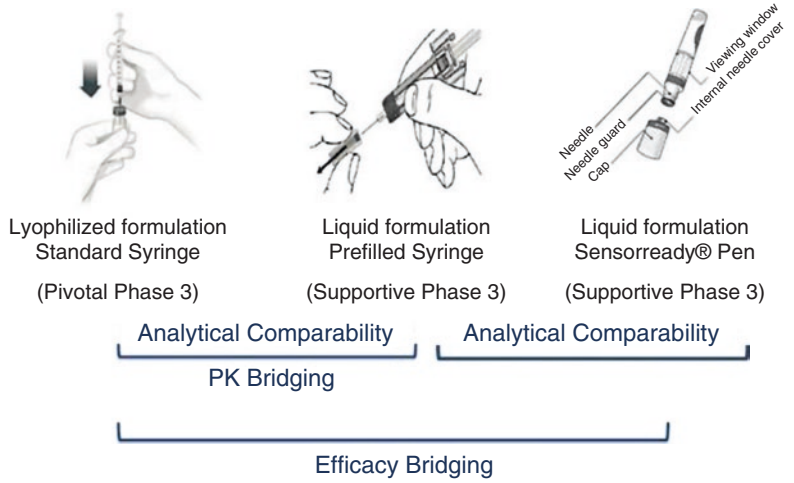

Fig. 2 Secukinumab: clinical bridging and analytical comparability from lyophilizate to autoinjector

Clinical bridging from LYO to AI was mainly based on comparison of efficacy, safety, and results from sparse PK sampling in a Phase 3 studies with multiple dosing schemes using LYO and AI in psoriasis patients. A detailed scheme of the Phase 3 program with different formulations and injection devices is shown in Fig. 3.

The composition of the LYO formulation and the composition of the solutions in the PFS and AI differed in the replacement of sucrose by trehalose dihydrate, the addition of L-methionine in the PFS and AI, and adjusted buffer and polysorbate concentrations (see Table 1).

No changes in secukinumab serum exposure accompanied these changes in formulations and injection devices, as confirmed in a PK comparability study that was powered to meet BE criteria using the LYO formulation and the PFS device with a parallel-group design in healthy subjects. In one arm, a dose of 300 mg reconstituted secukinumab in the LYO formulation was administered in two 1 mL subcutaneous injections, and in the other arm, 300 mg of the PFS formulation was administered subcutaneously with two prefilled syringes. An intersubject variability of 30% CV for C_{max} and AUC_{inf} was assumed, based on secukinumab PK in other clinical pharmacology studies. The sample size was calculated as follows: with 60 completers per treatment group, if the true ratio of geometric means of PK parameters C_{max} and AUC_{inf} would be 0.95, 82% power would be observed to conclude that the ratio of geometric mean of the PK parameter is within 0.8 to 1.25 limits for both C_{max} and AUC_{inf}. Considering a dropout rate of 20%, 150 subjects were enrolled into the study, 75 per arm.

As can be seen in Fig. 4, both arms in the study showed similar maximum concentrations (C_{max}) of 42.0 µg/mL for LYO and 43.2 µg/mL for PFS, respectively, reached at 5 days (median T_{max}) post-dose. Mean concentration-time profiles were nearly superimposable. The 90% confidence interval for ratio of means of AUC_{inf},

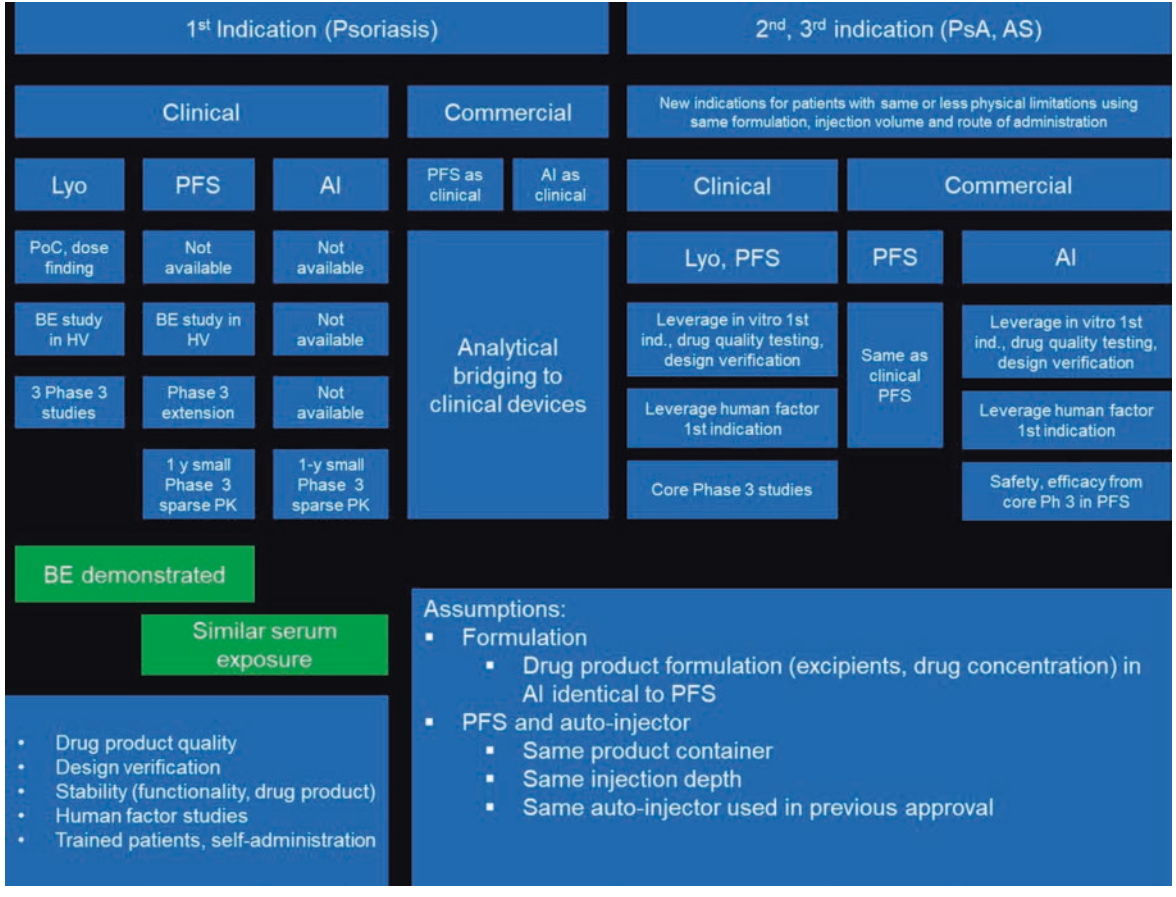

Fig. 3 Bridging from lyophilizate to autoinjector in Phase 3 studies. Legends: PsA psoriatic arthritis, AS ankylosing spondylitis, PoC Proof-of-concept, LYO lyophilizate, PFS prefilled syringe, AI autoinjector, BE bioequivalence

Table 1 Composition of formulations in LYO, PFS, and AI

Component	Composition	
	In one vial (LYO)	In (PFS, AI)
Secukinumab drug substance	√	√
Excipients		
Sucrose	√	–
Trehalose dihydrate		√
L-Histidine/L-histidine HCl monohydrate	√	√
Polysorbate 80	√	√
L-Methionine	–	√
Water for injection	–	√
Nitrogen	q.s.	q.s

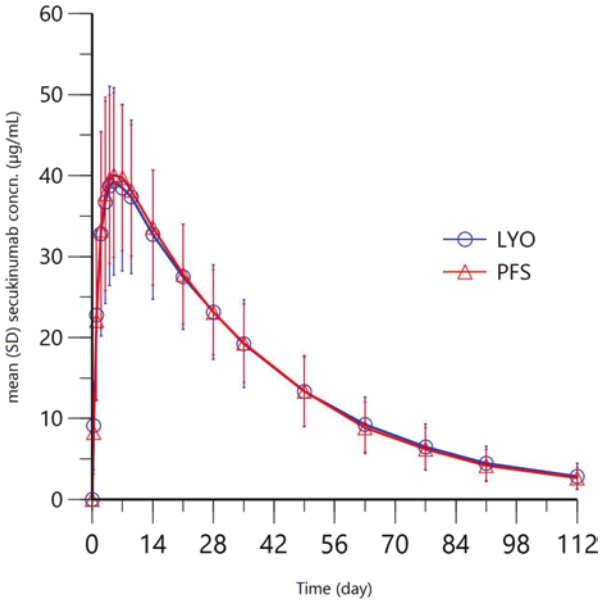

Fig. 4 Arithmetic mean (SD) secukinumab concentration-time profiles by formulation

AUC_{last}, and C_{max} were [0.92, 1.08], [0.93, 1.08], and [0.96, 1.12], which were all within the [0.8, 1.25] BE boundaries. For all pharmacokinetic parameters, the inter-subject variability was similar for the two formulations as shown by the %CV ranging between 24.5% and 27.7% for AUC_{last}, AUC_{inf}, and C_{max}. Demonstration of PK comparability, or more specifically bioequivalence between LYO and PFS in this case study, however, was not sufficient due to remaining knowledge gaps regarding mainly clinical safety and tolerability after multiple dosing scenarios with a PFS. Therefore, after regulatory feedback, a dedicated, 1-year Phase 3 study with the

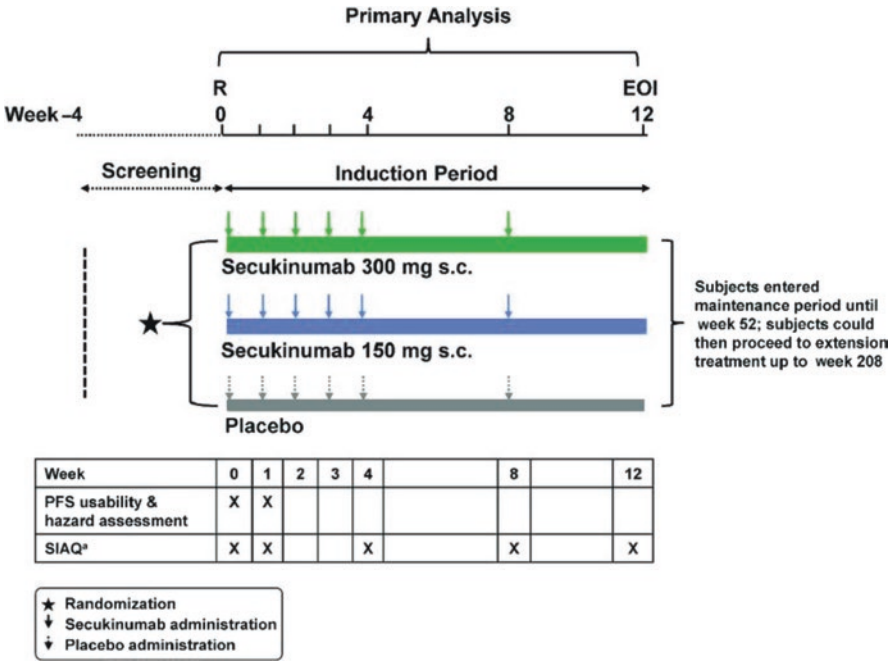

Fig. 5 Design (screening and induction periods) for PFS and AI Phase 3 studies. Legends: The study continued with maintenance treatment up to week 52, a treatment extension up to week 208, and an 8-week, treatment-free follow-up period. aSIAQ PRE module only at baseline (week 0); SIAQ POST module for all indicated time points. EOI end of induction, PFS prefilled syringe, R randomization, SC subcutaneous, SIAQ Self-Injection Assessment Questionnaire

intended therapeutic dosing regimen in moderate to severe psoriasis patients using the 1 mL PFS was conducted [13]. This study started later than the core Phase 3 studies with LYO. Interim safety, efficacy, and PK until 12 weeks were submitted for approval of the PFS. The design of the study is shown in Fig. 5. A second dedicated 1-year study, this time with a 1 mL AI as injection device, with exactly the same study design as described above for the PFS device, was conducted in the same time period as for the PFS study [14]. At the start of the treatment period, eligible subjects were randomized in a 1:1:1 ratio to receive secukinumab 300 or 150 mg or placebo. Both placebo and the active drug were dosed once weekly using the PFS or AI at baseline and at weeks 1, 2, and 3 and then every 4 weeks starting from week 4.

The PFS included a passive needle shield designed to avoid accidental needle injuries. Subjects in the secukinumab 300 mg arm received two 150 mg SC injections, and those in the 150 mg arm received one 150 mg SC injection and one placebo SC injection to maintain blinding. Randomization in each category was stratified by body weight (≥90 kg vs. <90 kg). Subjects were instructed at baseline by the site staff on how to self-inject using the PFS, using an injection pad as the training device. Self-injections were administered into one of the following body regions, with changing the injection site if possible at each time point: right thigh, left thigh, right abdominal area, and left abdominal area. As explained below,

assessment of immunogenicity, i.e., formation of anti-drug antibodies (ADA), was an important aspect that needed special consideration [15]. After having received FDA feedback at the end of Phase 2 meeting, it became clear that starting with LYO during a 1-year core study, followed by treatment continuation with either PFS or AI in extension studies, would not be optimal to evaluate long-term ADA incidence, because the impact of formulation and/or device changes from LYO to either PFS or AI on ADA incidence before the start of long-term extension studies cannot be properly evaluated with different formulations/devices in the core and extension studies. This was one more reason to conduct dedicated, relatively small, multiple dose, Phase 3 studies for to-be-marketed injection devices in patients to evaluate safety (ADA included), clinical efficacy, and PK. It can be noted here that in the case of use of platform technologies for AI devices with earlier, extensive clinical experience for previously marketed monoclonal antibodies, the program as described above could potentially be conducted with a more reduced Phase 3 program.

With all of the above in mind, serum exposures of secukinumab resulting from multiple administrations with LYO, PFS, and AI were compared between a large pivotal Phase 3 study with LYO formulation and two smaller Phase 3 studies with PFS and AI [12–14]. In all three studies with 1-year treatment duration, PK samples were drawn at week 4 during the induction period, at week 12, at weeks 24 and 52 during steady state, and at week 60 during washout, i.e., 12 weeks after the last dose. Typical mean time-concentration profiles in serum with the therapeutic regimen at 300 mg and at a twofold lower dose level of 150 mg in psoriasis patients are shown in Fig. 6.

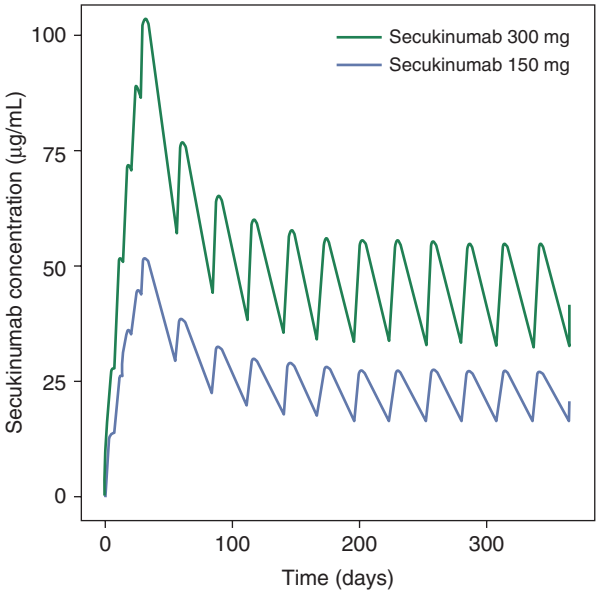

Fig. 6 Concentration profiles of secukinumab 300 and 150 mg with SC dosing regimens derived from Phase 3 trials in psoriasis patients. Legends: Reproduced from Ref. [4]. Patients received secukinumab at baseline, weeks 1, 2, and 3 and then every 4 weeks from week 4 to week 48

Table 2 Comparison of trough serum concentrations of secukinumab at steady state with PFS and AI in three indications

		PsO		PsA		AS
Week	Dose	PFS	AI	PFS	AI	PFS
Mean (%CV) secukinumab concentration (μg/mL)						
24	150	16.9 (44.9)	22.0 (43:1)	19.2 (51.7)	21.1 (46.1)	20.3 (49.2)
	300	33.2 (44.8)	45.8 (43.1)	39.4 (47.3)	40.8 (47.3)	–[a]
52	150	14.7 (33.7)	20.6 (47.6)	19.1 (50.4)	20.0 (43.2)	20.7 (41.7)
	300	30.6 (42.8)	40.6 (41.4)	34.2 (48.4)	41.5 (48.0)	–[a]

PsO psoriasis, *PsA* psoriatic arthritis, *AS* ankylosing spondylitis
[a]300 mg was not administered by AI in AS in clinical Phase 3 studies

As can be seen in Table 2, the pre-dose concentrations demonstrated a dose-proportional increase in exposure from 150 to 300 mg SC in all three studies. Pre-dose concentrations were similar with PFS and AI. The differences in concentrations between the AI and PFS were somewhat higher in psoriasis patients than in PsA patients, which could be due to slightly different instructions for use (IFU) in the psoriasis study with AI, with recommended site of administration at the front of the thighs in psoriasis patients as opposed to a more flexible choice on site of injection in the psoriasis study with PFS. It should be noted that the bulk syringe used in PFS and AI is identical and that these were between-study comparisons. Importantly, the differences were not clinically meaningful, which was supported by the fact that efficacy and safety across the three studies with LYO, PFS, and AI were comparable. In PsA patients, trough serum concentrations obtained with PFS and AI and in AS patients with PFS were very similar (Table 2).

Finally, it can be noted here that overall incidence of treatment-emergent ADA was only 0.4% in psoriasis patients in the Phase 3 program after 1-year treatment with secukinumab without any impact of formulation or device on incidence [16].

At the end of the development program, LYO, PFS, and AI were all simultaneously approved in psoriasis, first in the USA, Europe, and Japan, and in the two other indications (psoriatic arthritis and ankylosing spondylitis) only 1 year later in the USA and Europe. The final commercial devices, PFS and AI, are depicted in Fig. 7a, b.

2 The Technical Development Challenges for a New Device in a New Indication

The development of a stable and high concentration monoclonal antibody formulation can be a challenge for a quick entry into a first-in-human study and, as explained above, potentially leads to the development of different dosage forms, moving, for example, from a LYO in vial to a liquid in PFS and AI as the final product. In the secukinumab development program, the LYO formulation was used in all

Fig. 7 Commercial PFS (**a**) and autoinjector (**b**) for secukinumab

proof-of-concept studies with IV infusions, and the same formulation was used in the Phase 2B clinical studies with subcutaneous injections. Despite general progress in creating new bioprocesses for monoclonal antibodies, the development of a robust PFS formulation and manufacturing process for use in the Phase 3 study required a similar amount of time as needed for the commercial product. The PFS presents undoubtedly a more convenient administration form to patients than a LYO formulation in vial and potentially increases adherence. However, at the start of Phase 3 with secukinumab in psoriasis, the PFS development was too premature, and the LYO formulation in vial was therefore used for proof-of-concept studies, for dose finding, and for the pivotal core Phase 3 studies as well. The device components that were used for the drug preparation steps and injection with the lyophilizate formulation included standard off-the-shelf materials commonly used by physicians and patients for this type of application. The development and regulatory pathway for the LYO product followed the "Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use" in the EU.

During clinical development of secukinumab, injection systems such as PFS and AI were already commonly available for etanercept, peginterferon alfa-2a, darbepoetin alfa, golimumab, and adalimumab and used by patients for specific treatments and indications. The secukinumab program is intended to cover a wide range of patient populations in psoriasis, PsA, and AS, each with different strength, mobility, and manual dexterity. Patients with PSO, RA, PsA, and AS may experience dexterity difficulties due to impairments of joints. PSO and PsA patients may suffer additionally dermal problems affecting the use of an AI. Physical impairments may prevent to perform a self-injection or to correctly handle the AI and inject. For instance, patients with peripheral neuropathies or rheumatoid arthritis handling dexterity may experience difficulties in removing the cap from an injector, performing a one-handed injection, or activating a feature to trigger an injection. This aspect was addressed in the design of the Sensoready pen by developing a patient-centric

AI with shape and features suited to patients' physical and manual impairments to allow an intuitive and easy handling of self-injection. Additionally, the development of the AI followed a platform design approach by covering the requirements for a worst-case patient population with strict criteria on performance, dose accuracy, and enhanced convenience throughout the duration of the treatment. A platform AI approach compared to a narrowly defined intended use AI will enable a faster development of a future potential new therapy by using the same AI either with the same drug in new indications or with potential new drugs in development.

The selection of an AI for the market application of the secukinumab drug product was based on five basic interdependent product requirements:

1. Intended indications: including psoriasis, PsA, and AS
2. Liquid formulation characteristics: viscosity as for the LYO formulation
3. Dose of 300 mg as 2 × 1 mL, injected SC with a weekly dosing frequency during the first month and every 4 weeks during maintenance, administered either in a clinical/physician environment or by self-administration at home
4. Primary container in a prefilled syringe and delivery technology for self-injection
5. Target users including health-care professionals, caregivers, and patients

Combined together, these product requirements defined the input for the particular design features of the AI:

1. Device shape with an ergonomic design to ensure usability by all intended users
2. Needle cover with sharps protection to prevent potential needle stick injury
3. Actuation mechanism with acceptable force to prevent inadvertent injection of product
4. Indicator and sound clues (clicks) to provide user feedback for a specific handling step
5. Inspection window to view the drug product content before and after injection
6. Microbiological protection to ensure safe use
7. Tamper-proof feature to protect against counterfeits

A market assessment of devices available in the anti-TNF therapy area was conducted and provided insights on current market products in the USA and Europe as of June 2013 [17]. Novartis then undertook the development of different concepts of autoinjectors for the administration of secukinumab.

For the selection of the final device concept, a user preference study as part of the human factor program was conducted to assess different concepts of design features considering the above product requirements, including the design from the Sensoready® pen (the trademark of the secukinumab AI) and from competitors' products. Interviews were conducted in China, the USA, and Germany with over 200 patients and health-care professionals (HCPs) representing the intended users of secukinumab for the 3 intended indications psoriasis, PsA, and AS.

The study reported a clear preference both by patients and HCPs for the Sensoready pen compared with competitor products. The main reasons for the preferences were the following characteristics: triangular body shape for easy gripping, large window to view the drug content, twist-off cap design, convenient size and

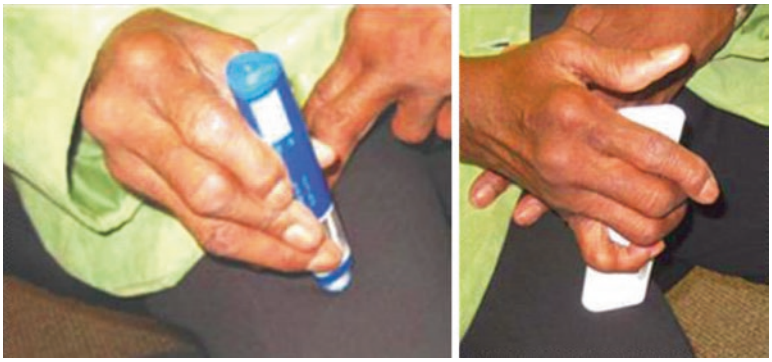

Fig. 8 Prototype Sensoready pen (left image) and competitor (right image) during user testing

cross section, and the convenient push-against-skin activation. An example of the testing can be seen in Fig. 8.

Study participants displayed fewer difficulties with the use of the Sensoready pen, which finally resulted in the selection of the Sensoready pen. It was selected as the new AI for secukinumab and other Novartis pipeline biologics. The PFS and Sensoready pen were then developed for secukinumab in psoriasis as the first indication. The two products were considered a drug-device combination product in the USA and followed the regulatory requirements for the FDA's 21 CFR Part 4 (Current Good Manufacturing Practice Requirements for Combination Products) [18].

In the EU, the PFS and the Sensoready pen assembled with the syringe-containing drug were governed as a medicinal product per Directive 2001/83/EC. When the solution for injection in an AI uses exactly the same primary container as the PFS presentation, according to European regulations, these device components form the primary packaging. They can be considered as a single integral drug-device product. The PFS and the Sensoready pen, therefore, followed the relevant essential requirements of Annex I to the European Medical Devices Directive (MDD) 93/42/EEC concerning medical devices, as amended by 2007/47/EC [19], as far as safety and performance-related device features are concerned. Further, a document, entitled "Clinical Evaluation: A Guide for Manufacturers and Notified Bodies Under Directives 93/42/EEC and 90/385/EEC" [20], was used as a guidance document to assess the clinical aspects and conformity of the device features, consisting of the following parts:

- Device constituent part: autoinjector including detachable cap
- Primary packaging: syringe included staked needle, rigid needle shield, and rubber stopper
- Secondary packaging
- Labeling

To demonstrate conformity of the secukinumab solution for injection in the PFS and Sensoready pen with the relevant essential requirements of Annex I to the MDD [19], different technical standards were considered: glass barrels for

injectables [21], sterile hypodermic needles for single use [22], needle-based injection systems for medical use [23], biological evaluation of medical devices [24], and sharp injury protection [25].

Dose, injection time, reliability, injection depth, activation and overriding forces, and cap removal torque were also assessed during design verification. The stability of the drug product secukinumab in the PFS was tested in a long-term stability test under various storage conditions to establish a shelf life of 24 months in the USA when stored at temperature between 2 and 8°C. The durability of the components, subassemblies and assembled PFS and Sensoready pen (2 years of storage simulation for components and subassemblies and 3 years for final assembly), was demonstrated by conducting accelerated aging studies at 50 °C and 40–75% room humidity. Dose accuracy and functional stability of the PFS and Sensoready pen were tested during registration stability studies following the ICH guidelines for drug product stability. In all instances, the PFS and Sensoready pen functioned as intended, and the observed results were as expected.

Safe use of the device was demonstrated in non-interventional simulated use studies following the standard IEC 62366:2007 Medical devices [26] and FDA draft guidance on Applying Human Factors and Usability Engineering to Optimize Medical Device Design [27]. Following formative evaluations and a summative study with trained and untrained users, the system was found to be safe and effective for the intended users and use environments. The two formative studies were observational, simulated use studies. The first study assessed the core dose administration task, including preinjection checks and disposal after use. The second study assessed effectiveness of the IFU, i.e., whether participants could readily achieve correct use of the Sensoready pen with the IFU. A validation human factors study (HFS) with trained and untrained participants was then conducted on both the to-be-marketed PFS and Sensoready pen across all three indications (moderate to severe psoriasis, AS, and PsA). Across the trained (1 week and 4 weeks decay) and untrained cohorts combined, high levels of successful use were observed with 93% (n = 153/165) of participants delivering their first unsupervised injection into a dummy skin pad successfully. There were no observed cases of use errors or close calls, i.e., cases with intervention needed to avoid an injury caused by physical usability issues. All participants were physically able to complete a successful simulated injection, and there was reason to believe that the participants would not repeat errors after multiple-dose administration. Comprehension of most other required task steps, such as correct storage, site preparation, and medication checks, was also demonstrated to be effective.

Additionally, a usability assessment of the Sensoready pen was conducted as part of the 1-year clinical study in psoriasis patients. Study patients were trained in the use of the Sensoready pen by clinical investigators by following the IFU and using an injection pad. Successful and safe use of the Sensoready pen was then assessed. A high rate of successful self-injection was observed during this clinical study with 100% (n = 178/178) of subjects successfully injecting following a 1-week training decay. Further, the device performance for the AI was tested to demonstrate robustness, suitability, and patient safety during actual use in the clinical setting.

Based on the results of human factors evaluations, the Sensoready pen was found to be safe and effective for the intended users and user environments. Modification to the design of the device was not needed and would not further reduce any residual risk that remained after the validation testing. At the end, minor "cosmetic" changes have been made to the final instructions for use (IFU) to enhance the clarity and identification of information within the IFU.

A risk management process was executed according to the ISO standard EN ISO 14971:2012 for the application of risk management to medical devices to facilitate the development and maintenance of safe and effective medical device designs by identifying and appropriately mitigating risks associated with the use, design, and manufacturing of medical devices [28]. Appropriate risk controls were implemented to mitigate existing risks as far as possible. Those risk controls were verified, and their effectiveness was validated. Furthermore, it was assessed that all implemented risk control measures did not introduce new hazards or hazardous situations. Any residual risk that remained after the validation testing was outweighed by the benefits that may be derived from the device's use.

To summarize, based on the programs described above, with a thorough integration of clinical, drug, and device development, secukinumab was approved for the psoriasis indication in December 2014 in Japan and in the USA and EU in January 2015 in three dosage forms simultaneously: LYO, PFS, and AI (Sensoready pen).

3 Leveraging to New Indications

The clinical submission of secukinumab for the second and third indications, PsA and AS, was done with results from Phase 3 studies using LYO and PFS only. The performance, functionality, and usability of the secukinumab AI (Sensoready pen) in the second and third indications were leveraged from data generated in the psoriasis indication and from AI user-level testing across several indications. Since PK, efficacy, and safety comparability between the PFS and the AI (Sensoready pen) could already be demonstrated in the first indication, only additional PK results for LYO and PFS from Phase 3 studies in PsA and AS were submitted for approval of secukinumab in these indications. It can be noted here that population PK models in psoriasis, PsA, and AS demonstrated very similar PK characteristics across these indications. Cross-study PK comparisons resulting from PFS and AI administrations after submission and approval in PsA and AS showed very similar serum exposure of secukinumab across formulations/devices (see Table 2).

4 Conclusions

This case study describes the development of secukinumab as a new monoclonal antibody in a new device (AI) including the associated development challenges for the first indication and development opportunities for nonclinical bridging studies

in later indications. With dedicated Phase 3 studies with PFS and AI and interim analyses at appropriate time points in these studies, no compromises had to be made in overall timelines for submission and approval.

A fast track development of an existing combination product in a new indication or variant of an existing combination product, for example, targeting a different dose or fill volume, may be available by executing a risk-based development approach during the product development cycle from early phase through to the to-be-marketed combination product. Moving from a LYO drug product formulation in vial to a liquid in PFS and AI can be potentially accelerated by leveraging prior experience from an established drug or device constituent part in combination with a new device or drug. Of course, availability of the to-be-marketed device before the start of Phase 3 studies and using such a device in the Phase 3 program would be the preferred option and probably speed up development timelines. Technical development bridging studies, accompanied by a strong analytical comparability program, are the cornerstone of a successful bridging strategy and may include drug-device compatibility, design verification, simulated use human factors studies, and risk analysis (design, process, human factors, and product), and may not warrant additional clinical studies.

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Chapter 35
On-Market Prefilled Syringe and Autoinjector Studies

David A. Post, Sherwin Shang, Shweta A. Raina, and William Szechinski

1 Introduction

The autoinjector (AI) is a popular combination product delivery system that offers value to the patient and/or care giver. This type of presentation allows for ease of use and has needle hiding features for the needle phobic user. There are many AIs in the global market; each has its own specific design and functionality but generally offers similar performance. Humira 40 mg (50 mg/mL adalimumab) was commercially launched in 2003 as a ready to inject prefilled syringe (PFS). The final PFS used by the patient also included an added-on plunger rod and finger flange backstop. As an example to provide an alternative presentation for patients, the Humira AI (Pen) was launched in 2006. The Pen uses the same PFS as used by the patients, but instead of the plunger rod and backstop, the PFS is assembled with two subassemblies to produce the Humira Pen (Fig. 1).

The Humira AI is assembled from three constituent parts: two subassemblies (syringe housing and firing mechanism) and the PFS (Fig. 2).

The assembly of the Humira Pen is performed on fully automated lines as briefly described in Fig. 3. Throughout the assembly process, online controls are used to ensure that the assembly meets specifications.

The subassemblies consist of four molded parts and one spring each as described in Fig. 4.

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Fig. 1 Humira Pen

Fig. 2 Constituent parts in the assembly of the Humira Pen

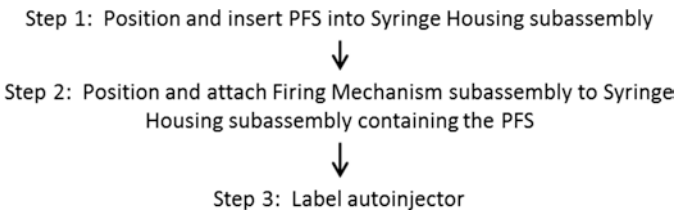

Fig. 3 Assembly of the Humira Pen

For use in an AI, there is a set of specifications for the PFS which ensure proper functionality of the AI such as:

- Breakout force for the stopper
- Glide force during dispensing
- Stopper position

Component	Subassembly	Primary Function
Syringe Housing	Syringe Housing	Housing for subassembly
Boot Remover	Syringe Housing	Removes PFS needle shield
Lockout Shroud	Syringe Housing	Needle stick protection
Syringe Carrier	Syringe Housing	Support for PFS
Syringe Carrier Spring	Syringe Housing	Energy source for needle stick protection
Firing Body	Firing Mechanism	Housing for subassembly
Locking Pin Cap	Firing Mechanism	Protects from actuation until ready
Firing Button	Firing Mechanism	Press for actuation
Plunger	Firing Mechanism	Dispenses PFS
Main Spring	Firing Mechanism	Energy source for dispensing

Fig. 4 Components in the Humira subassemblies

- Needle dimensions
- Drug product quality characteristics such as viscosity

A thorough understanding of the stack interactions within each subassembly and the capabilities of the AI design and PFS physical characteristics are essential to launch and support on-market needs. These understandings which can be rooted in a single element or multi-interactive system elements are essential for product support and can lead to the opportunity to identify and implement improvements to mitigate use confusion and device malfunction.

1.1 Insight into On-Market Support

Information for on-market support comes through several channels and informs about the reliability of the product for users and use by and interaction with the patient or care giver (see Fig. 5).

Characterization in the context of on-market support is driven by the following inputs such as:

- Complaints
- Commodity variability
- Post-approval changes

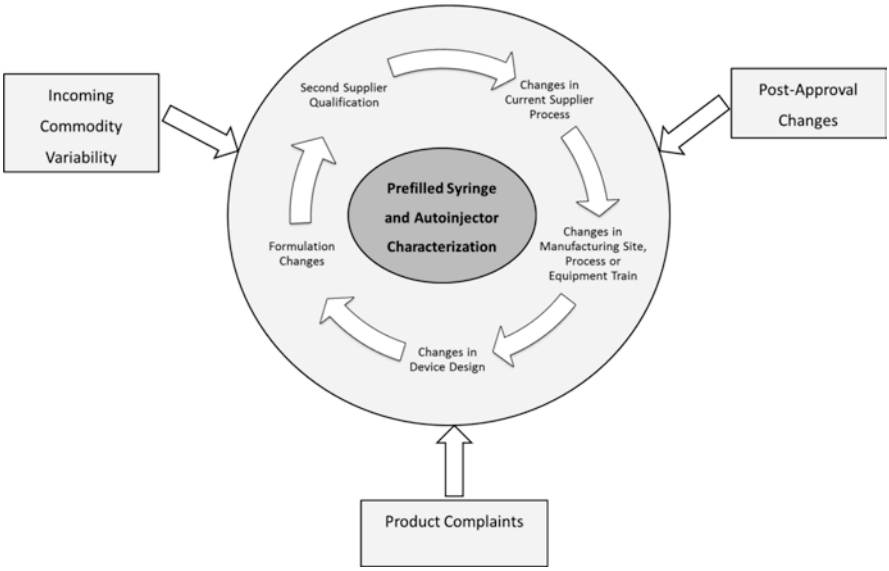

Fig. 5 On-market support characterization

On-Market Support

For a combination product such as the Humira Pen, one of the best sources of information for the on-market product about the reliability of the device and use of the device by the patient/care giver is through user feedback. Several methods can be used to receive feedback from patient/care giver including direct phone contact, leaving of complaint information in a voicemail, as well as written/audio-visual information sent in by the patient/care giver. Prior to the launch of a device, both medical and nonmedical complaint categories should be identified as part of the risk management process. These categories should include potential device malfunctions, use errors, and medical complaints. Questionnaires should be developed to better help both the patient/care givers and the person receiving the complaint to identify the root cause of the complaint. These questionnaires then should be continually updated to reflect the received information and to better communicate with the patient/care givers. When assessing the information from a complaint, understanding the accuracy of the information is important in order for the quality and technical teams to assess whether:

- The combination product has malfunctioned
  - Can the patient/care giver observe and correctly interrupt the device malfunction?
  - Even with an incomplete description by the patient/care giver, can the technical team identify potential failure modes which could result in what was observed by the patient/care givers?

  - If a complaint category is composed of both use and device malfunction root causes, can the contribution to the complaint be identified from the received information?
  - Can the complaint be linked to a specific constituent part?
- Use error has occurred
  - Is the use error related to device complexity or supplied instructions?
  - Are the patient/care givers aware that a use error has occurred?
  - Are the patient/care givers willing to admit to a use error?

Complaints are tracked and trended to establish baselines for each category and then the data used to assess whether a continuous improvement program could be applied to reduce the observed frequency of a particular complaint category or correlated categories. Though the goal is to receive no complaints, the insights into the use and reliability of the combination product as gained from user feedback are invaluable. A critical success factor for the Humira Pen having a strong history of low and steadily decreasing complaints over its product life cycle is the diligent process established by AbbVie to track feedback from the field, analyze the data from a technical and risk perspective, and implement changes using sound engineering principles.

Commodity Variability

At the highest level, subassemblies and the PFS are incoming commodities for the final assembly of the AI. The functionality space of the AI should be understood such that normal variation in these commodities will not impact the reliability or the use by the patient/care giver. These are initially obtained in development and human factors studies, and then once on-market, variability and its impact on outputs such as dispense time or use operations such as holding the AI during dispensing further enhance understanding. During development of the AI, it should be demonstrated that there is sufficient capability of the design that direct interactions of the AI with the user as described in the instructions for use are not impacted. Variation in commodities such as the following needs to be considered:

- Resins used in the molding of the plastic components. Do different lots of material, material from different manufacturers or different sites of same manufacturer, affect functionality? Affects could range from changes in component dimensions to physical properties.
- Energy source variation for the AI – can the source or process affect the available energy such as a spring over the shelf life of the AI?
- Impact of the physical characteristics of the PFS over the shelf life of the combination product. How does the variability of the breakout and glide forces of the PFS impact the AI? Since the energy force capability is typically fixed in an AI, as the glide forces change over time, will they directly affect the dispense

time? Other variations in physical characteristics such as needle inner diameter, glass/flange geometry, and sterile barrier components' impact on the assembled combination product need to be fully understood.

Post-approval Changes

After approval and the combination product is in the market, changes to the production of the combination product can be driven by:

- Assurance of supply/alternate supplier qualification
- Supplier changes such as elimination of used resin grades
- Supplier manufacturing changes
- New/updated regulations or standards
- Change in design of the combination product

The risk and potential impact of these changes need to be identified and plans executed to obtain results demonstrating that the changes do not impact the combination product functionality or use by the patient/care giver. For example, it has been observed that even changes thought to be initially low-risk such as change in colorant or carrier for the colorant (because they are a small percentage of the final molded component) ended up being the cause of a significant change in physical properties of the component, thus affecting the AI functionality.

Reliability and Scalability of Processes

The AI platform is typically built on the system integration that includes design, materials, process, and equipment, as shown in Fig. 6. The platform is set up to apply science and engineering to understand device design robustness, design for

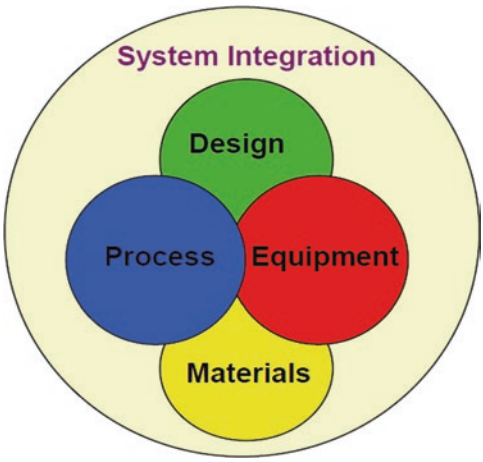

Fig. 6 System integration

functionality, design for manufacturability, reliability and scale up for commercial production. This systems approach enhances the probability of success to meet the patient's satisfaction throughout the life cycle of the product.

Scalability of design development impacts commercial manufacturing in several ways. These include:

- Scalability of component molding from development to commercial tooling
- Scalability of assembly from manual/semiautomated to fully automated systems
- Confidence that testing conducted during development is predictive of on-market performance

Using a platform or predicate device design is preferred as this provides more robust information on impact of variability to the functionality of the device compared to a new design. At commercial-scale variation of components can be introduced through differences in steel cavity dimensions, molding process parameters/equipment used, and/or differences in resin lots. These variations can affect functionality of the device in subtle ways, but larger affects may be observed if these variations are multifactorial and synergistic. In these instances, the mechanism which leads to malfunction may be well known, but which factor is the primary contributor for a given device may be more difficult to predict. Thus, it is highly desirable to assess multifactor impacts during development, allowing for better definition of dimensional tolerances. These tolerance ranges will then define the complexity in industrialization. However, many development programs for new device designs rely on single cavity tooling. This limits assessment within the design space for understanding the robustness of the device design and potential impact to industrialization. One option is to produce components with critical dimensions at their tolerance limits and then assess impact on functionality or industrialization. However due to cost and time limitations, this approach may be not always practical.

As an example, an improvement project was initiated to modify actuation and cap 2 removal forces of the Humira Pen. During feasibility/development, single cavity tooling was used to mold the plunger and cap 2 components involved in the force to actuate and the force to remove cap 2, respectively. For the plunger, a tool design was built which removed split lines from functional features of the component as well as changing the injection gate position, thus reducing variation introduced through molding of the component. However, later on it was decided that the design of a commercial tool which mimicked the single cavity tool would be too complex and introduce too much risk for the industrialization of the tool. Thus a different design for the commercial tool was used, but this led to slightly different properties of the functional features and additional design verification testing. Regarding the cap 2 design, the thickness of the wall changed between the pilot scale (single cavity) and commercial tooling. This in turn changed the cap 2 force to remove profile, and again the design of the commercial tool was changed to accommodate this difference requiring repeating of verification studies.

Another aspect in industrialization of processes to commercial scale is the difference in testing performed during development versus commercial use. A significant

difference between development design verification testing and commercial monitoring of device function/interaction with the users (which is primarily through on-market support) is on the amount of devices which are assessed. In most cases the complaint rate for a given category is lower than what can be statistically assessed in design verification testing. During development a product sampling approach may be used to assess test results versus verification criteria. This approach can use current product attribute tests with a previously defined sampling strategy or a quantitative measures. The following recommendations are provided for collecting necessary information to complete design verification activities:

Reference draft or current product limits/specifications governing testing/evaluation of the device for recommended sample sizes and confidence levels for verification activities.

- Use quantitative (e.g., numeric) vs. qualitative (e.g., pass-fail) information, if available, for verification activities.
- Use nonparametric statistics when quantitative data do not meet required statistical assumptions.
- Increase product sampling if none of the above applies.

Occasionally, too few samples will be available to use an attribute/pass-fail sampling plan. Since variable/numeric data provide stronger discriminatory power than attribute data, it is beneficial to verify a design output using quantitative data. For example, quantitative data may be collected, and a variables sampling plan approach may be used to compare results with a verification criterion. In the end, identifying key functional tests and applying appropriate acceptance criteria are essential to build confidence for the commercial product.

Lastly, the impact of assembly on the function of the AI needs to be understood. The assembly of an AI consists of an ordered sequence of steps of the various constituent parts such as subassemblies and/or individual components and the PFS. During development of an AI, typically, the assembly is either manual or semi-automated for a new device or could be completed at commercial scale if a platform device is being used. In most cases, lessons learned from the assembly and critical quality attributes identified during development are used to establish an optimized commercial assembly process. The automation of an AI assembly may require multiple steps to move the individual components or subassemblies into the correct position for successful production. For loose components, they can be bulk fed into the process, while complex items or ones that have a delicate nature are tray fed. To ensure proper presence and placement of the components or subassemblies by the machine onto a pallet, in-process checks are required. These checks can be simple to ensure a part is in place or complex with dimensional measurement to ensure the part is correctly positioned. Some assembly steps can be finite, due to known dimensional interactions with the parts such that a control assembly mechanism (CAM) driven station can snap or seat parts together, whereas others can be complex, due to specifications or product requirements that require more inspection of the assembly step. Online, 100% checks aid in allowing automation to proceed without personnel and reduce the chances of potential known manual human assembly errors.

When assembly stations define a product as a reject, the machines are capable to stop that device assembly process for that specific device while maintaining the production on the next ones, allowing for less scrap/waste of good components. An example of a simple check would be a physical touch of product for presence to ensure the part is loaded in the assembly pallet. A complex one would be a visual inspection of product identity by a camera system, such as differentiating from different dosage forms or products using the same shape of part. A more complex type of quality check would be monitoring the forces of the part engagement to ensure the expected assembly has occurred. For the Humira Pen, all of the above in-process checks are used, and, especially where constituent part variability plays a role in the assembly, more sophisticated mechanisms are used to ensure proper assembly.

Summary

There are many aspects which need to be considered before and after launch of a combination product. Many of these are the same topics, but the source and quantity of the data are different, and impact of the change on the patient is also different. Further discussion on some specific areas of impact of the constituent parts is presented in the next section.

2 Autoinjector: A Combination Product

The design of the Humira AI at the highest level is the assembly of the constituent parts (subassemblies and PFS) to produce the final combination product. Variability of the PFS has been observed, and physical characteristics of the PFS need to be taken into account when designing the subassemblies. These include syringe needle inner diameter and length, siliconization process, stopper design, flange design to sustain high impact and mitigate glass breakage, minimization of scratching during handling, and barrier properties to protect the drug product. For subassemblies, specifically in the design of the firing mechanism, the following attributes need to be considered: robustness of the energy source to deliver the drug product at the desired dispense time of a given drug solution volume and viscosity with no risk of glass breakage and use-related functions such as force of actuation and safety mechanism to minimize accidental actuation. Additionally, the AI must be able to be stored at cold temperatures and the impact of mis-use wherein patients do not wait for the AI to reach room temperature as specified in the instructions for use (IFU) be anticipated. In the case of use at cold temperatures, the increased viscosity of drug product (DP) will impact the functionality of the AI by elongating dispense time which needs to be understood. Other supporting techniques for the design of an AI include modeling such as Moldflow, Finite Element Analysis, and computational fluid dynamics. Impact of industrialization including component molding tolerances, subassembly, and final assembly processes also must be considered.

2.1 Primary Container

AI combination products have been broadly accepted as delivery devices for use with biopharmaceuticals and commonly used syringes as the primary container for the biological drug products. Syringes can be made of either glass or plastic materials. Each has its own features with advantages and challenges when used in an AI. The drug product is filled into the syringe, and the PFS then becomes a constituent part of the AI. The physical characteristics of the PFS over the shelf life of the AI need to be fully understood and sufficient functionality tolerance margin established. In most cases, the syringe is a purchased commodity; thus changes to the manufacture of the syringe to adjust to a given AI are not practical, and the AI design and functionality need to have the capability to encompass syringe variation impact on the functionality.

2.2 Drug Product

Optimal formulation of the DP which provides the desirable stability profile is first determined along with the compatibility and stability of the DP in the primary container such as a syringe over the target product shelf life. Understanding potential change in the physical properties of the PFS over shelf life of the combination product is essential. There is potential for the physical properties of the PFS to change over time, and this may be dependent on the DP either the physicochemical characteristics of the API or the formulation composition. The biopharmaceutical industry trend is progressing toward higher doses of biologic drugs which require higher concentration formulations, with implications being higher viscosity formulations and higher injection volumes of drug product. This impacts AI design such as a strong energy source mechanism to dispense drug product subcutaneously within delivery time expectations. From human factor perspective, a patient can accept drug product delivery up to 15 seconds for a 1 mL syringe and slightly longer for a 2.25 mL device.

2.3 Subassembly

There are two AI subassemblies used in the Humira Pen. The subassemblies consist of molded plastic components and springs. The primary functions of the firing mechanism subassembly are to provide the energy source and functional mechanism to actuate the Pen and dispense the DP. The primary functions of the syringe housing subassembly are to remove the syringe needle guard from the syringe, ensure correct needle depth for dispensing, and provide needle stick protection after completion of injection. These design elements are verified in development, but as with

the PFS, potential issues typically only arise at very low frequency and are not observable until the higher commercial volumes. For example, there are many complaint categories which are reported at less than a rate of 10 per million devices used, thus are only observable during commercialization.

3 PFS Characterization and Interaction with Biologic Formulations

A PFS is a disposable syringe comprised of a cylindrical barrel made of either glass or plastic fitted with a Luer lock or staked needle capable of delivering a fixed dose of drug solution. A schematic representation of a PFS is depicted in Fig. 7.

A PFS offers several advantages [1] as a primary container such as (a) a high accuracy of dose-volume administration, (b) lower overfill requirements, (c) lower microbial risk owing to fewer manipulations prior to dosing, and (d) ease of self-administration either using the plunger rod or in conjunction with a device such as an AI. However, a PFS has several DP contact materials, viz., the stainless steel needle, silicone oil that may be present on the inner glass barrels or the inner needle wall, the soft needle shield which is placed over the needle in addition to the resin from the plunger stopper, and glass or plastic barrel. These drug product contact materials in tandem with PFS interactions with the AI device components warrant extensive characterization of such systems with respect to:

- Material properties and their interactions with biologics
- Container closure integrity
- Extractable and leachables
- Dimensional testing of various components such as flange, barrel, shoulder and cone, stopper, needle gauge, inner diameter of the needle, needle bevel design, and syringe needle shield
- Needle and barrel lubrication/siliconization

A schematic illustration of PFS characterization is provided in Fig. 8.

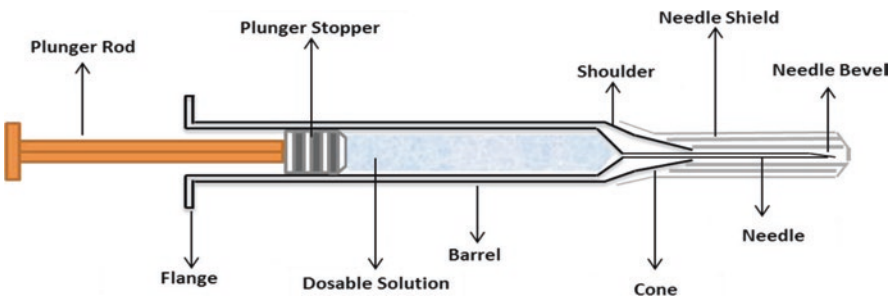

Fig. 7 Components of a prefilled syringe

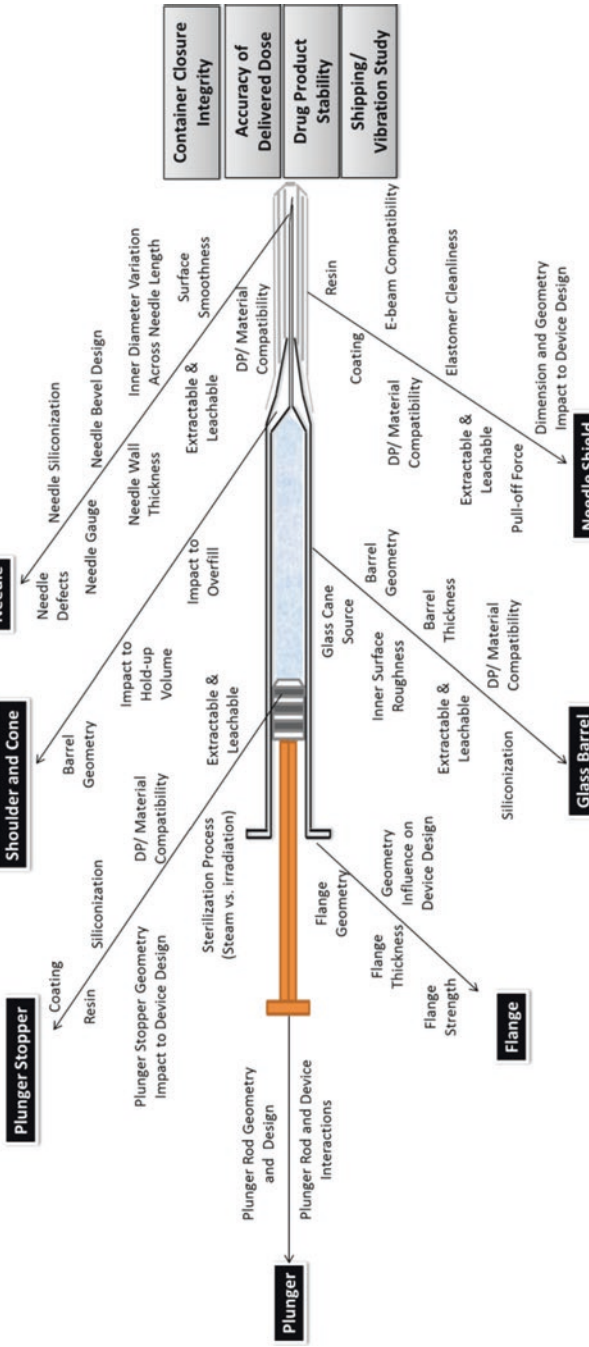

Fig. 8 Schematic representation of the characterization of prefilled syringes

Understanding the impact and change of the functional properties of the PFS with the drug product over the shelf life is essential to ensure robust AI design. For example, it is typical that a slight increase in glide force is observed over the shelf life of the PFS. This increase is typically due to increased friction within the PFS on account of the loss of silicone oil from the wall of the glass syringe. This silicone oil loss can come through interactions with the drug product formulation or other mechanical stress encountered through normal handling. This increase in glide force needs to be aligned with the typical decrease in the energy source of the AI. Even if delivery times are within specification, if they become longer than what is typically experienced by the user, it could result in a complaint.

Life cycle or post-approval changes such as changes in formulation components including the concentration of the protein/monoclonal antibody (mAb), changes in needle gauge, etc. require not only updates to drug product filed specifications but also re-characterization of an array of attributes such as:

- Changes in glass cane – Cane can impact the syringe-forming process as well as the texture/surface energy of the inner glass walls. These differences may in turn impact the movement of the plunger stopper along the glass barrel and the overall functional performance of the PFS.
- Changes in syringe needle shield – Depending on the design of the AI, interaction of the syringe needle guard and the AI needs to be assessed; thus risk of changes in the syringe needle guard needs to be evaluated.
- Changes in the drug product manufacturing site or a manufacturing process change can trigger extensive PFS characterization and product stability and compatibility studies. For example, although it is desirable during qualification of alternate manufacturing sites to keep the equipment process train identical or similar, changes in manufacturing sites can lead to changes in equipment train.
- Lastly, lot to lot variability in the quality attributes of incoming commodities which may have hitherto not been assessed during product development owing to limited number of lots available can have implications on manufacturing, cycle time efficiency, and device/product compatibility.

As previously mentioned, the PFS characterization landscape is vast and complex. This section will provide a high-level overview of characterization of three major components, i.e., (1) syringe, (2) stopper, and (3) needle, in the context of on-market support. Lastly, a discussion will be presented on the challenges associated with the siliconization of the syringe barrel and its impact on AI functional performance.

3.1 Syringe

The dimensions of the flange and barrel are defined in ISO 11040-4 [2]. Syringe geometry, dimensions, thickness, as well as glass surface texture or roughness can have implications on functional performance. On the other hand, changes in combi-

nation product design may trigger evaluation of existing flange/cone thickness and strength so as to ensure combination product component flange/cone interaction stresses do not cause cracks or breakages leading to a malfunction. For example, delivery time improvements in AIs may require increased energy and lead to changes in spring geometry or spring constant to accommodate changes in the syringe physical characteristics. An increase in spring constant can lead to higher stresses on the flange/cone during actuation of the device, and in such cases, flange/cone strength must be adequately demonstrated.

Glass defects such as cracks/chipping or broken flanges can also occur during manufacturing, i.e., syringe forming or in fill/finish, and these could lead to complaints. For example, it is well known that an existing scratch or crack in the glass could result in breakage of the syringe due to an outside stress such as dispensing from an AI, therefore handling of the syringe throughout the syringe manufacturing and drug product manufacturing process needs to be considered. Glass defects would typically be flagged during visual inspection in a syringe manufacturing process. Thus syringes with resilient cones/flanges are essential to minimize glass defects during fill/finish in a DP manufacturing facility. Becton Dickinson, a leading manufacturer of PFS, offers a syringe platform Hypak for Biotech which exhibits higher mean flange resistance for pharmaceutical applications. Their newer platforms such as Neopak and Neopak Xsi continue to offer higher flange resistance as well as tighter tolerances for biotech/AI applications. Other PFS manufacturers such as OMPI, Schott, etc. have similar syringe platforms with reinforced thickness for AI applications.

Susceptibility to glass defects may change when incorporating life cycle/post-approval changes such as site transfers or changes in equipment train. Changes in filling/handling/machinability during processing and manufacturing of drug product need to be verified and validated to ensure no downstream impact to PFS syringe quality, container closure integrity, or AI performance. Further, any changes in the syringe supplier manufacturing processes, e.g., forming and annealing steps, changes in glass cane supply, changes in temperature curing in various ovens, washing, drying and siliconization cycles, sterilization cycles, etc., must be evaluated for impact.

3.2 Stopper

The function of the stopper is twofold: (a) to provide suitable barrier/closure properties to ensure integrity of seal and (b) to enable delivery of the drug solution by sliding along the inner syringe barrel surface. The stopper is a product contact surface and thus must be evaluated for potential extractable and leachables as well as any impact to drug product stability. Changes in elastomer resin formulation, elastomer coating, as well washing/drying and sterilization cycles can all cause physical changes to the stopper which may impact functionality of the AI.

As in all molded products, stopper dimensions are controlled by the molds. These molds may undergo wear over time. A lack of timely replacement of worn molds from the supplier end can cause small shifts in stopper dimensions. For example,

during the routine incoming testing for the Humira DP process, changes in stopper dimensions were observed, and the wearing of stopper molds was identified as the root cause for the change. Most incoming requirements in manufacturing plants assess stopper identity using IR (infrared spectroscopy) or other suitable methods but not stopper dimensions. It is not typical across industry for certificates of analysis of incoming stoppers to depict stopper dimensions with acceptable ranges; however, if applicable, dimensional features of incoming commodities can be measured to ensure downstream capability. In addition of impact to functionality, out of specification dimensions of the stopper could impact CCI (container closure integrity). As with stopper dimensions, stopper design or geometry is quite critical to CCI. Stoppers typically used in a PFS have two or more sealing ribs. Although a higher number of sealing ribs ensure seal integrity, it may lead to higher resistance and elevated glide forces. Changes to stopper design post-launch to enhance container closure must be carefully evaluated to ensure that no excessive glide forces are now generated which could in turn require changes in device constituent design. For example, W.L. Gore & Associates, Inc. offers a fluoropolymer-coated stopper suitable for use in silicone oil-free syringes. The design and geometry of these stoppers are different from, e.g., the fluoropolymer-coated stoppers offered from West Pharmaceutical Services, Inc., which are intended for use with siliconized syringes. It may be desirable to pursue a post-approval change with stoppers that offer silicone-free system incorporation especially when dealing with formulations that are silicone oil sensitive or in cases wherein lot to lot variability in siliconization of incoming syringes has significant impact in functional performance of AI.

Stoppers are also available with varying amounts of silicone oil. A study carried out at AbbVie demonstrated that given a certain threshold of siliconization in incoming syringes, the level of siliconization of stoppers had no significant impact on glide forces or delivery times of a pipeline monoclonal antibody (see Fig. 9).

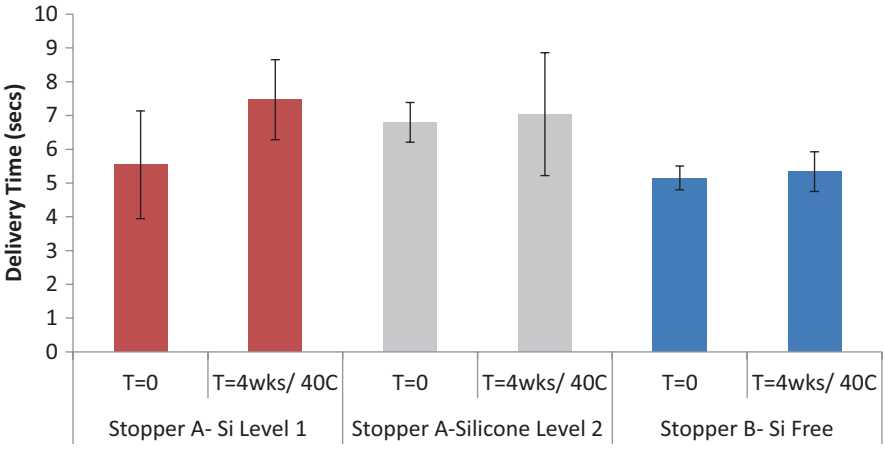

Fig. 9 Comparison of stoppers with varying silicone oil amounts on AI performance

In general, if stopper silicone oil amount has no impact on functional performance, it is recommended to eliminate or reduce this amount so as to further reduce Si oil contribution to particulate load in drug product solutions.

On account of post-approval or alternate supplier qualifications of stoppers as part of assurance of supply, one might end up with changes in stopper manufacturing process, e.g., changes in stopper washing, drying, siliconization, and even sterilization steps. Changes in sterilization, i.e., steam sterilization process to a gamma sterilization process, may have an impact to the stopper resin which could impact gliding properties of stoppers. In general, gamma radiation is regarded as a harsher process than steam sterilization; however, the authors of this paper have found no evidence of changes in AI functional performance between stoppers sterilized with either process.

3.3 Needle

The needle design variables such as needle length and the diameter of the outer versus inner wall impact functional performance or delivery time. The Hagen-Poiseuille equation [3] below (see Eq. 1) illustrates that the delivery time or plunger force required to deliver a dose is inversely proportional to the 4th power of the needle inner diameter but directly proportional to the length of the needle. In other words, the larger the inner diameter, the lower the delivery time and glide forces. The longer the length of the needle, the higher the glide force and delivery times.

$$F = \frac{128QuLA}{\pi D^4} \tag{1}$$

wherein:

F = Plunger force
Q = Flow rate
L = Length of needle
D = Inner needle diameter
A = Plunger area

For the Humira presentations, the needle underwent a change from 27G to 29G TW to further reduce pain at the injection site. The consequence of reducing needle inner diameter is advantageous in terms of patient compliance but can elevate glide forces and delivery times in the AI [4]. In this case for the Humira AI, an increase in about 1 second for delivery was observed; however, this was still well within the specification for delivery time. Changes in needle gauge must be assessed to ensure compatibility with AI design and overall delivery times. In addition to changes in gauge, lot to lot or vendor to vendor variability in incoming needles, i.e., needle

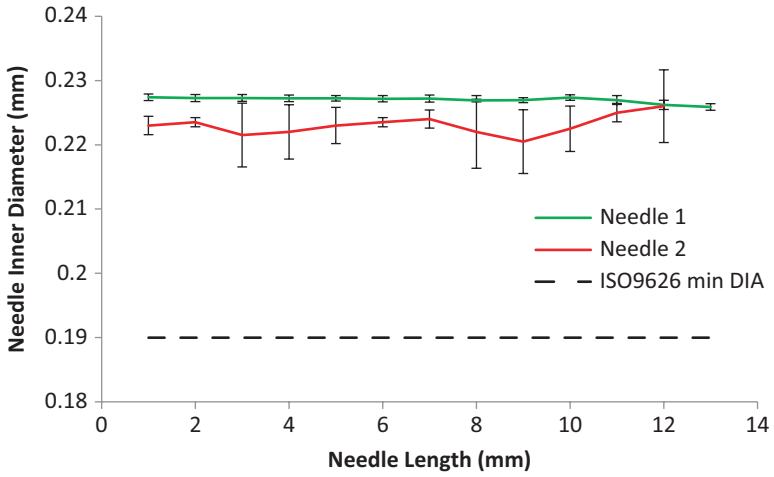

Fig. 10 Comparison of needle inner diameter variability

inner diameter as a function of needle length, must also be evaluated. Figure 10 depicts two different needles from two different needle vendors. Both sets of needles comply with the 29G ISO standard on needle inner diameter. However, Needle 1 exhibits a tighter control on diameter as a function of length, whereas Needle 2 exhibits a slightly more variable path. Higher glide force and DT on average were observed in Needle 2.

Other factors such as diameter of the outer wall, bevel design, lubrication, and surface smoothness may impact injection site experiences such as perceived pain. Becton Dickinson offers a regular bevel typically with three bevels as well as a five-beveled needle angular tip [5]. The latter on account of two additional flatter bevels has been shown to be less painful upon injection [5, 6]. Changes in the thickness of the needle wall, e.g., reduction in wall thickness from regular to thin wall (usually flow rate equivalent to 1 gauge larger) to extra thin wall, lead to lowering of glide forces and delivery time. Newer platform needles are available that utilize a sharply decreasing needle diameter principle from needle base to tip, to reduce delivery times and glide forces. For example, Terumo Pharmaceutical Solutions, Japan, offers a tapered needle system wherein the needle base may have a significantly larger diameter as compared to the needle tip which may have an effective diameter of 27 or 29G. Such a system presents significantly lower delivery times when compared to a standard 27G or 29G needles.

Needle defects including axial deviations, surface roughness, burrs, or hooks may increase delivery time as well as pain during injection [1]. In addition to characterizing the geometry of the needle, extractable/leachables testing and product stability are key. Several components of needles such as metal ions, glue/adhesive from the staked needle, tungsten from the cone-forming process, etc. can leach into

the drug product solution. Overall, changes in supplier needle manufacturing processes must be thoroughly evaluated as they can impact downstream drug product filling and manufacture and drug product stability.

3.4 Siliconization

Lubrication of certain components of the prefilled syringe system, namely, syringe barrel, stopper, and external needle wall, is essential to reduce friction and minimize glide forces. A certain threshold of lubrication and thereby glide forces is essential in the optimal functioning of an AI. Since lubrication is achieved by using pharmaceutical grades of silicone (Si) oil, i.e., polydimethylsiloxane, this process is also termed as siliconization, and its complexity in terms of performance attributes to both the PFS and the AI should not be underestimated. The Si oil application process differs between glass barrels, stoppers, and the external needle wall. The stoppers typically are siliconized during the final washing step. The external needle wall is typically siliconized using a wiping process, and the siliconization of glass barrels involves a spray nozzle process. The uniformity of Si oil application, the thickness of the silicone oil layer, the droplet size, the amount of silicone oil applied, its molecular weight as well as the amount of silicone oil lost during shipping/handling/filling, and subsequent storage can interact with the drug product as well as impact functionality of the AI. Limited syringe lot history during development can pose a challenge to fully vetting the influence of various formulation factors as well as silicone oil migration kinetics. There are numerous industry-wide examples of the challenges of silicone oil migration, i.e., movement of silicone oil from the glass surface into solution containing DP. The removal or migration of silicone oil is influenced by the presence of salts, excipients, pH, as well as surfactants. Shi et. al. [7]. have demonstrated that presence of acetate- or histidine-based buffering systems may influence or accelerate silicone oil migration over stability and this corresponds to a marked increase in glide forces over time. Presence of surfactants in solution can heavily influence removal of silicone oil from the hydrophilic glass surface [8]. These surfactants may be present in solution as a formulation component. However, on occasion, inconsistencies in the syringe manufacturing process with respect to washing/drying and annealing may lead to residual amounts of surfactants on the glass surface, and this may accelerate silicone oil migration into PFS solution, thereby causing elevated glide forces and functional AI challenges upon stability.

3.5 Summary

Overall there are several significant physical characteristics of the PFS which need to be considered in the development of an AI. A thorough understanding of these physical-chemical characteristics played a role in the development and on-market support of the Humira Pen.

4 Product Enhancements: Approach to Making Changes to a High-Volume Product

As previous chapters in this book have discussed the development and design of combination products, here we focus on the approach to making a design change to a high-volume product already launched into the market. Through development and human factors testing, a design can be verified and validated and fully demonstrated that it functions as intended and that the user can understand how to use the device. However, this testing is typically limited in scale compared to the number of users and the use conditions once a product has launched commercially. Feedback from the field whether it is directly from a user such as a complaint or compliment, through an assistance program, or from a health care giver provides a deeper insight into how the device is used, ease of use, and challenges if any. From these insights potential improvements to aid use may be identified. Some of these improvements may be obvious to the user, while others are not. For Humira, a vigilant process was implemented to analyze feedback from the field and to prioritize potential improvements based upon the risk assessment. This process has been key in maintaining overall very low complaint rates.

With the development of complaint trending tools early within the introduction of the Humira AI, two complaint categories were observed which were identified as likely use error but if true still had a direct link to design features of the device. These complaint categories were:

1. Activation with cap 2 intact
2. Activation with both caps removed

The description for activation with cap 2 intact is a premature dispensing of product prior to removing cap 2 (which exposes the actuation button). The design of the Humira AI prevents the initiation of the injection until cap 2 is removed and the plum-colored injection button is pressed, as described in labeling. If the user partially removes or recaps cap 2 to the point where the locking pin has been removed, the Humira AI can actuate if pressure is applied to the plum-colored injection button. This is considered to be activation with cap 2 intact as the cap is still partially on the device and actuation has occurred. In the instructions for use, it is stated that if cap 2 is removed, it should not be replaced; thus if the contrary is done, then the instructions are not being followed as intended.

The description for the complaint category activation with both caps removed encompasses premature dispensing of the product prior to use after removal of cap 2. Based on design and approved labeling, once cap 1 and then cap 2 have been removed, the Humira AI is ready for actuation. If the patient inadvertently touches the plum-colored activator button, the Humira AI may actuate and dispense the drug product. The Humira AI has a product requirement to ensure that the force to actuate the device is not too difficult as based on formative human factor studies or too low to cause autoactivation due to design. Humira is used in 13 different indications to date, and there is a large range of associated potential human abilities. For the use with a rheumatoid arthritis patient, a lower activation force is desirable due to potential

hand impairment, while with many of the other indications, hand impairment is not a typical condition. Thus in the design of the device, the entirety of the population needs to be considered. In the initial launch of the Humira AI, a low actuation force was used since the original indication targeted rheumatoid arthritis; however it was observed that unintended activation was occurring. The design was assessed, and a small increase in actuation force was determined, verified with extensive engineering confidence and design verification testing, and validated by human factor studies.

5 Design Improvements

An example of a design changes and manufacturing improvement identified and implemented as part of the Humira Pen on-market sustaining engineering support process is described here.

5.1 Actuation Force Improvements

FEA modeling in solid mechanics has been employed to assess the AI design and predict experimental testing results. The modeling is based on physics to simulate the assembled parts of an AI. Figure 11 shows the modeling results of different actuation forces as compared to the nominal design with an early version of simplified FEA model. The FEA model has been gradually improved to match the actual molded part geometry during the course of assessment. The FEA results of the actual part geometry match fairly well with the experimental observation. Once built, the model

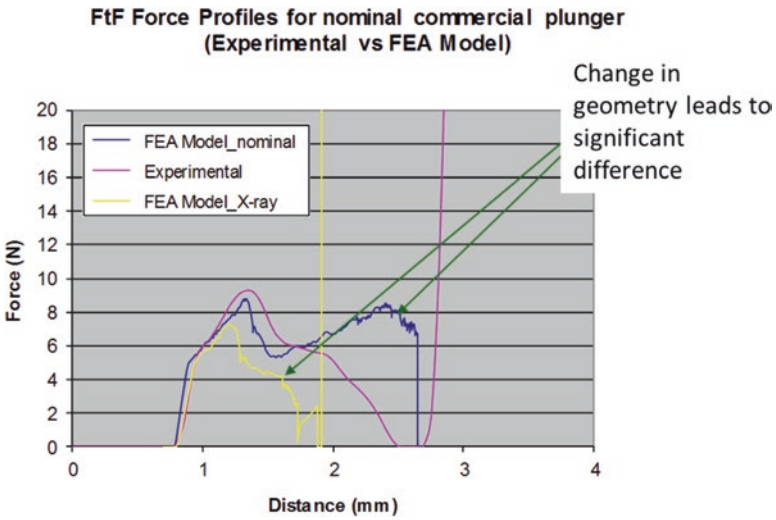

Fig. 11 FEA modeling

can not only be used to analyze at nominal design of product performance but also extended to actual parts from X-ray CT-scanned dimensions. This would allow the team at development phase to assure the product reliability via FEA modeling assessment.

To achieve a slight increase in the actuation force, a design change was made to the plunger rod component which interacts with the firing button, thus increasing the actuation force. Additionally other design changes were assessed to improve manufacturability and address other low-level complaint categories.

An example of plunger design to explore the initial contact surface (ICS) angle on force to actuate was studied. Figure 12 shows the plunger designs at three sets of ICS angles for analysis.

Figure 13 shows the effect of ICS angles on the force to actuate. The ICS angle design seems to have a pronounced impact on the force to actuate. A high force up to 42 N at 48 degrees is established as compared to 15 N at 28 degrees.

It is also noted at the same degree of angle, the force could vary up to fourfold depending on the material used. Material study in Fig. 14 shows that the force to fire is increased with material flexural modulus (rigidity).

The design is expected to balance between product performance and human factors. The preferred force is to be high enough to minimize the risk of unexpected premature actuation but low enough to meet the requirements of the patient with compromised dexterity. Accordingly, material selection and dimensions become critical to fulfill the design space of the preferred force window.

Figure 15 shows another modeling example of detailed fine-tune of the plunger part geometry design. The ICS angle has been designed in two different approaches.

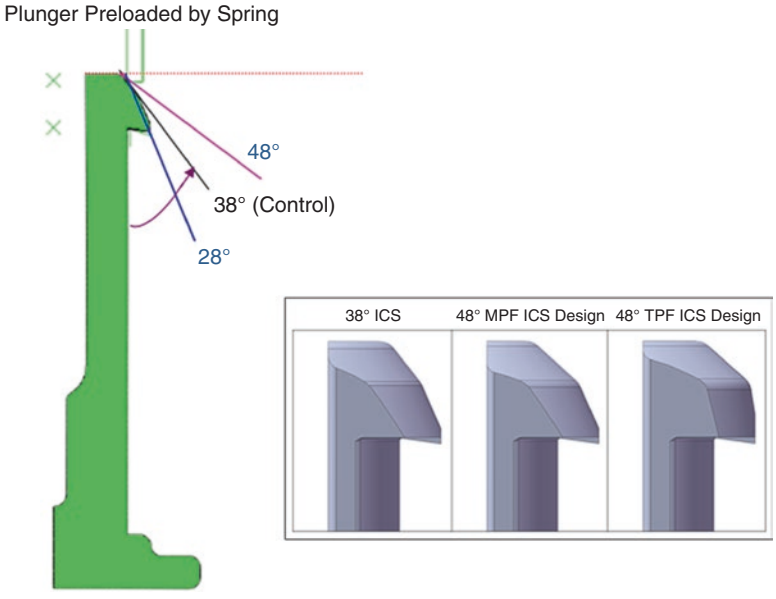

Fig. 12 Contact angle

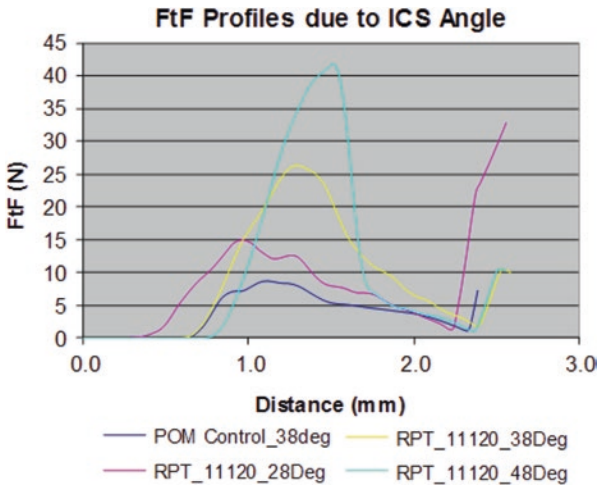

Fig. 13 Force to actuate

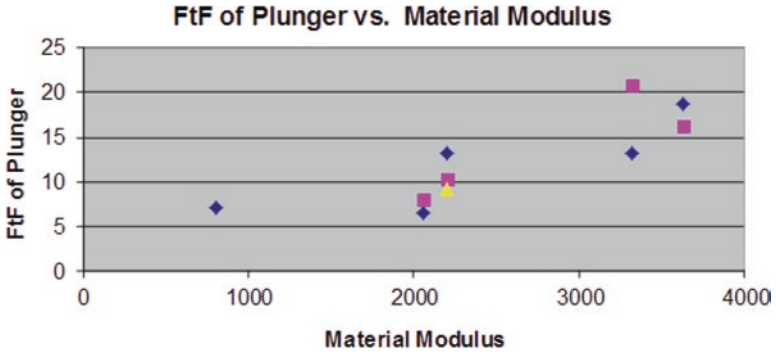

Fig. 14 Effect of material

One is the midpoint fixed design and the other is top-point fixed design. The modeling shows that the fine-tune design can also play a role on the force to activation. Again, the modeling in the fine-tune designs can guide the optimization of an autoinjector design and minimize the experimental work to shorten the product development cycle.

5.2 Assembly Improvements

Initial concept for inserting a syringe into the Humira Pen relied on a CAM to move to a fixed position where the syringe would engage in the AI subassembly. With a fixed move, the accuracy of placement is assured to the equipment position, but the

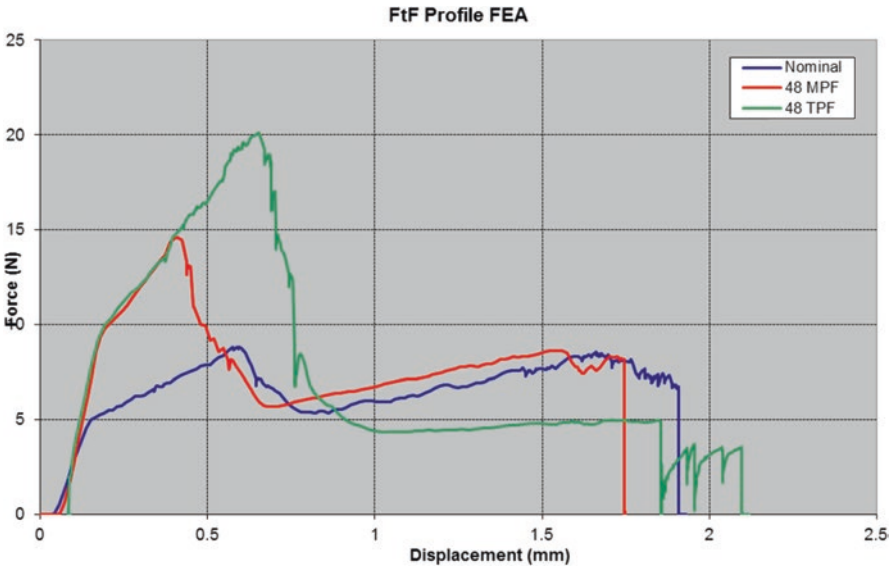

Fig. 15 Force to activate with fine-tune of plunger designs

device components interacting with the features of the syringe and needle guard also need to be considered. When considering the types of interactions within the device assembly process, the station design should be considered to ensure robust mechanism is used. For engaging two device parts together, if CAM linear motion design tolerance is within the needed part engagement tolerance and there is no risk to excessive force on the device, then this common assembly practice should be used. This movement can be in the form of a pick and place unit that moves a part from one position to the next or could represent a press motion to engage two parts together. The motion is a fixed distance for travel and will typically accommodate release overload pressure to prevent damage to the machine. This prevents movement any further than the set distance but can stop short if the action requires too much force. Subassembly/component variability contributes to the placement of the syringe into the AI. If a specific depth of insertion is required due to necessary interactions/engagements, then a CAM may not provide the most robust mechanism for the assembly process due to variation in the constituent parts. For the Humira Pen, the initial assembly used a CAM mechanism for placement of the syringe into the subassembly; however complaints were observed where the root cause for the AI malfunction was related to correct PFS placement in the syringe housing subassembly. Differentiating the unique constituent part engagements to ensure the correct PFS/subassembly interaction was needed to devise a solution. Since there is interaction of constituent parts during assembly, then the process can be monitored using a force profile in real time. Understanding which forces of engagement relate to the syringe position within the AI design can be useful to creating a force monitoring process. This process can be used to assure proper insertion by allowing one to

"see" into the insertion process and "monitor" the distances that may not be finite, but variable based on the incoming variability of the constituent parts. In some instances, a force response by the monitoring system to the moving servo can be used to stop the process, allowing for precision engagement of the syringe within the AI. With this information an improved assembly process syringe insertion step was implemented which resulted in the elimination of the previously reported AI malfunctions.

6 Conclusion

During the commercialization of the Humira Pen, a number of changes have been made to address feedback received from the field as well as other post-approval changes made to various manufacturing changes upstream for the Humira Pen processes. Constant monitoring of the product has been in place and provides data to direct the program and product support. The success of the product will to continue to build on the learned lessons, and these approaches and knowledge gained will also be applied to other new and upcoming product lines.

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Chapter 36
Considerations in the Development, Approval, and Commercialization of On-Body Delivery Devices Used in Combination with a Biologic

Donald Busby and Kesley Gallagher

1 Introduction

Amgen currently has two commercially available combination products including an on-body drug delivery device, the Pushtronex® system as shown in Fig. 1 (single-use on-body infusor with prefilled cartridge) for the delivery of Repatha® (evolocumab) and the Neulasta® (pegfilgrastim) Onpro® kit as shown in Fig. 2.

The electromechanical devices included in these combination products are coupled to the body of the user via an adhesive patch and deliver the Amgen biologic through a needle or soft cannula. Both on-body delivery devices are co-packaged with the biologic. However, each is designed to meet differing user needs. This chapter covers topics at a high level from development through commercialization that are applicable to both products and are not intended to be an in-depth discussion of the overall development process.

2 Requirements Development

The dosing strategy for Repatha® offered a bi-weekly and a monthly option for users. An autoinjector with a 1 mL syringe was optimal for the 140 mg bi-weekly dose; however, as the monthly Repatha® dosage was 420 mg, i.e., a much larger dose, the resulting volume was too large for the current autoinjector. Thus, there was a need for an autoinjector capable of delivering this volume. Amgen entered into a development agreement and partnership with our key partner, to develop an on-body delivery device capable of delivering 3.5 mL.

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Fig. 1 Pushtronex® system for the delivery of Repatha® as labeled for the United States' market

Fig. 2 Neulasta® Onpro® kit as labeled for the United States' market

For Neulasta®, the drug must be delivered approximately 27 hours after chemotherapy, which requires the patient to return to the physician's office the following day. This unique need to delay an injection led to a development agreement and partnership with our key partner to develop an on-body delivery device to deliver a dose of Neulasta® approximately 27 hours after application to the body. The use of this pre-programmed device is preferential since the user does not need to return to the HCP's office for the injection the day following chemotherapy.

For both products, the time or speed (rate) to deliver the drug volume would be based upon tolerability or convenience rather than clinical efficacy. In other words, the time or speed to deliver the drug volume does not impact the clinical efficacy of the drug being delivered.

Using a risk-based product development process, the teams leveraged the risk management process in ISO 14971 to identify potential failure modes for the product. As much as possible, the teams identified requirements within consensus standards as mitigations to the failure modes.

The use of consensus standards can streamline regulatory reviews since they generally define a level of safety and quality that has been agreed upon through a Standards Development Organization such as ISO, IEC, or ASTM. Although consensus standards can be leveraged, it is still the manufacturer's responsibility to assure the requirements specified in the standards are appropriate and provide the required risk reduction for the device.

During initial requirements development for the Pushtronex® device, it became apparent that a single consensus standard directly addressing on-body injection systems did not exist. Infusion pump consensus standards primarily focused on risks related to the rate of delivery. Ultimately, the team leveraged consensus standards to address risks associated with dose delivery, as well as a risk management process to identify the potential failure modes unique to on-body systems (see chapter 30). Where applicable, the teams identified and leveraged portions of consensus standards to address design considerations. For example, for the Pushtronex® device, ISO 9626 was utilized for needle tube materials or ISO 7864 for needle/tubing bond strength. Where failure modes resulted in unique mitigations not covered by any consensus standard, the mitigations were identified in risk management documents and translated into design input requirements. Although this may be standard practice for medical device development, it may not be readily apparent from a combination product development perspective where the device is not the primary mode of action.

Understanding the use environment is essential to identifying requirements. For example, although the drug delivery devices in the Pushtronex® system and Onpro® kit are both adhered to the body via an adhesive patch, the Pushtronex® device remains in place for approximately 10 minutes; however, the Onpro® device needs to remain in place over 27 hours and could be subject to exposure to water via shower or bath and should be identified as a use case. The patient's age or disease state may have accompanied comorbidities, which will drive requirements and design preferences such as device ergonomics or error/status light patterns that can easily be understood by color-blind patients. Devices designed primarily for use in the home environment may have more strict requirements pertaining to immunity to electrostatic discharge compared to a healthcare office.

3 Risk Management

The development of risk management documentation is time-consuming and tedious work. It involves cross-functional participation from medical safety, device engineering, complaint handling, packaging engineering, human factors, container engineering, quality assurance, and drug product departments (to name a few!). The time spent during the development process to create and maintain a robust risk

management file provides significant value beyond the product development cycle. Complaints management will utilize risk documents to develop complaint intake codes, and trending will be assessed against the identified failure modes within those documents (see chapter 29). Regulatory requests for information may reference back to the risk documents in support of the risk/benefit of the product.

The risk management documentation provides the basis for risk/benefit analyses to support clinical and/or commercial introduction of the product. Risk management provides scope to complaint handling questions used to define the compliant and information to aid root cause analysis. Even with the most comprehensive risk document file, new failure modes will be identified throughout the life cycle management of the device, and risk management must be updated accordingly.

3.1 Use of Consensus Standards

When leveraging consensus standards within the risk management process per ISO 14971, remember the standards only specify a minimum level of safety. The risk management process must assess the risk associated with the failure mode to determine if the minimum requirements specified in the standards are acceptable to appropriately mitigate the risk.

As an example, for electromechanical devices, IEC 60601-1-2:2014 specifies an immunity test level (V/m) based on an assumption that the device will have a minimum separation distance of 30 cm from the radiating source (cell phone, WiFi router, etc.) in the use environment. The manufacturer's risk management process should determine if higher immunity test levels should be specified to lower the separation distance and reduce risk to the patient.

4 Human Factors Usability

With a new device, usability testing is an essential component of early development. That is, design changes later in development or after clinical studies take significantly longer and cost substantially more to implement. The credo "fail early and fail fast" applies to early development and formative human factors testing. By mapping the predicted user process flow and identifying all the points where the user interacts with the product provides a testing road map. Most early issues are observed when it's something that the user does with the device or to the device and/or where the device provides some type of feedback to the user. Also note that devices intended to be used in the home environment may be used infrequently. Therefore, evaluating the frequency of use is an integral part of the early usability assessments.

With any usability study, the number of subjects enrolled in the study typically ranges between 15 and 20 users per group. Small signals observed during these

studies can manifest into measurable complaint signals once the product is commercially introduced to the market. Even if the subjects are successful in performing the tasks associated with administering a dose during the study, any observed difficulty can result into complaints related to the use of the device.

One of the more important interfaces for on-body devices is the placement of the device on the skin with an adhesive. For the Pushtronex® and Onpro® devices, an adhesive patch is utilized. Most usability studies are performed using skin pads, some of which may be placed over the intended injection site on the body. However, skin pads will not provide sufficient data to assess the functionality and durability of the device/skin interface as these scenarios are mutually exclusive. There are several different skin models available for verification testing, but none can simulate all the various conditions experienced in real-world applications given the diversity of use environments. Evaluating the effectiveness of the adhesive with actual use on body HF studies early in development will potentially mitigate costly and time-consuming redesign during clinical trial or launch.

5 System Considerations

With the implementation of FDA's CGMP Final Rule on Combination Products per 21CFR Part 4b, there is increased awareness on product development from a system perspective. Risk management forms the foundation from which the biologic/device combination product is developed, with a specific emphasis on interfaces and interactions between the biologic and device as well as the device and user.

5.1 Integration and Interface of System Components

Interfaces with other components of the final medical product should also be considered. These interfaces between the device constituent part, the primary container, biologic, and packaging (including the sterile device packaging) all need to be assessed as a system from both a functional and usability aspect. As an example, if the biologic is light sensitive and there are multiple doses of a product in a single carton, then understanding how the user interacts with the packaging is key to assuring the packaging functionally protects the biologic up through the use of the last dose in order to protect the quality of the biologic.

The concept of interface design should be applied broadly, and not just to the final medical product, for example, to the full supply chain process. For instance, bulk palletized devices in cartons with the pallet stack height optimized for sterilization may not fit in pallet racks at the final manufacturing site. Understanding the entire product flow helps prevent surprises later during the development of the commercialization process.

5.2 Extractables and Leachables

Amgen has formal internal procedures based on ICH Q9 Quality Risk Management and the FDA guidance for the industry (May 1999) on Container Closure Systems for Packaging Human Drugs and Biologics developed to assess extractables and leachables (E & L) created by the interaction between the biologic and the assembled primary container.

In addition, a device developer must also test for biocompatibility of those materials that will contact the user according to ISO 10993 *Biological Evaluation of Medical Devices* series. The scope of the standard is related to the materials of the device and the relative safety of those materials to the patient. ISO 10993 series does not cover the effect of the materials on the biologics stored in the device nor delivered through the device. Historically, pharmaceutical companies assessed the impact of the primary container on the biologic but not the impact of the device on the patient. For on-body devices, the fluid path and/or reservoir within the device comes into contact with the biologic and therefore to the user. This interaction must be assessed through extractables and leachables and ISO 10993 series.

5.3 Biologic Stability and Device Shelf Life

The introduction of on-body devices required new requirements for the overall expiry of the combination product. The biologic in its primary container is historically assessed for stability. The stability program should also assess the interaction between the biologic and device by evaluating product quality after delivery through the device.

Within a combination product, the device constituent part must be assessed for shelf life as well as stability of the drug.

For Pushtronex® and Onpro®, the requirements to assess device shelf life were identified through a combination of primary function and risk management evaluation. The primary function of dose delivery was derived from the scope of ISO 11608, with dose accuracy identified as the key requirement to demonstrate that the primary function of the device constituent part was met. Additional requirements were also identified through risk assessment and identified mitigations associated with higher risk.

Devices utilized in shelf life testing were manufactured using commercially representative units and exposed to transportation simulation per ASTM D4169 to simulate real-world conditions (stressors). As the majority of on-body devices are terminally sterilized, the device developer should consider exposing the device to the sterilization cycle before entering into shelf-life testing. Shelf life is assessed in both accelerated and real-time aging conditions. In both cases, multiple intermediate time points are assessed, and the same requirements are tested at each time point (as calculated by the Arrhenius equation) for both accelerated and real-time protocols.

The expiry of the combination product will be the lesser of its constituent parts, e.g., device and drug, at time of co-packaging. For the device constituent part, the overall device shelf life is a combination of the warehouse storage time and the time co-packaged with the biologic in its primary container. The warehouse storage time is mainly driven by supply chain requirements with longer times preferred in order to minimize risk of scrap. The co-packaged shelf life is driven by the expiry of the biologic such that the device should never be the expiry-limiting constituent component of the combination product.

5.4 Software Classification

For electromechanical devices containing software, a software classification is assigned based on assessment of risk as defined in FDA document Guidance for the Content of Premarket Submissions for Software Contained in Medical Devices as well as in IEC 62304 Medical Device Software – Software Life Cycle Processes. The FDA guidance assigns a level of concern (minor, moderate, major) to the software based on the potential severity of the potential harm to the patient, and IEC 62304 assigns a safety classification (class A, class B, class C) also based on the severity of the potential harm. Although similar in classification based on potential harm, there are some differences between the two documents.

IEC 62304 does not explicitly take into consideration the delivery of the biologic through the device, it generally speaks to the device constituent part of a combination product only. To determine the appropriate classification, the risk of not delivering the biologic is needed to be taken into consideration.

The FDA guidance does take into consideration that the device may be used to deliver a biologic; however, it leans to defining any software within a device that delivers a biologic to have a level of concerned specified as major.

5.5 Software Privacy Concerns

On-body devices generally include embedded software. Even though the device supplier owns the proprietary code and programs, the legal manufacturer is responsible for the integrity and privacy of the final product.

Consider the following. Does the device:

- Have wireless communication capabilities (programming or data transmission)?
- Store any patient identifiable information on the device (including from clinical studies)?
  - Health or healthcare professional-related information?
  - Government-issued identifiers?

  - Cultural or social information?
  - If yes, will a third party have access to this information?
- Have externally accessible data or programming ports that can be accessed while in the market?

A risk assessment should assess the risk associated with integrity and privacy of the software. Examples of risks include access to patient data, integrity of data stored on the device, or willful hack or corruption of device functionality. Additionally, the FDA has published guidance titled Content of Premarket Submissions for Management of Cybersecurity in Medical Devices that provides recommendations for decreasing risk associated with such things as patient data, device functionality, and connectivity.

5.6 Consensus Standards

The use of consensus standards streamlines requirements development and risk management process, establishment of acceptance criteria, and identification of test methods. Part of the challenge in selecting the appropriate standard is highly dependent on the intended markets for the product as they can vary by region. The FDA maintains a Recognized Consensus Standards list through their Standards and Conformity Assessment Program and encourages manufacturers to leverage standards within this list. The list can be found online at https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfstandards/results.cfm.

The EU maintains a list of standards that are harmonized and accepted by all 28 Member States. Japan may use consensus standards as a baseline but modify or add requirements and then publish under a country-specific standard such as JIS. If the product is intended to be global, regional standard variation can present a significant barrier to market acceptance. Global regulatory strategy, in light of standard variations and differing requirements, is essential to the development of the device constituent part of a combination product.

For instance, although ISO 7864 is harmonized across the EU and recognized by the FDA, Japan has introduced modifications to the standard, as documented in JIS T 3209. Identifying these regional differences early will minimize negative impact to regulatory approval timelines.

5.7 Transfer to Manufacturing

Most think design transfer occurs late in the development process. Experience in this area will dictate manufacturing requirements must be developed much earlier in the process. Design transfer is bi-directional, and if a development partner, i.e., supplier, is involved, the transfer is much more complex but also more essential to be

developed early. Equipment specifications may need to be developed while the device is being designed. Input to these specifications may be device-specific requirements such as assembly forces or torque limits which may come from the device development partner. These limits need to be documented and controlled in order to be referenced in the equipment specifications.

6 Regulatory Considerations

As previously described, a single consensus standard did not exist directly applying to on-body type devices at the time of development. (Note: at the time of this writing, ISO 11608-6 for on-body delivery systems is being drafted).

FDA published two guidance documents: *Infusion Pumps Total Product Life Cycle* and *Technical Considerations for Pen, Jet, and Related Injectors Intended for Use with Drugs and Biologics.* During the approval of Pushtronex®, FDA indicated the infusion pumps guidance would be applied to submissions related to on-body devices. Application of this guidance may drive additional design requirements and/or additional assessment of specific risks identified within the guidance, and development of a safety assurance case is required as part of the submission.

Unlike the United States, combination products are not recognized in the European Union per se. In the EU, should the product not meet the requirements for a medicinal product, the device constituent part must comply with the MDD and receive a CE mark from a Notified Body. This is followed by submission to EMA for review and approval of the medicinal product.

For a combination product where the CE marked device is designed for or used with a specific medicinal product, this two-part approval process creates unique and obscure challenges. For instance, consensus standards such as IEC 60601-1 specify labeling requirements for the unit packaging and in the IFU. The MDD encourages manufacturers to use symbols defined in ISO 15223 on packaging and labeling to address translation issues across the multiple languages in the EU. If a manufacturer decides to use a third party to certify the device to IEC 60601-1, the manufacturer must comply with all the applicable requirements of the standard in order to receive the certification. This certification is provided within the technical file to the Notified Body to show conformity to the essential requirement checklist per Annex 1 of the MDD. This CE mark certificate is submitted to the EMA as part of the medicinal product submission.

Because the biologic has the primary mode of action, the EMA has purview over all labeling including the instructions for use (IFU). Interestingly, the EMA does not endorse symbols per ISO 15233, and symbols are not allowed on any labeling for the medicinal product. This created a bit of confusion and conflict since it could invalidate the certification to IEC 60601-1 and may impact the Notified Body certification to the MDD. Therefore, it is imperative to work closely with the testing houses and notified body to justify why symbols are not included in the submitted labeling on behalf of the device constituent part.

Also under the MDD, devices can be classified as class IIa or class IIb. The implication for class IIb devices, essentially a higher risk, is more stringent Notified Body obligations for review of device changes as well as regulatory reporting requirements for complaints attributed to device failure that has potential for serious injury or harm. A Medical Device Vigilance (MDV) report will be filed with the competent authorities for each device failure with the potential for serious injury or harm. Some competent authorities will send the manufacturer a request for information for each MDV report submitted.

Japan has a similar process as Europe in that it requires a Notified Body assessment of the device prior to the submission to PMDA. Japan does recognize consensus standards; however, in some instances, there are Japan-specific requirements incorporated. For instance, JIS T 3226-1 is the Japanese version of ISO 11608-1.

7 Sustaining Engineering

When considering the total product lifecycle, development of the product up through market approval represents only a portion of the total lifecycle; maintaining the commercialized product on the global market also represents a significant portion. When the product is marketed globally, configuration management can create regulatory submission challenges. A simple post-market design change can easily take months to implement, and if the change requires pre-approval from global regulatory bodies, approval times by region can vary between months to years for the same change. Robust device design change management is essential to control costs and manage global inventory.

One of the bigger challenges to electromechanical devices is electronic component obsolescence. The medical device industry has always been at the mercy of consumer electronics and the tech industry. Microprocessors are introduced and obsoleted in a matter of a year or two. Given that medical devices can take 3–5 years to develop and have long shelf lives, it's not uncommon to have components become obsolete before the device is even introduced to the market. Replacing microprocessors post-market can be an expensive and time-consuming change. Mitigations to this include identifying all the critical components of the system and ensuring they are not single-sourced. For microprocessors and other major electronic components, have early discussions with the supplier. Determine their life cycle plan for the components and make sure there is a clear succession plan for easy upgrades or replacement in the future.

In Europe, for products falling under the Medical Device Directive, there is an expectation the device is acknowledged state of the art. For example, an electromagnetic device should claim compliance to IEC 60601-1-2:2007 for electromagnetic compatibility to satisfy essential requirement 2 in the Essential Requirements Checklist. However, when the 2007 version was withdrawn on December 31, 2018,

there was a presumption of conformity that the device on the market will comply with the latest 2014 version. Manufacturers are given a 3-year window between publication of the new version and withdrawal of the previous version in order to give time to comply.

8 Device Development Partnership

As is well documented, a successful project is highly dependent on team dynamics; specifically, team interaction and management oversight to engender positive behavior and more reliable project outcomes. As challenging as that is to manage internally, imagine adding an external development partnership into the normal program challenges. A development partner will bring different dynamics to the effort, and to have a successful development relationship, inter-company teamwork and goal setting is essential.

A strong collaboration between the device developer/manufacturer and the pharmaceutical/biopharma company is critical to delivering innovative, patient-centric combination products. As an example, Amgen and West Pharmaceutical Services Inc. created an integrated, cohesive, single team with co-executive sponsorship. This overarching program leadership enabled successful development and launch of the Pushtronex® system.

With the alignment on an overarching vision – to serve patients – Amgen and West built a collaborative and integrated team through:

- Alignment and partnership from corporate leaders
- Empowered, co-executive sponsorship
- Strong program management and planning
- Single points of contact by function at each company
- Establishment of a shared goal
- Shared visions for "what does good look like?"
- Clear decision-making and escalation pathways
- Joint meetings once a week at the team level to discuss issues

The structure utilized in these development partnerships is illustrated in Fig. 3.

This cross-company team shared common goals and milestones throughout the project. Goal progress and/or roadblocks were reported on a weekly basis. By building a one-team philosophy, success was anticipated rather than thwarted.

Within each function, dual points of contact within each company were equally responsible for reporting and presenting. The most important single team aspect was the face-to-face meetings and time spent with each other. Within the project, there were some challenging times, but there were also many great accomplishments. It is important to note that teams must use time effectively within a predetermined structure to develop and introduce a new product in the market.

Fig. 3 Device development partnership model

8.1 Leverage Internal Experts

Remember to leverage the technical expertise within the organizations. Larger companies tend to have experienced resources that have significant knowledge across diverse fields of expertise. Too often, project teams attempt to solve problems or work through unique aspects of a design themselves; after all, that's what engineers like to do! However, this can result in a lost opportunity for the team to leverage experts within their company and resolving issues more effectively.

9 Conclusion

Development and commercialization of a combination product involving an on-body delivery device is a complex journey. Teamwork and clear, shared goals between the sponsor and supplier were invaluable to the initial success and for life cycle management of the devices after launch. The efforts taken by these teams, and the strategies developed by these collaborations, have ensured state-of-the-art drug delivery systems for Amgen's patients and blazed new pathways for combination products using on-body delivery devices.

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