Chemical & Thermal Denaturation

Application Note NT-PR-011

Getting the Full Picture: Predicting the Aggregation Propensity of mAbs Using Chemical and Thermal Denaturation on a Single, Fully Automated Platform

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Abstract

One of the most important parameters in the development of therapeutic biologics is their long-term stability. While after purification being seemingly stable in a variety of formulations, many antibodies display very slow aggregation kinetics over time. This gradual aggregation could thus far only be evaluated by monitoring monomer contents and aggregates over months or even years. Predictive methods are therefore urgently needed to speed up the development of biologics.

Here we demonstrate that the Prometheus NT.Plex by NanoTemper Technologies can be used for predicting long-term stability in only a few hours. The approach uses a combination of thermal and chemical unfolding analysis in a high-throughput setting. We show that chemical denaturation is a tool which can determine folding enthalpies of monoclonal antibodies (mAbs) to predict their long-term stability in formulation screenings. Using the Prometheus NT.Plex nanoDSF instrument with aggregation detection optics, we screened 5 formulations at different mAb concentrations for their thermal and chemical stability. The obtained unfolding data correlates with long-term turbidity and monomer content over time, showing that the Prometheus NT.Plex can be used to rapidly predict the long-term stability of biologics within 1 day.

Introduction

Biologics belong to the fastest growing group of drugs, and an ever growing number of complex molecules such as mAbs, antibody-drug conjugates (ADCs) and biosimilars demands novel, predictive analytical methods to streamline the development process. An important aspect during drug development is the long-term stability of the medicinal product. (Geng et al, 2014; Seeliger et al, 2015).

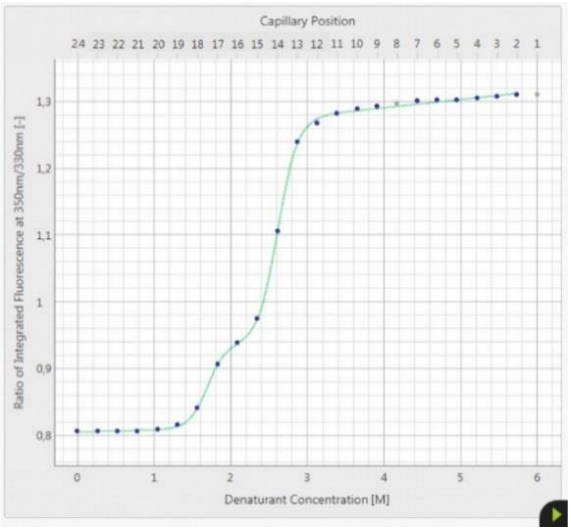


Figure 1: Chemical denaturation curve of a mAb, analyzed by detecting changes in the fluorescence emission ratio using the Prometheus NT.Plex. The fit was performed using a three-state unfolding model.

The most critical parameter is the formation of protein particles, especially at high protein concentrations. Thereby, protein particles can serve as nucleation seeds and might promote

protein aggregation. Consequently, particle formation does not only reduce the amount of active native-like protein, but can also cause unwanted effects which render the drug ineffective, or even harmful to patients (Garidel, 2014; Roberts, 2014).

In order to predict the aggregation propensity of biologics, the assessment of their relative thermal stability and the measurement of ΔG° of unfolding are becoming invaluable tools during formulation development and protein engineering (Temel et al, 2016).

The principle behind the chemical denaturation approach is the following: By increasing the concentration of a chaotropic salt, here guanidine hydrochloride (GuaHCl), protein conformation equilibrium is shifted from a folded to a partially unfolded up to a completely unfolded state. The fraction of unfolded protein at each measured GuaHCl concentration can be determined, e.g. by monitoring changes in the F350/F330 fluorescence ratio. From this GuaHCl concentration dependence of unfolding, ΔG° can be calculated (Figure 1). Importantly, based on the Gibbs-Helmholtz law and van´t Hoff equation (ΔG° = - RTlnK), ΔG° can also be used to very precisely determine the amount of fraction of -folded protein, which can then be used to calculate back the amount of unfolded protein at [D] = 0 (Table 1).

Fraction denatured	ΔG° (kJ/mol)
0.1	5.4
0.01	11.2
0.001	16.8
1E-4	22.5
1E-5	28.1
1E-6	33.7
1E-7	39.3
1E-8	44.9
1E-9	50.5
1E-10	56.1
1E-11	61.7
1E-12	67.4
1E-13	73.0
1E-14	78.6

Table 1: Calculated values illustrating the correlation between ΔG° and the fraction of denatured protein at [D] = 0.

Different aggregation mechanisms have several effects on protein stability, and thus on ΔG°. Since aggregation depends on the overall protein concentration, additional information about the aggregation mechanism can be extracted by measuring ΔG° at different protein concentrations. There are three possible outcomes of this approach (Rizzo et al, 2015):
i) ΔG° is <u>independent</u> of protein concentration, which means that there are no intermolecular interactions and no aggregation propensity.
ii) ΔG° <u>increases</u> with increasing protein concentrations, which means that the folded state of the protein is stabilized by “native-state”-aggregation. And
iii) ΔG° <u>decreases</u> with increasing protein concentration, which means that the unfolded state becomes more populated by irreversible “denatured-state” aggregation. The latter can also be accelerated using thermal unfolding in temperature ramps, which can then be detected with the Prometheus NT.Plex with aggregation detection optics, thereby providing direct feedback on conformational and colloidal stability of proteins.

In this study we performed proof-of-concept chemical denaturation experiments with lysozyme, which is very well characterized in terms of aggregation pathways. Moreover, we analyzed $\Delta G°_{app}$, aggregation onset temperatures (T_{agg}) and unfolding transition temperatures (T_m) of a mAb in different formulations, and compared the results with turbidity and monomer content over time as assessed by HPSEC for long-term stability data. For this, we used automated liquid handling in conjunction with capillary-chip filling using the NT.Robotic Autosampler and automated measurement execution by the Prometheus NT.Plex. The results show that this combinatory approach of thermal and chemical denaturation allowed for the identification of the formulation with the best long-term stability.

Results

In order to test the capability of chemical denaturation to predict protein aggregation, we first investigated lysozyme unfolding in presence of GuaHCl at different buffer pH values. Lysozyme is known to undergo irreversible, denatured-state formation of fibrils and aggregates at pH values ≥ 12.2 (Homchaudhuri et al, 2006; Kumar et al, 2009; Ravi et al, 2014), whereas it is stable at low salt condition at pH 5.2. Therefore, we measured changes in $\Delta G°_{app}$ at these two pH values at different lysozyme concentrations. Figure 2 shows

a strong lysozyme-concentration dependent decrease in $\Delta G°_{app}$ in phosphate buffer pH 12.2, whereas $\Delta G°_{app}$ is independent of lysozyme concentration in acetate buffer pH 5.2. These results indicate that chemical denaturation is suited to identify buffer conditions which promote protein aggregation, and are in good agreement with studies using the osmotic second virial coefficient to predict colloidal stability (Le Brun et al, 2010).

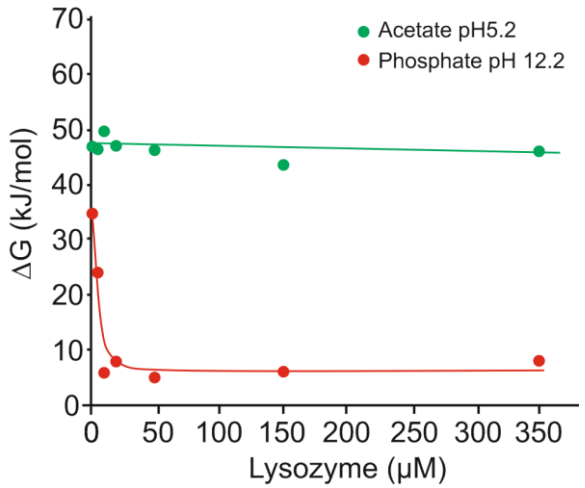


Figure 2: Chemical denaturation of lysozyme at different pH-values. The decrease in $\Delta G°_{app}$ at pH 12.2 indicates denatured-state aggregation. Lines are guidance to the eye.

Next, we investigated a monoclonal antibody, mAb1, in 5 different formulations. Preparation of dilution series, filling of capillary chips and assay execution were fully automated using the Prometheus NT.Plex in conjunction with a liquid handling system and the NT.Robotic Autosampler. For each condition, 24 GuaHCl concentrations between 0 and 6 M at mAb concentrations of 20 mg/ml, 5 mg/ml, 2 mg/ml and 0.5 mg/ml, respectively, were prepared and incubated over night to ensure sample equilibration.

In 4 out of 5 formulations a marked decrease of $\Delta G°_{app}$ could be observed at increasing mAb concentrations, suggesting that the denatured state of the mAb has a strong tendency to form irreversible aggregates (Figure 3). Only formulation F04 showed no change in $\Delta G°_{app}$, suggesting that the colloidal stability is maximal under these conditions, and that neither aggregation of the folded nor unfolded state occur. In order to test this hypothesis, we compared the chemical denaturation data with long-term stability measurements. Here, monomer content and aggregation were monitored by HPSEC and turbidity measurements over a time period of up to 17 months at different temperatures, respectively. An increase in aggregation over time results in a decrease in monomer content and an increase in sample turbidity. Formulation F04 was identified by both methods to provide best long-term stability, as it showed the highest monomer concentrations and lowest turbidity values over time. This demonstrates that $\Delta G°_{app}$ -measurements can robustly predict the long-term aggregation propensity of mAbs.

In addition to chemical denaturation experiments, we investigated the conformational and colloidal stability of the mAb by nanoDSF with backreflection detection optics. F03 and F04, which were shown to be most stable in the long-term turbidity measurements, had the highest unfolding transition temperatures (75.3 °C and 73.8 °C, respectively) when compared to the least stable formulations F01 and F02 (69.7 °C each) (Figure 4). F03 and F04 also had the highest aggregation onset temperature (86.0 °C and 86.4 °C, respectively) (Figure 5). Notably, the backreflection signal intensity scaled directly with the long-term stability measurements, with F04 showing the smallest signal at 95 °C (225.2 mAU), while F01 and F02 showed the highest degree of aggregation (313.4 mAU and 312.0 mAU, respectively). These data suggest that the temperature-induced unfolding and subsequent aggregation detection using the backreflection optics of the Prometheus NT.Plex can be used as a predictive method to evaluate the degree of aggregation propensity of the unfolded state of biologicals. Thus, quantifying the degree of aggregation using backreflection detection could be beneficial when compared to conventional light-scattering approaches: Light scattering can typically only be used for T_{agg} determination when using concentrated samples, since the signal is typically lost once strong aggregation occurs. In contrast, the backreflection signal quantifies the overall loss in backreflected light intensity, and can thus qualitatively measure the overall degree of aggregation even in highly concentrated samples.

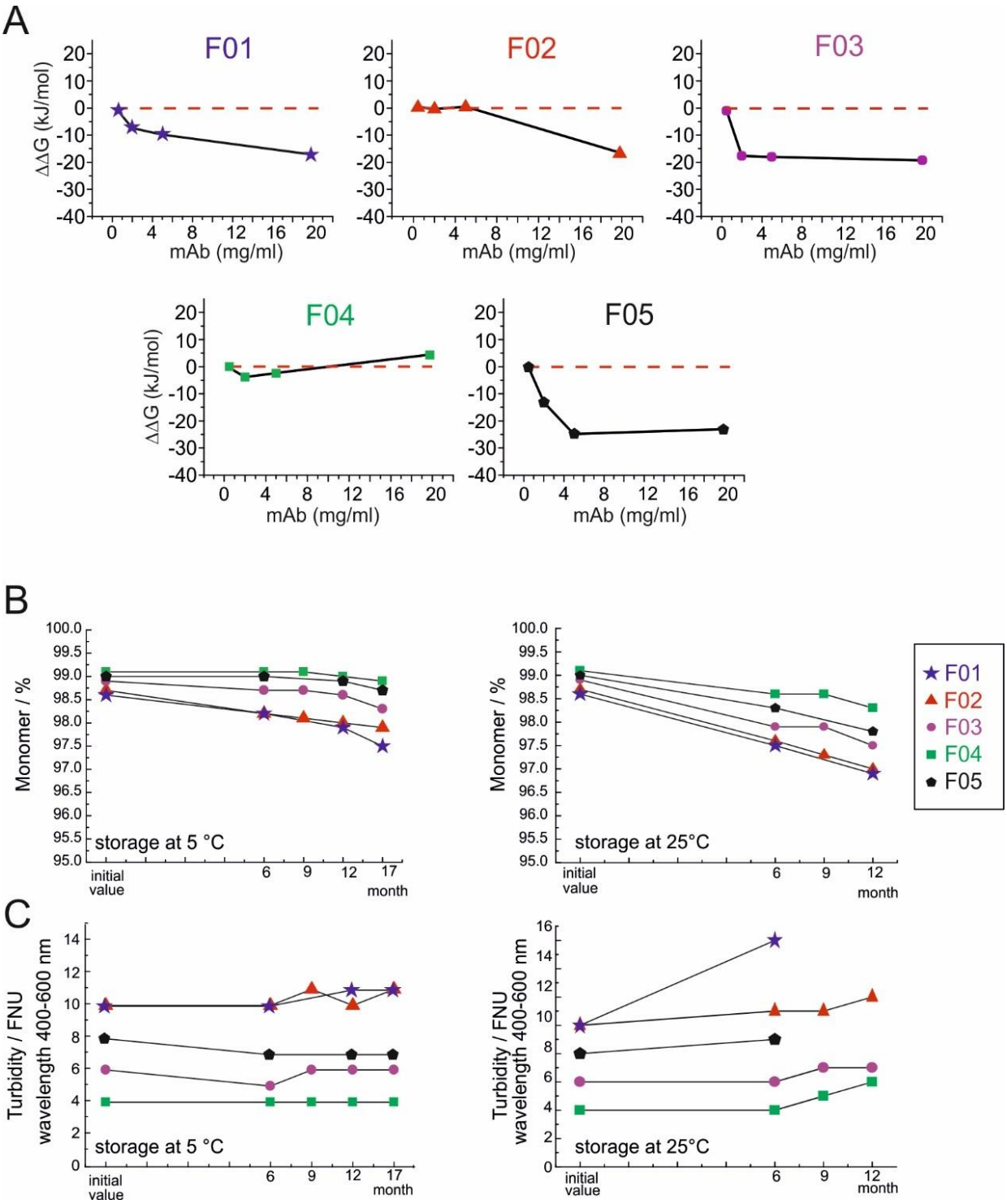
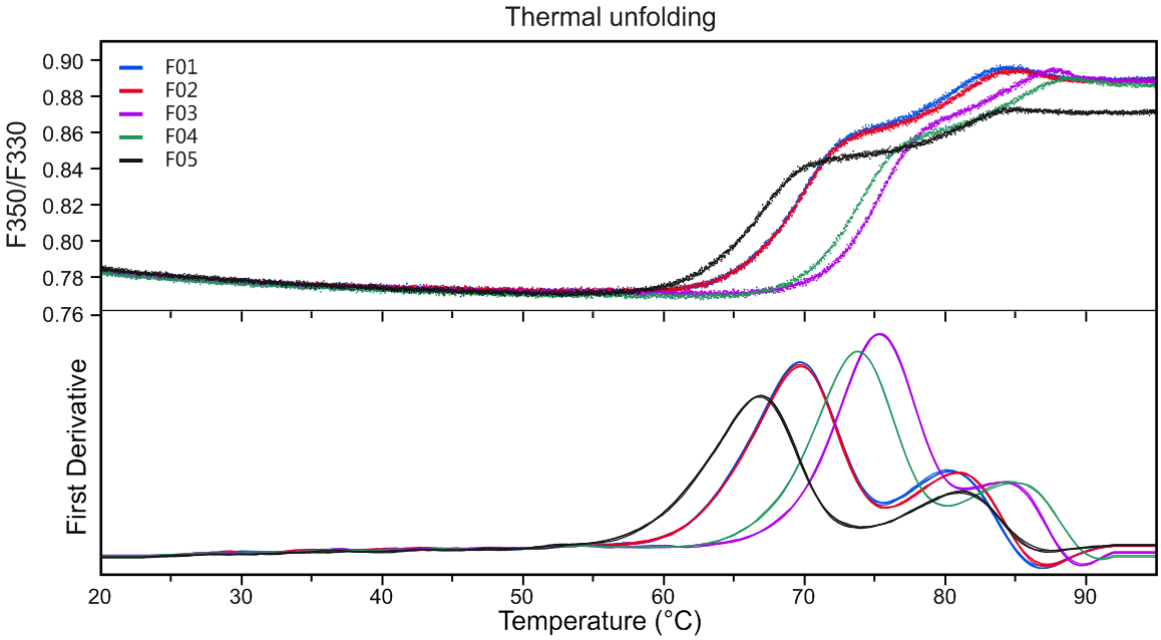
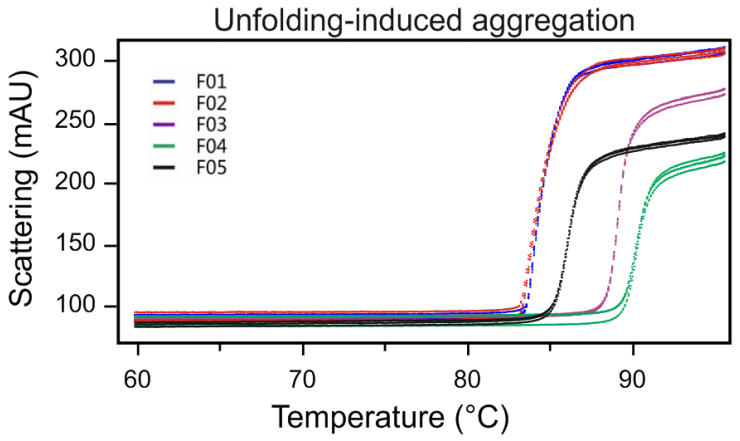


Figure 3: Comparison of aggregation prediction by chemical denaturation and long-term stability measurements. (A) Chemical denaturation data of mAb1 in formulations 1-5. Shown are the changes in ΔG° (ΔΔG° values) relative to the ΔG° of the lowest mAb concentration. (B) Determination of monomer contents of mAb1 in formulations 1-5 by HPSEC at 5 °C and 25 °C over a time period of 17 and 12 months, respectively. (C) Turbidity measurements of mAb1 in formulations 1-5 by nephelometry at 5 °C and 25 °C over a time period of 17 and 12 months, respectively.



	T_m1 onset (°C)		T_m1 (°C)		T_m2 (°C)	
F01	59.46	+/- 0.25	69.69	+/- 0.02	80.36	+/- 0.22
F02	59.31	+/- 0.10	69.74	+/- 0.07	80.83	+/- 0.22
F03	66.06	+/- 0.18	75.33	+/- 0.09	84.28	+/- 0.06
F04	64.24	+/- 0.01	73.82	+/- 0.05	84.80	+/- 0.55
F05	55.98	+/- 0.11	66.86	+/- 0.08	81.13	+/- 0.15

Figure 4: Analysis of mAb stability by nanoDSF. Thermal unfolding curves of mAb1 in formulations F01-F05. Changes in the F350/F330 fluorescence ratio (top) and the corresponding first derivative are shown. T_m - and T_m onset values represent averages with standard deviation from triplicate measurements.



	Tagg (°C)		Agg at 95 °C (mAU)
F01	81.19	+/- 0.05	313.46
F02	80.82	+/- 0.03	312.06
F03	85.96	+/- 0.07	279.32
F04	86.41	+/- 0.02	225.24
F05	82.77	+/- 0.00	243.26

Figure 5: Analysis of mAb stability by nanoDSF with backreflection aggregation detection. Aggregation detection of the samples shown in Figure 4. Aggregation was monitored in parallel to the intrinsic fluorescence. T_{agg} values represent averages with standard deviation. from triplicate measurements, the degree of aggregation at 95 °C represent averages from triplicate measurements.

Conclusion

The presented data demonstrates that chemical denaturation is a feasible approach to predict the colloidal long-term stability of biologicals. Moreover, changes in ΔG° in dependence of the protein concentration can give immediate feedback whether protein aggregation occurs, and can also be used to discriminate between native- and denatured-state aggregation. Knowledge about the aggregation mechanism is vital to design further steps in the development process: Denatured state aggregation can be reduced by excipients which further stabilize protein conformation, while native state aggregation might require different optimization strategies, such as excipients that shield surface-exposed hydrophobic patches in aggregation-promoting sequences, or protein engineering to eliminate such critical sequences entirely (Courtois et al, 2016; Seeliger et al, 2015).

In summary, we show that the Prometheus NT.Plex is a uniquely flexible and easy-to-use device that can be used to extensively characterize the chemical (ΔG°), thermal (T_ms and T_m onsets) and colloidal stability (T_{agg} and degree of aggregation) of biologicals. The modular design of the fully automated nanoDSF solution, comprising the Prometheus NT.Plex in conjunction with the NT.Robotic Autosampler and a liquid handling system of choice allows to perform meaningful chemical denaturation experiments with sufficient incubation times in-between sample preparation and measurement. It also ensures maximum flexibility, since no just-in-time sample preparation by the liquid handler is required, and since multiple Prometheus NT.Plex instruments can be combined with a single NT.Robotic Autosampler.

The Prometheus NT.Plex is a unique and valuable tool to get the full picture about biologics stability and aggregation propensity with unprecedented ease-of-use, flexibility and precision.

Methods

For chemical unfolding experiments, GuaHCl dilution series were prepared with concentrations from 6.0 M to 0 M GuaHCl. For this, two stock solutions with 0 and 6.75 M GuaHCl were prepared using 5 x formulation buffer which was adjusted to 1 x with water after GuaHCl-addition. Arithmetic dilution series with a final volume of 40 µl were prepared in 384-well plates (Corning 3820 non-binding) using a Hamilton Starlet liquid handler. Preparation time for 16 chemical denaturations with 24 dilutions each was 1.5 hours. Subsequently, 5 µl of protein were added to each dilution to reach the desired final concentrations, mixed, sealed and incubated over night to ensure equilibration at each concentration. Prepared MTPs were loaded into the MTP stacker, and capillary chips were filled automatically from MTPs using the NT.Robotic Autosampler. Filled capillary chips were then automatically transferred to the Prometheus NT.Plex and chemical denaturation was measured. Chemical denaturation data were fitted unattended by a three-state unfolding model to yield $\Delta G°_{app}$. $\Delta G°_{app}$ values were plotted versus protein concentration to identify trends in $\Delta G°_{app}$. All ΔG° values presented in the manuscript are $\Delta G°_{app}$ values.

For nanoDSF measurements, mAb1 was diluted into the respective formulations to reach a final concentration of 5 mg/ml, and subsequently filled into nanoDSF standard treated capillaries. Thermal unfolding and aggregation was monitored in a temperature ramp with 1 °C/min from 20 °C to 95 °C with a resolution of ~ 20 data points/min. Analysis of unfolding and aggregation was performed using the PR.Control Software.

For long term stability measurements, mAb1 was stored at a concentration of 20 mg/ml at 5 °C and 25 °C, respectively. HPSEC was performed using a TSK Gel G3000 SWXL 7.5 mm x 300 mm column (Tosoh, Bioscience, Tokyo, Japan) on a Waters 22675 Alliance HPLC system connected with an absorbance detector Waters 2487 (Milford, MA, USA). Turbidity was measured in formazine nephelometric units (FNU) using photometry of 90° scattered light at 400-600 nm (2100 AN Laboratory Turbidimeter, Hach, Loveland, CO, USA).

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