

Unfolding and Aggregation of mAbs

Application Note NT-PR-005

Analysis of formulation-dependent colloidal and conformational stability of monoclonal antibodies

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Abstract

The growing number of biological drugs such as monoclonal antibodies (mAbs), as well as the wealth of heterogeneity between mAb variants requires a thorough development process to maximize mAbs compliance with regulation. Therefore, biophysical analytical methods are required already at early stages of the development process to guide and streamline further antibody processing and to predict antibody developability.

In this case study, we demonstrate how the Prometheus NT.48 can be used to predict long-term mAb stability in a formulation screen by simultaneous quantification of both, conformational and colloidal stability of biologicals in thermal gradients.

Introduction

Monoclonal antibodies (mAbs) constitute the majority of therapeutic biologicals today. Owing to their high specificity and potency, they are used to treat a number of diseases, ranging from different cancer types to autoimmune defects. Novel protein engineering approaches lead to a growing number of therapeutic mAbs, which can additionally be modified to be bispecific, conjugated with other biologicals or modified with small molecule drugs.

The conformational and colloidal stability of antibodies are key parameters to predict their stability and also developability, since they affect the long-term storage stability which is critical for

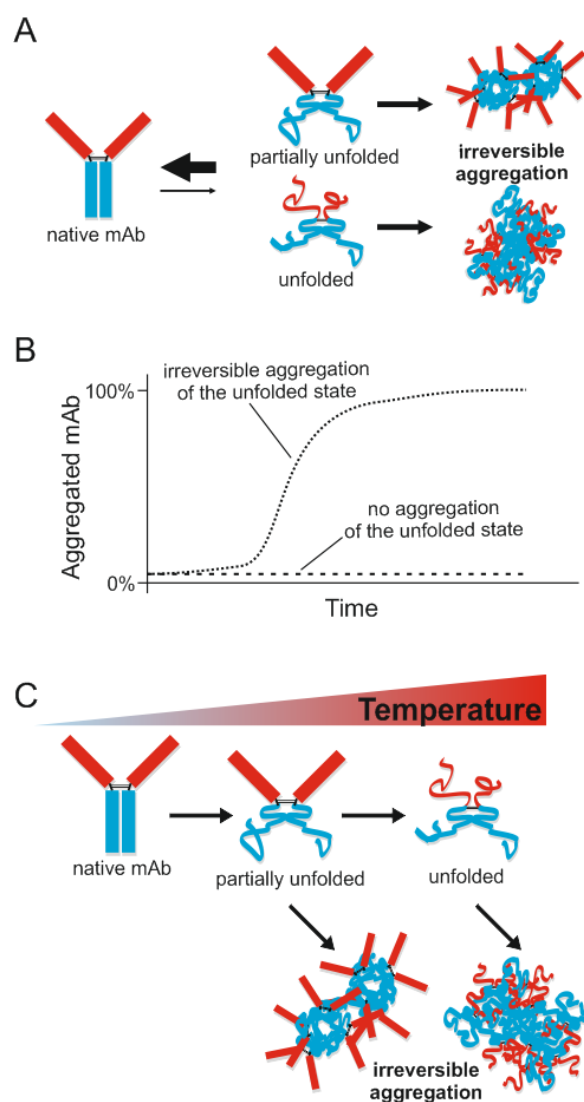


Figure 1: Relation between conformational and colloidal stability of antibodies. (A) Equilibrium and non-equilibrium states between folded and unfolded antibodies and aggregates. (B) Schematic plot showing the consequence of strong aggregation of the unfolded state. Irreversible aggregation leads to an accumulation of aggregates over time, eventually resulting in complete aggregation of the mAb. (C) Schematic representation of directed unfolding and aggregation of antibodies in a thermal gradient.

drug approval [1, 2]. The growing number of mAbs and mAb variants in development pipelines calls for biophysical methods which can quickly assess those parameters [3, 4].

Screenings for conditions and antibody constructs in early stages of development aim to identify most promising candidates to meet regulatory requirements for drug approval. Here, we use a small scale formulation screen for a therapeutic monoclonal IgG1 antibody to demonstrate the capability of the Prometheus NT.48 in determining critical stability parameters.

One approach to determine the conformational stability of proteins is to follow their unfolding in temperature gradients [5-9]. Increasing the temperature of the sample results in a transition of proteins from the folded to the unfolded state (Figure 1C). The temperature at which this transition occurs, the T_m , is used as a surrogate parameter for the thermal stability of the protein.

The Prometheus NT.48 uses nanoDSF to assess mAb conformational stability in thermal gradients by following changes in tryptophan fluorescence emission [8, 9]. Owing to the high sensitivity of the fluorescence detection in capillaries, even minute changes in antibody conformation can be detected, allowing for the unambiguous identification of multiple unfolding events which can be attributed to the different mAb subdomains.

At the same time, the Prometheus NT.48 can detect changes in colloidal stability and temperature-induced aggregation. This is achieved by detection of the backreflection intensity of a light beam that passes the sample twice (Figure 2).

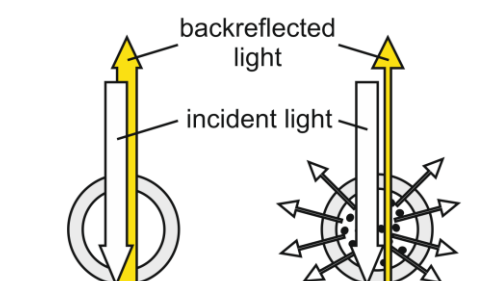


Figure 2: Schematic representation of the backreflection principle to detect protein aggregation. (Left) light passes the capillary, is backreflected into the detector and light intensity is quantified. (Right) particles scatter light, leading to an extinction of the incident light and thus a reduction of backreflected light, which is a direct measure for protein aggregation.

Upon aggregation, the intensity of the backreflected light decreases due to light scattering, and thus serves as a measure for total aggregation in a sample. Importantly, backreflection and fluorescence are detected simultaneously, allowing for maximal scanning speed and data point density.

A simultaneous assessment of conformational and colloidal stability is a powerful approach to predict long term stability. Every antibody – irrespective of its conformational stability – is in equilibrium between native (folded) and unfolded conformational states (Figure 1A). If the unfolded state of the antibody shows a strong tendency to form aggregates, it precipitates out of solution, resulting in more antibody becoming unfolded driven by the law of mass action (Figure 1B). Thus, strong aggregation of unfolded antibodies has to be prevented, e.g. by identifying optimal formulation compositions or molecular modifications that prevent aggregation.

Simultaneous backreflection and fluorescence analysis using the Prometheus NT.48 provides several important information: Firstly, it is possible to directly correlate thermal and colloidal stability, meaning that aggregation-causing unfolding events can be identified, and more importantly, aggregation onset temperatures can be determined. Secondly, the overall degree of aggregation of the unfolded state of an antibody can be determined, which can vary significantly between different formulations and antibody types.

Results

We tested the thermal stability of an IgG1 antibody at concentrations of 1 mg/ml in acetate buffer with pH values between 4 and 6.5, and in presence and absence of 130 mM NaCl. For this, 10 μ l of each sample were loaded into standard-treated Prometheus NT.48 capillaries and subjected to a thermal ramp from 40 $^{\circ}$ C to 95 $^{\circ}$ C at a heating rate of 1 $^{\circ}$ C/min.

The resulting unfolding signals in the F350/F330 ratio showed two clearly defined unfolding transitions (Figure 3A). A decrease in pH resulted in a shift of the unfolding transition midpoints to lower temperatures (Figure 3A and 4A). This effect was even more pronounced in presence of 130 mM NaCl, showing that the addition of salt leads to a conformational destabilization of the antibody. In

general, pH- and salt-dependence was most pronounced for the first unfolding transition. Parallel analysis of antibody aggregation was performed by measurement of light extinction using backreflection detection. Interestingly, the aggregation data revealed that aggregation was most pronounced under buffer conditions with higher pH values (Figure 3B and 4C). These results indicate that shifts in T_{agg} do not correlate with the large, pH-dependent shifts in the first unfolding transition temperature T_{m1} (Figure 3 and 4B), suggesting that unfolding of the corresponding mAb domain is not responsible for aggregation.

In contrast, no aggregation was detected in acetate buffer pH 4 in absence of salt, showing that aggregation of the unfolded state is largely suppressed under these conditions (Figure 3B, 4B and C). Since an increase in pH resulted in an increase of the overall aggregation signal, increasing the pH-value seems to promote the formation of unfolded-state aggregation. In addition to sample pH, sodium chloride greatly affected temperature-induced mAb aggregation: The addition of 130 mM NaCl led to strong aggregation under all conditions tested, as well as to a shift of the aggregation onset temperature to lower values.

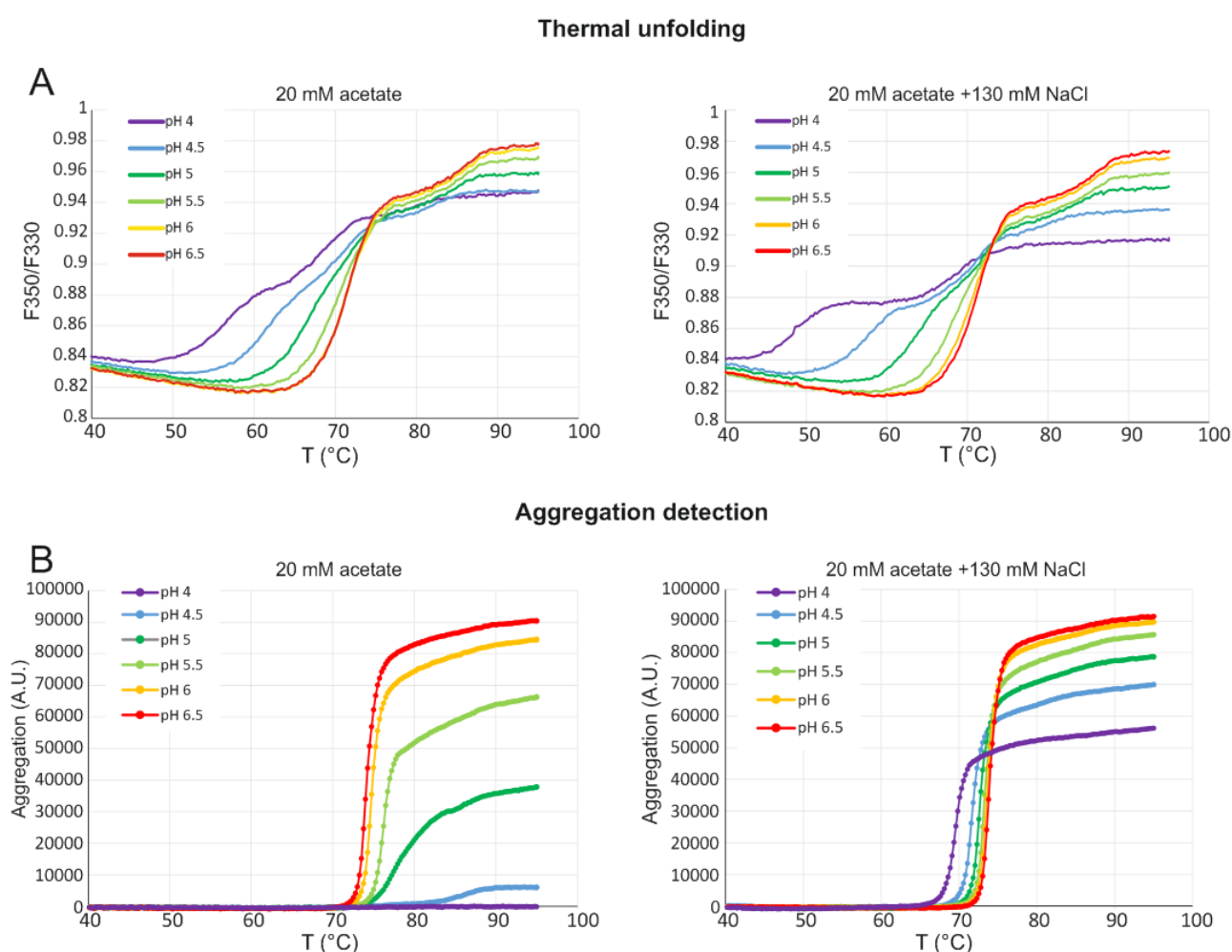


Figure 3: Conformational stability and aggregation of a mAb under different buffer conditions. (A) Thermal unfolding monitored by detection of shifts in the fluorescence ratio (F350/F330) in dependence of different buffer pH values and NaCl concentrations. (B) Aggregation is detected by changes in backreflection.

Since partial unfolding of proteins can be reversible under certain conditions, we next tested for reversibility of the first unfolding transition. For this,

the samples were heated to temperatures either near at or ~ 10 °C beyond T_{m1} , respectively, and subsequently cooled back to 20 °C at 1 °C/min

while the fluorescence was monitored to detect conformational changes by nanoDSF. These experiments showed that the first unfolding transition was indeed reversible at pH 4 (Figure 5A), which is highlighted by a clear refolding signal in the F350/F330 ratio when heating was limited to slightly above T_{m1} (60 °C). In contrast, heating beyond the first unfolding transition to 70 °C resulted in irreversible unfolding. At pH 6, the sample was heated to 70 °C (within the first unfolding transition) and 80 °C (beyond the first unfolding transition) (Figure 5B). In contrast to the experiments at pH 4, no re-folding was observed at pH 6. Albeit the antibody is thermally less stable at pH 4, it is able to re-fold under these conditions to a certain extent, whereas unfolding is irreversible at pH 6.

Conclusion

The results from our combined investigation of thermal unfolding and aggregation using the Prometheus NT.48 have multiple implications: First and foremost, although pH values > 5.5 thermally stabilize the antibody, colloidal stability is compromised, since strong aggregation of the unfolded state occurs. Moreover, even though being thermally less stable at low pH values, unfolding of the antibody is reversible at temperatures up to 60 °C. Taken together, these data suggest that a slight thermal instability might be acceptable and even favourable for long-term stability of the antibody, due to reduced aggregation under these conditions and the ability of the antibody to re-fold. Future screening approaches could therefore be designed to find excipients which thermally stabilize the mAb at lower pH values while maintaining low aggregation of the unfolded state.

The presented data demonstrate the versatility and precision of the Prometheus NT.48 which can be used to facilitate formulation development processes. The Prometheus NT.48 detects several stability parameters in an entirely label-free approach. 48 different samples can be analyzed simultaneously, with customizable heating and cooling rates from 0.1 to 7 °C/min. Typical

unfolding experiments take just 60 minutes, employing 48 samples, heating rates of 1 °C/min and a temperature ramp from 30 to 90 °C. The on-the-fly detection results in very high datapoint densities (20+ datapoints per minute and capillary) for most precise unfolding analysis. Moreover, sample requirements are low, since only 10 µl of sample with possible protein concentrations between 10 µg/ml and > 250 mg/ml are used per capillary.

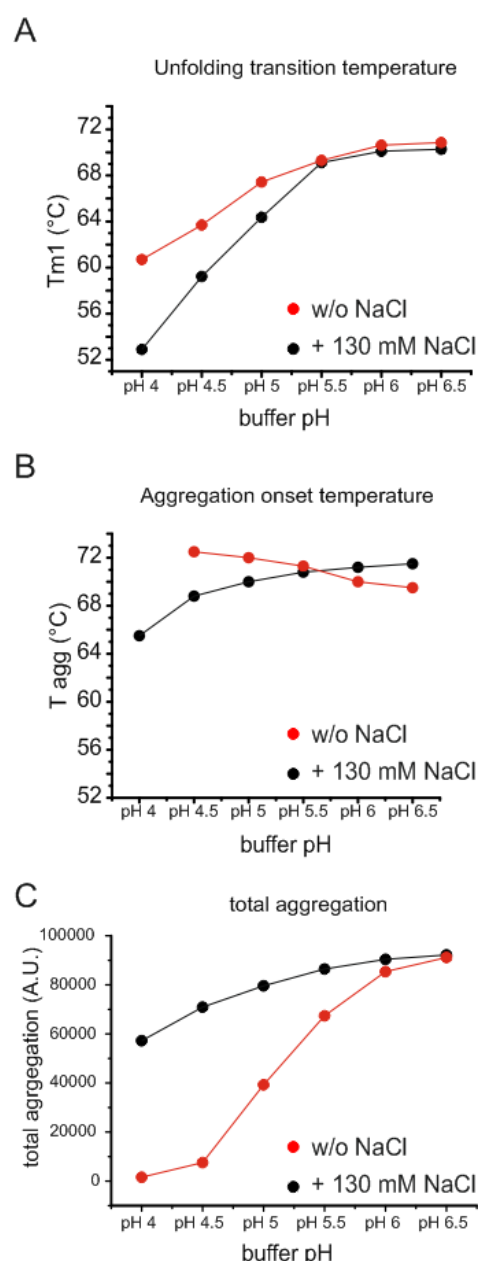


Figure 4: Summary of T_{m1} and T_{agg} values for all tested conditions

unfolding reversibility

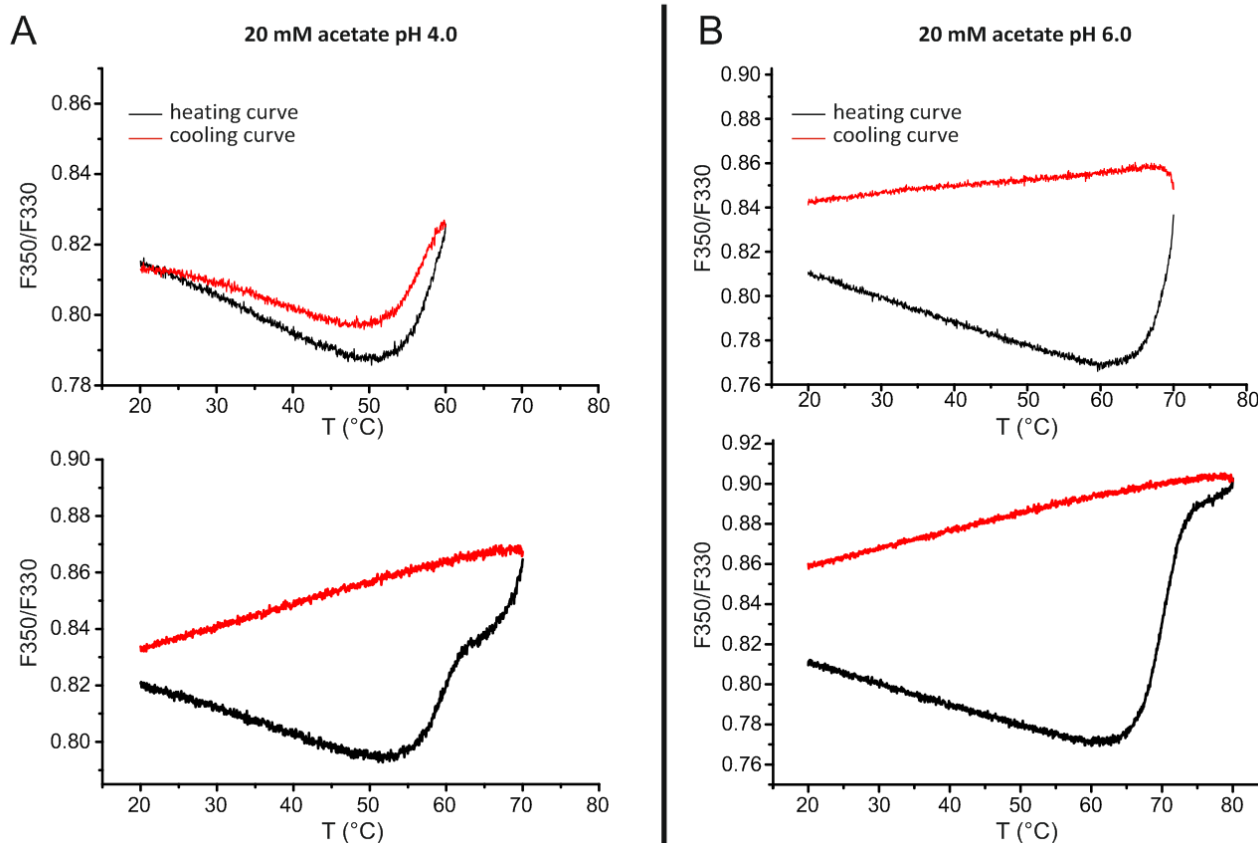


Figure 5: Testing the reversibility of the first unfolding transition event of a mAb at (A) pH 4 and (B) pH 6. mAb re-folding can be observed after incomplete unfolding in acetate buffer pH 4 (top left).

Methods

Sample preparation:

The purified mAb (49.8 mg/ml in PBS pH 6.2) was diluted into buffer to reach a final concentration of 5 mg/ml mAb, 20 mM acetate at indicated pH values, and optionally 130 mM NaCl. Prior to thermal stability experiments, the solutions were incubated for 1 h at RT.

Thermal unfolding and aggregation experiments:

20 μ l of each sample were loaded into 2 Prometheus NT.48 standard treated capillaries. Capillaries were placed into the instrument and thermal unfolding and aggregation was monitored over a temperature range from 40 to 90 $^{\circ}$ C, with a heating rate of 1 $^{\circ}$ C/min. Fluorescence at 330 and 350 nm as well as light extinction were detected in parallel. For refolding experiments, 20 μ l of each sample were loaded into 2 Prometheus NT.48 standard treated capillaries. Capillaries were

placed into the instrument and thermal unfolding and aggregation was monitored over a temperature range from 20 to 60, 70 or 80 $^{\circ}$ C, respectively, with a heating rate of 1 $^{\circ}$ C/min, and subsequently cooled down to 20 $^{\circ}$ C at 1 $^{\circ}$ C/min.

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