APPLICATION NOTE

AN4003: Influence of heating rates on the thermostability characterization of antibodies by DLS and SLS

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Summary

Formation of aggregates of therapeutic proteins can lead to reduced clinical effectiveness and potentially a severe immunogenic response. Thermostability screening by means of a temperature ramp is a common approach to assessing the aggregation propensity of different candidate molecules and formulation conditions. Such screening is usually performed in a high-throughput manner using dynamic light scattering (DLS), intrinsic fluorescence and/or other techniques to quantify key attributes like the melting temperature (T_m) or the onset temperature of aggregation (T_{agg}).

Reliable determination of T_{agg} is critical for risk assessment of biotherapeutic products. As multi-domain proteins, monoclonal antibodies are subject to multiple unfolding steps and subsequently different aggregation kinetics during thermal screening. This application note showcases the effect of thermal ramp rate on the unfolding and aggregation behavior of trastuzumab (Herceptin®). A DynaPro® NanoStar® cuvette-based DLS/SLS instrument is utilized to show that the choice of heating rate during thermostability screening is critical to obtaining meaningful results.

Introduction

Monoclonal antibodies (mAbs) are the most commonly exploited class of biotherapeutics because of their ability to target cells and tissue for direct treatment or precise delivery of small molecular drugs. Indications of mAb-based drugs include cancer, infectious diseases, asthma and cardiovascular diseases. Since mAbs typically consist of two light chains and two heavy chains, organized into three domains, with differences in structure and stability, their unfolding generally leads to numerous intermediate states that aggregate with different likelihoods.

The effects of mAb aggregation in pharmaceutical products range from simple loss in efficacy to strong immunogenicity in patients. Therefore, precise and reliable characterization of antibody stability has become a key requirement for successful screening and approval of mAb-based therapeutics.[1]

The DynaPro NanoStar utilizes an intuitive on-board app, DYNAMICS Touch, for guided measurements and walk-up operation. Advanced analyses such as onset temperature are carried out using the full-features DYNAMICS software, which runs on a standard Windows PC.

The importance of ramp rate

Thermostability screening is a method that evaluates physiochemical properties of a protein over a temperature ramp. This type of experiment often includes screening different formulation conditions such as salt concentrations, pH values and excipients. Experiments may be automated in standard microwell plates for high throughput analysis by using a DynaPro Plate Reader. Although for

simple proteins thermostability experiments may be independent of the heating rate, there are exceptions like mAbs—with their multistep unfolding—where the choice of heating speed matters. Excessively high heating rates might cause relatively slow unfolding or aggregation events occurring at lower temperatures to be assigned to a higher temperature onset. As a result, a higher stability would be assumed than is actually the case. To this end, it is not only good practice but of uttermost importance to compare results obtained at the same experimental settings, and to assess the impact of temperature ramp rate on the measured T_{agg}.

DLS is a powerful technique that determines hydrodynamic radius (R_h), a parameter that increases relative to the native state with both unfolding and aggregation. The combination of DLS with temperature ramps enables the determination of T_m or T_{agg} by measuring size changes over the temperature range. If static light scattering (SLS) is recorded in parallel, unfolding may be distinguished from aggregation by assessing changes in the weight-average molar mass (M_w) for the analyte.

Measuring DLS and SLS over a temperature ramp

The DynaPro NanoStar measures DLS and SLS simultaneously using dedicated, optimized detector modules for each type of measurement. It also provides exquisite temperature control which accounts for the lag in solution temperature relative to instrument temperature that arises from the thermal mass of the quartz cuvette and liquid. This results in a precise ramp over a broad temperature range. As little as 2 µL of sample can be analyzed for each condition or candidate.

The NanoStar was used to study the impact of ramp rate on the apparent thermostability of trastuzumab (Herceptin), a commercially available mAb applied in the treatment of breast cancer. The study examined the effects of heating rate on the measured value of T_{onset}, the temperature at which unfolding or aggregation leads to an increase of R_h and M_w.

Materials and Methods

Trastuzumab obtained in powder form (Sigma-Aldrich) was resolubilized in standard PBS buffer to a final concentration of 1 mg/mL and filtered through a 0.02 µm syringe-tip filter. For each thermal ramp experiment, 2 µL of the trastuzumab solution were transferred into a clean, calibrated quartz cuvette and a drop of silicon oil was placed on top to prevent evaporation. Thermostability was then analyzed by DLS and SLS in a DynaPro NanoStar. Both R_h and M_w were measured over a temperature range from 25 °C to 90 °C with ramp rates from 0.1 °C/min to 2.0 °C/min. Data were acquired at each temperature point over 3 acquisitions of 3 seconds each. Cumulant analysis and Onset analysis were performed by DYNAMICS® software to calculate R_h and T_{onset}. Distribution plots were generated from the NNLS regularization analysis.

Results and Discussion

DLS analysis reveals R_h of 5.5 nm for trastuzumab at 25 °C, which is typical for a monomeric, natively folded mAb.[2] M_w of ~140 kDa measured by SLS underlines that the mAb is monomeric as it is close to the molar mass calculated from sequence of ~150 kDa.

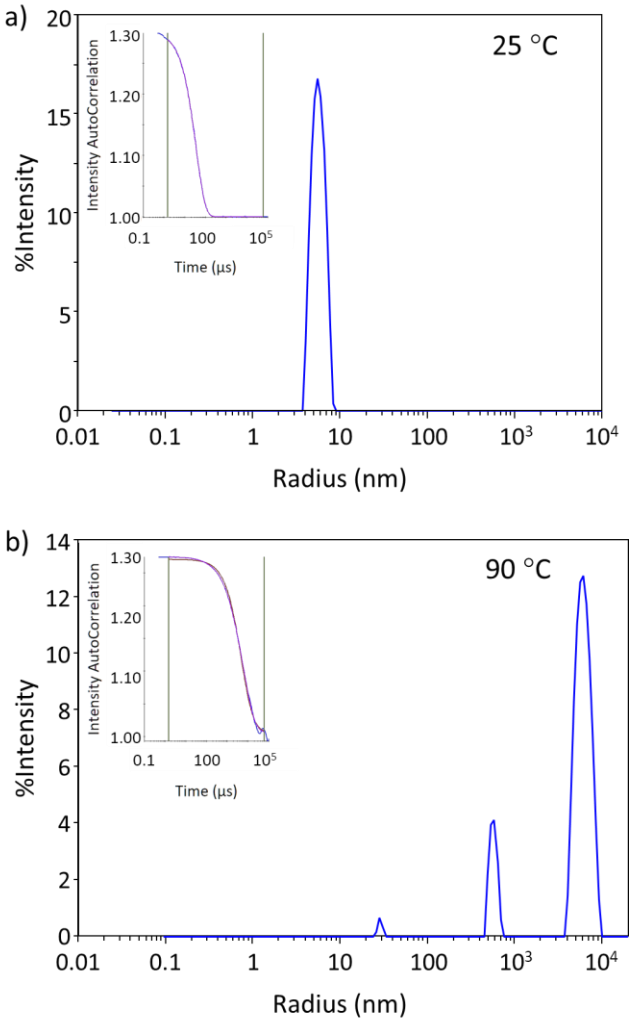

Figure 1. The autocorrelation functions and regularization histograms of trastuzumab at 25 °C and 90 °C.

Figure 1a shows the regularization histogram with a single narrow peak, suggesting that only monomeric antibody is present at 25 °C and therefore, the samples are initially free of higher-order species or aggregates. Figure 1b showcases how thermal stress leads to aggregation, presented by the regularization histogram at 90 °C.

Thermostability in slow and fast ramps

The influence of heating rate on the apparent thermostability of trastuzumab was studied by recording DLS and SLS data while performing temperature ramps at different rates. Table 1 lists the T_{onset} for each heating rate and measurement type while Figure 2 shows a comparison of the thermostability measured during the slowest heating rate (0.1 °C/min) and the fastest heating rate (2.0 °C/min) applied in this study.

Both R_h and M_w of the mAb remain unchanged up to 70 °C (Figure 2). Above this threshold an increase in both radius and molar mass takes place. T_{onset} from DLS is 71.8 °C for the slowest and 78.2 °C for the fastest heating rate. A similar result is obtained when calculating T_{onset} from SLS: 71.0 °C and 77.8 °C for slowest and fastest heating rate, respectively. Increase in R_h and M_w takes place at roughly the same onset which shows that aggregation takes place rather than just unfolding.

Table 1. Trastuzumab temperature of onset (T_{onset}) calculated from DLS (R_h) or SLS (M_w) recorded during different heating rates.

Heating Rate (°C/min)	T_{onset} from DLS (°C)	T_{onset} from SLS (°C)
0.10	71.8	71.0
0.25	74.3	73.5
0.50	75.6	74.8
1.00	77.2	76.8
2.00	78.2	77.8

The mAb appears to aggregate at lower temperatures if the heating rate is slower. This indicates that initial unfolding occurs as early as ~71 °C, which in turn causes slow aggregation. The combination of slow aggregation at 71 °C and the fast transition in temperature for the high ramp rate leads to an apparent increase of 7 °C in T_{onset} at 2.0 °C/min. Clearly the choice of ramp rate impacts the results: too fast of a ramp rate leads to significant error in thermostability assessment.

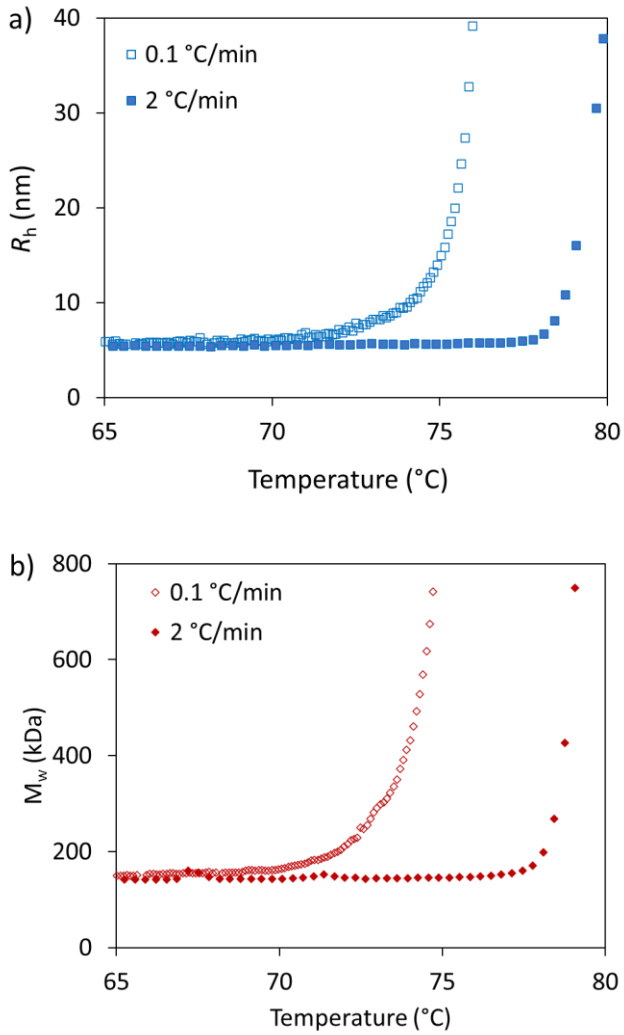

Figure 2. Influence of heating rates on the thermostability characterization of trastuzumab by a) DLS (R_h) and b) SLS (M_w).

T_{onset} from DLS vs. SLS

When comparing T_{onset} from DLS and SLS, it becomes apparent that the onset temperature from SLS is always slightly lower than the onset from DLS (Table 1). Figure 3 compares R_h and M_W versus temperature during 0.1 °C/min and 2 °C/min heating rates. While the onsets at the highest ramp rate are nearly identical, there is a notable earlier onset for M_w observed at the lowest ramp rate compared to R_h.

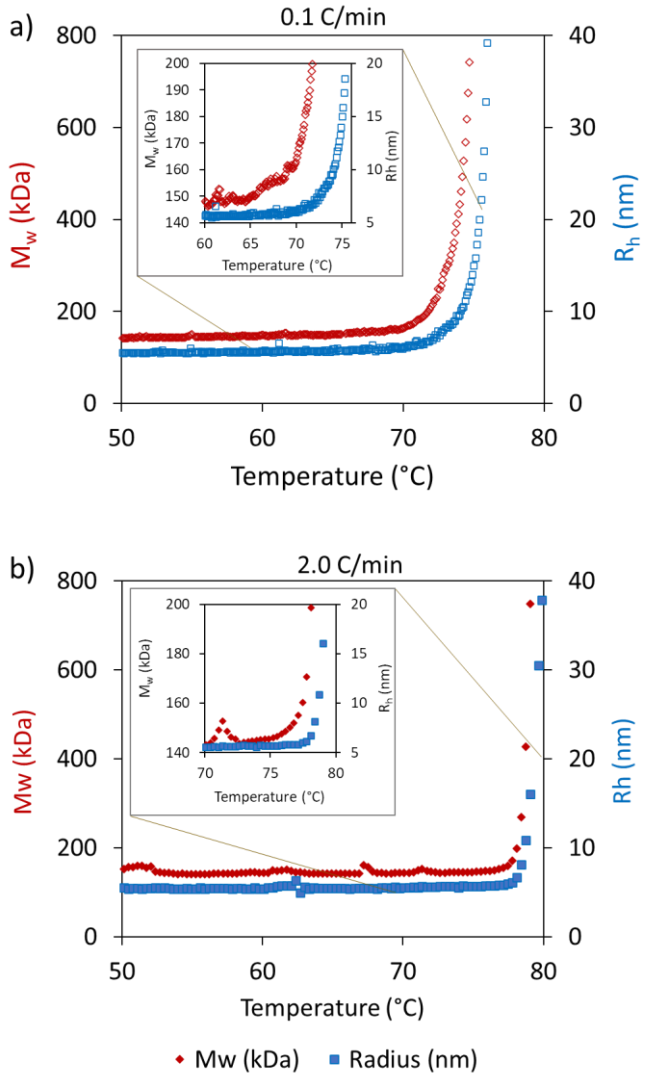

Figure 3. Differences are observed in the onsets of M_w and R_h between heating rates of a) 0.1 °C/min and b) 2.0 °C/min.

SLS is in general more sensitive to the formation of aggregates, so even if the unfolding could not be observed by DLS, aggregation was markedly registered by SLS. It is also possible that the increase in apparent M_w without a corresponding increase in R_h is the result of transient self-association, which increases upon conformational changes taking place in the mAb that do not impact R_h.

Mapping ramp rate versus apparent T_{onset}

A plausible explanation for the absence of the earlier onset at the fastest ramp rate might be that unfolding around ~71 °C causes a slower aggregation process than the one observed at presumably complete unfolding and subsequent aggregation at ~78 °C. Therefore, multiple heating rates between 0.1 °C/min and 2 °C/min were applied to determine the onsets for R_h and M_w (Table 1).

Figure 4 shows how apparent T_{onset} from DLS and SLS increases with increasing ramp rate. Aggregation during a thermostability experiment occurs when unfolding reveals hydrophobic residues normally hidden inside the protein, resulting in (usually) irreversible self-association. The aggregation process is temperature dependent, meaning that with increasing temperature the rate of aggregation increases. On this premise, the earlier onset observed during the slower heating rates is caused by a much slower aggregation process connected to partially unfolded protein.

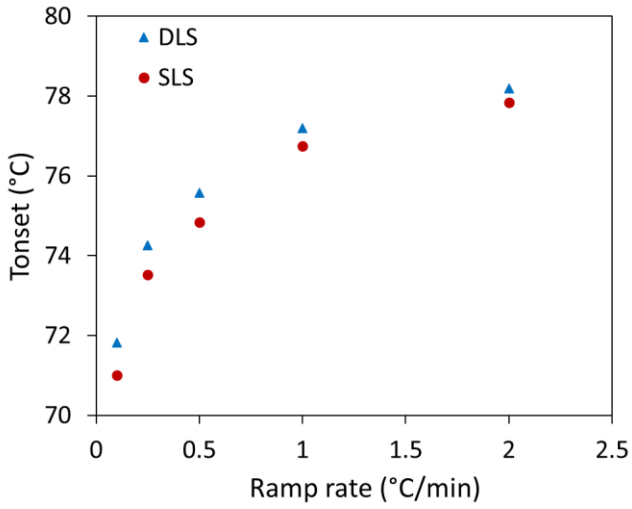

Figure 4. Influence of heating rates on T_{onset} of trastuzumab observed for DLS (R_h) and SLS (M_w).

When the heating rate is increased, the time spent at each temperature step decreases, reducing the chance of aggregation taking place, while higher temperatures boost the speed of the aggregation process. These counteracting effects cause a shift towards higher onset temperatures with increasing heating rates until the complete unfolding of the mAb becomes the predominant source of aggregation at the highest heating rates. In conclusion, aggregation caused by partially unfolded mAb is a much slower process than aggregation of completely unfolded protein explaining the absence of an earlier onset at the highest ramp rates.

Conclusions

Thermostability studies are of key importance for biotherapeutic characterization and the optimal selection of candidates and formulations. Although often considered to be independent of heating rate, fast ramps may hide slow,

early-onset aggregation if unfolding is a stepwise process. The impact of ramp rate can be readily assessed with DLS and SLS measurements in a DynaPro NanoStar.

The NanoStar implements precise temperature control covering a large temperature range from -10 °C to +120 °C yet with astonishingly low sample consumption—as little as 2 µL. The instrument stands out as the instrument of choice when addressing challenging thermostability studies on just a few samples. For high throughput screening, the DynaPro Plate Reader can perform thermostability testing in standard microwell plates.

Though the DynaPro Plate Reader cannot perform temperature ramps as quickly as the NanoStar, this application note shows that faster ramps can lead to significant misjudgment of thermal stability of mAbs and other biotherapeutics.

References

1. Le Basle, Y. et al, "Physicochemical Stability of Monoclonal Antibodies: A Review", *J. Pharm. Sci.* 109(10):169-190 (2020). https://doi.org/10.1016/j.xphs.2019.08.009
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