Multi-day biologics stability experiments with Uncle

Introduction

Thermal ramp stability measurements (T_m and T_{agg}) are well-established methods to quickly rank order the stability of protein constructs and formulations. Ranking may be more difficult if T_m or T_{agg} values are similar among tested conditions. Alternate testing and characterization may be required to further refine and understand stability differences.

One approach to differentiating proteins with similar T_m's is to use isothermal methods. Rather than ramping through a wide temperature range, the temperature is raised to a point just below the measured melting point, and held at that temperature for hours or even days (Figure 1). This allows you to get a more detailed picture of unfolding and aggregation events as they occur.

Uncle is an all-in-one stability platform that enables 12 different applications with one instrument (Figure 2). Fluorescence, static light scattering (SLS) and dynamic light scattering (DLS) detection methods are used to characterize protein stability. Temperature control (from 15–95 °C) and sealed samples provide greater flexibility in how that characterization can be performed. Multiple measurements, such as thermal melting, aggregation and sizing are possible with the same set of samples. And since you can run low volumes of 48 samples at a time, you can thoroughly characterize more biologics and formulations earlier than before.

Uncle can measure isothermal stability changes with two different measurement modes:

- Fluorescence and SLS. Use intrinsic fluorescence to monitor conformational changes in your proteins over time, and SLS to monitor aggregation of small and large particles.
- DLS. Monitor changes in hydrodynamic size and polydispersity with DLS.

Arginine can be a useful additive for protein formulations, as it can reduce aggregation during storage.

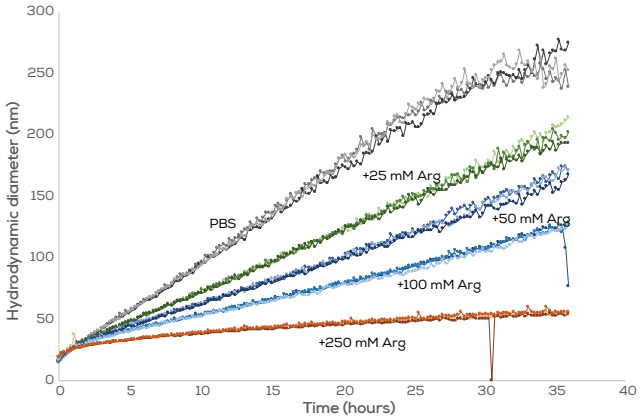

Figure 1: Measuring isothermal stability continuously over 36 hours on Uncle.

Arginine can also have a destabilizing effect on proteins, likely through interactions with hydrophobic residues. This series of experiments on Uncle probes the effects of increasing arginine concentration on both the stability and aggregation of an antibody.

Methods

Thermal melting and aggregation

Human polyclonal IgG (Lampire Biological Laboratories) was prepared at 5 mg/mL in PBS or in

Figure 2: Uncle: a one-stop stability platform.

PBS with arginine added to a final concentration of 25, 50, 100 or 250 mM, and 9 µL of each was loaded in duplicate in a Uni. The samples were run on Uncle with a thermal ramp from 15–95 °C, with a ramp rate of 0.5 °C /minute. Uncle Analysis Software calculated the T_m using the first derivative of the ratio of fluorescence at 350 and 330 nm and the T_{agg} using the onset of aggregation at 266 nm.

Isothermal stability

For the fluorescence and SLS isothermal experiment, the same IgG was prepared at 1 mg/mL in PBS or in PBS with arginine added to a final concentration of 25, 50, 100 or 250 mM. Nine µL of each sample was immediately loaded in triplicate in a Uni and the sealed samples were held at 58 °C for 36 hours, with a scan from 250–720 nm collected every 10 minutes.

For the DLS isothermal experiment, the same samples were loaded in triplicate and held at 58 °C for 36 hours, with four DLS acquisitions of 5 seconds each, collected every 13 minutes. Uncle Analysis Software automatically calculates the hydrodynamic diameter of each sample at every time point.

Results

T_m and T_{agg}

The melting temperature of the tested human IgG formulated in PBS was determined to be 66.5 °C (Figure 3). This preliminary experiment was done to guide the isothermal stability experiments. Adding arginine to protein formulations has been shown to stabilize the protein denatured state and lower their melting temperature; therefore, we chose 58 °C for subsequent isothermal experiments, to ensure that the incubation temperature would be lower than the T_m in all conditions tested (Table 1).

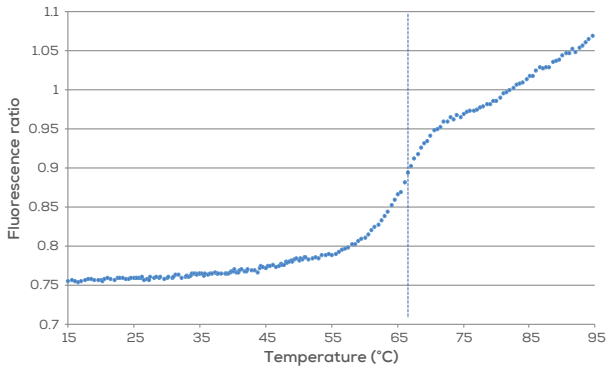

Figure 3: 350:330 nm ratio of intrinsic protein fluorescence over a thermal ramp. The dotted vertical line indicates the T_m of human IgG in PBS, which undergoes one major unfolding transition.

Formulation	T_m (°C)	T_{agg} (°C)
PBS	66.5	57
PBS + 25 mM Arg	65.5	61
PBS + 50 mM Arg	64	63.5
PBS + 100 mM Arg	62	65
PBS + 250 mM Arg	59	n.d.

Table 1: The T_m and T_{agg} were measured over a thermal ramp and averaged for each set of samples. T_m values decreased while T_{agg} values increased with increasing arginine concentrations. There was no significant onset of aggregation observed in the presence of 250 mM arginine.

Isothermal stability

After a 36-hour incubation at 58 °C, there were distinct differences in raw spectral data due to the presence and absence of arginine (Figure 4). Measuring the intensity of the SLS intensity at 473 nm is useful for detecting aggregation of larger particles, while the scattering intensity at 266 nm has higher sensitivity and can detect the formation of smaller particles. Both are collected simultaneously on Uncle. In PBS, at T_0 there were very small peaks at 266 and 473 nm, signifying no or very little aggregated protein. There was also a significant fluorescence signal, contributed mainly from tryptophan residues in the protein (Figure 4A, blue trace). At the end of the 36-hour incubation, there were large peaks at 266 and 473 nm, and the intensity of the signal from folded protein was significantly reduced (Figure 4A, red trace). In contrast, with 250 mM arginine added to the formulation, the final trace was almost identical to the initial one, with only a very slight increase of intensity at 266 nm (Figure 4B). Additionally, the intensity of the initial protein fluorescence peak was slightly lower than in PBS alone, which could indicate a difference in the

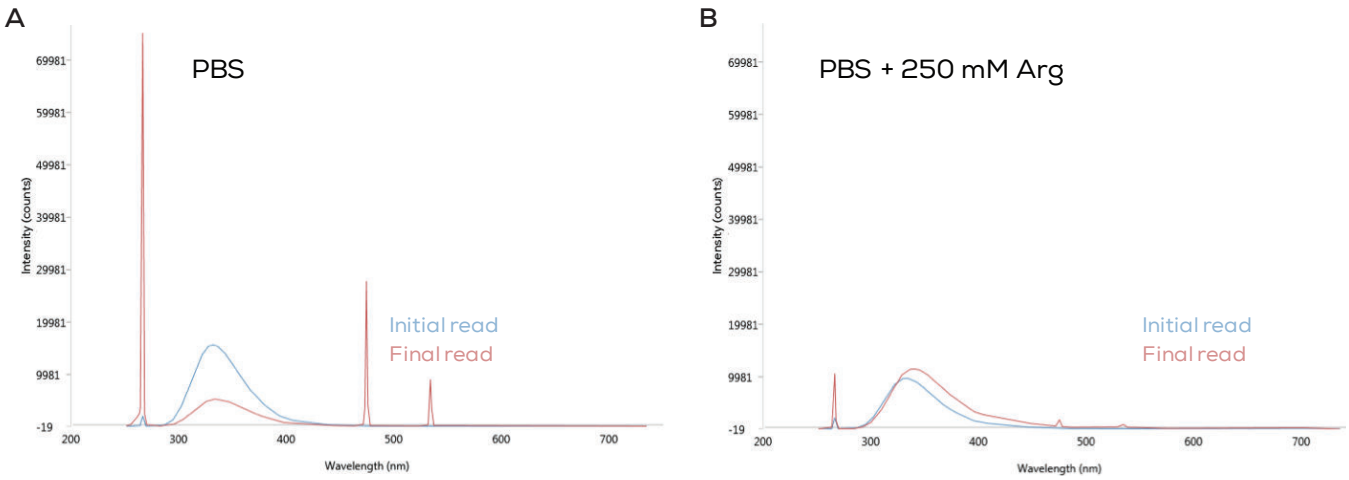

Figure 4: Raw fluorescence and SLS data. Only the first and last reads are shown for clarity. **A:** The IgG was formulated in PBS. **B:** The IgG was formulated in PBS with 250 mM arginine.

initial conformational state of the protein in the presence of arginine.

The fluorescence spectrum and SLS intensity at 266 and 473 nm were monitored continuously over 36 hours. Similar to the raw spectral data in Figure 4, total scattering intensity decreased with increasing arginine concentrations (Figure 5). In the presence of high concentrations of arginine (250 mM) there was no measurable protein aggregation after a 36-hour incubation at 58 °C. The sample formulated in PBS appears to reach a maximal intensity after 25 hours of incubation, at which point the large aggregates presumably begin to precipitate out of solution and the scattering intensity decreases. This highlights the value of continuously monitoring the samples over the incubation period, as one can distinguish differences in samples more accurately than snapshot data at 0 and 36 hours.

The intrinsic fluorescence signal can also provide information about the relative conformational state of the protein in different formulations. All formulations tested show a similar pattern: a gradual increase in the 350:330 nm ratio, signifying unfolding over time (Figure 6). It appears that in the presence of increasing concentrations of arginine, the protein is in a different state at the beginning of the incubation, which confirms previous work showing that arginine can slightly destabilize some proteins.

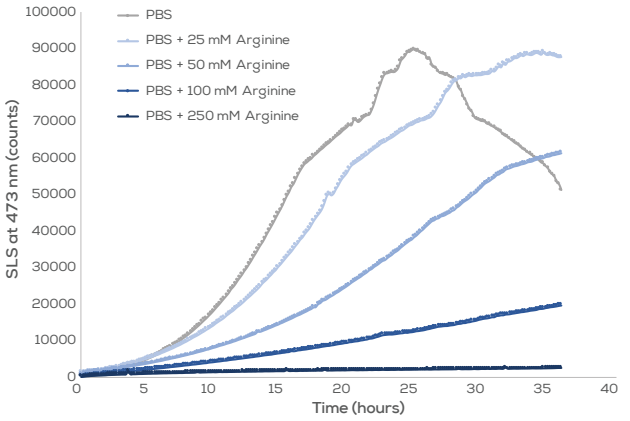

Figure 5: Scattering intensity at 473 nm over 36 hours at 58 °C.

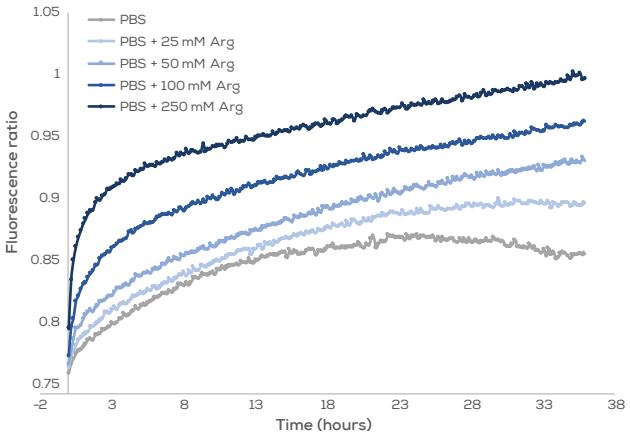

Figure 6: 350:330 nm ratio of intrinsic protein fluorescence is shown over the 36-hour time course of the experiment for samples with increasing arginine concentrations.

DLS data collected from samples undergoing the same incubation (36 hours at 58 °C) can provide additional valuable information about the size of the molecules. Looking at the raw data in the form of correlation functions, it is clear that there is a significant size difference between the IgG at the beginning and end of the incubation in PBS (Figure 7A). At the end of the incubation period, the curve remains correlated for longer – takes more time for the correlation to decay – signifying that the average size of the particles is larger than they were initially. In contrast, when 250 mM arginine is added to the formulation, the average size of the particles at the beginning and the end of the incubation are similar (Figure 7B).

Tracking the calculated hydrodynamic size over the course of the incubation for all samples, the same trend is seen, but with more detail (Figure 8). For the IgG in PBS alone, the average particle size increases by over 10-fold, with a starting diameter of about 20 nm, and a final diameter of over 250 nm. With each increasing concentration of arginine, the size change is less dramatic, and at 250 mM of arginine, there is less than a two-fold increase in average particle size throughout the course of the 36-hour incubation. Triplicate data is shown in the graph, and demonstrates the high reproducibility of the measurements.

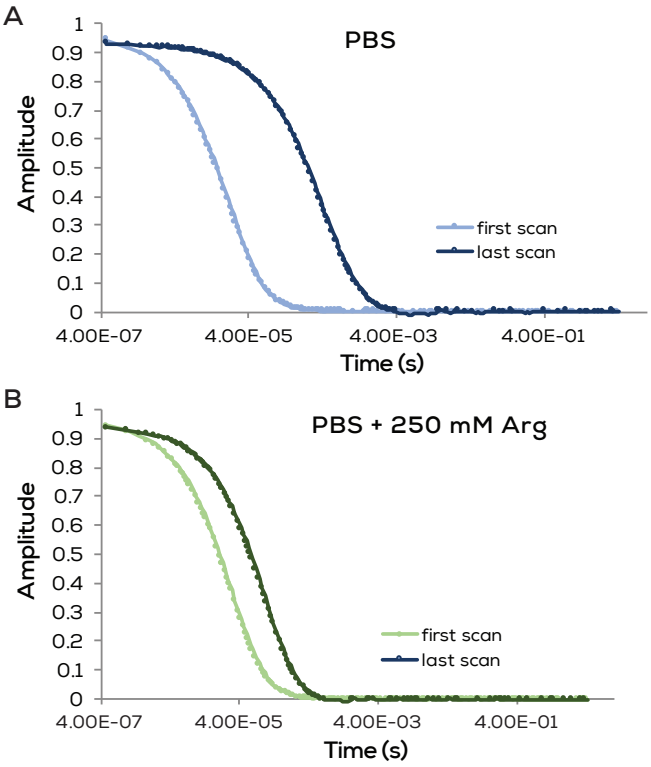

Figure 7: Individual correlation functions from the beginning and end of the DLS isothermal experiment. **A:** For the IgG in PBS alone. **B:** In PBS with the highest concentration of arginine tested.

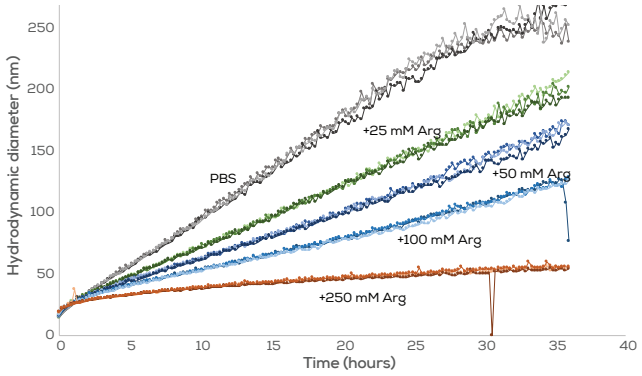

Figure 8: The average hydrodynamic diameter for IgG samples with increasing amounts of arginine, over a 36-hour DLS isothermal experiment.

Conclusion

This series of experiments with an antibody formulated in increasing concentrations of arginine illustrates how Uncle can facilitate screening for stability and aggregation, with hands-off isothermal experiments that are easy to set up and analyze. With a single platform, we are able to obtain thermal melting (T_m), aggregation onset (T_{agg}) and hydrodynamic radius. We could also monitor for changes in folding, aggregation and particle size over 36 hours.

With all of this data collected on a single platform, researchers can gain more insight into protein stability and aggregation under various conditions to move with confidence to the next steps of characterization.

References

1. Arakawa T, Ejima D, Tsumoto K, Obeyama N, Tanaka Y, Kita Y, and Timasheff SN. 2007. Suppression of protein interactions by arginine: A proposed mechanism of the arginine effects. *Biophysical Chemistry* 127(1-2): 1–8.

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