Get the whole story: combine T_m and T_{agg} with sizing and polydispersity on Uncle

Introduction

Thermal ramp stability measurements (T_m and T_{agg}) are well-established methods for ranking proteins and formulations for stability. This data is critical for stability determinations, and ensures that researchers are focusing on winning constructs and formulations. While valuable, there is additional information that is not easily gleaned from T_m and T_{agg} experiments. Sample quality should be assessed prior to a thermal ramp experiment. This is often done with dynamic light scattering (DLS) techniques to gain information on hydrodynamic radius and polydispersity. Another potentially interesting piece of data comes from DLS measurements at the end of a thermal ramp. In some instances, aggregation is not observed by static light scattering (SLS) methods for T_{agg} determination. The DLS measurement post-thermal ramp could potentially be used as verification when SLS shows unexpected results, or for determining if the average aggregate size exceeds 100 nm (0.1 microns).

This application note describes how you can use Uncle to get even more information out of a simple T_m and T_{agg} screen, by opting to collect DLS data at the beginning and end of a thermal ramp. Without adding any more sample volume or set up time to your workflow, get high-resolution information about the size and polydispersity of your samples from the same instrument. Learn about any red flags with your formulations or unstable constructs, and use the sizing information as an additional clarification for samples that display odd behaviors in the thermal ramp measurements.

Uncle is an all-in-one stability platform that enables 12 different applications with one instrument. Fluorescence, SLS and DLS detection methods are used to characterize protein stability. Temperature control (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.

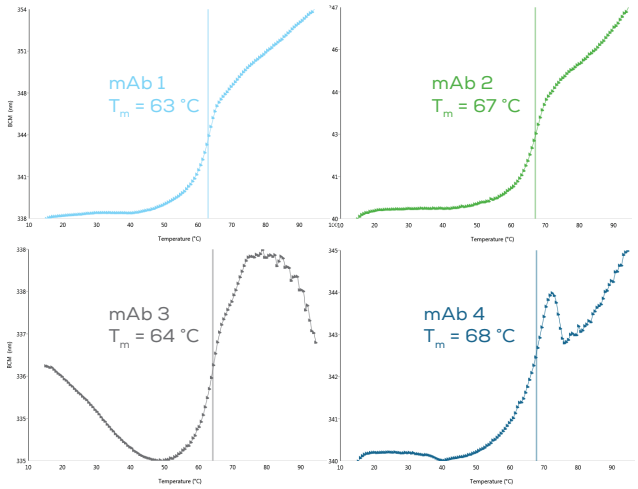


Figure 1: Melting curves for four mAbs over a thermal ramp from 15–95 °C. The T_m as determined by Uncle Analysis Software is indicated by a solid dropline on each graph.

Methods

Four monoclonal antibodies (mAbs) were reconstituted in PBS at 0.5 mg/mL and stored at 4 °C overnight. Nine µL of each sample were loaded in triplicate in a Uni, and run with a thermal ramp from 15–95 °C, with a ramp rate of 0.3 °C /minute. Initial and final DLS measurements were taken with four acquisitions of 5 seconds each. Uncle software calculated the T_m of each sample using the first derivative of the barycentric mean (BCM) of fluorescence intensity. The T_{agg} for each sample was calculated using the intensity of scattered

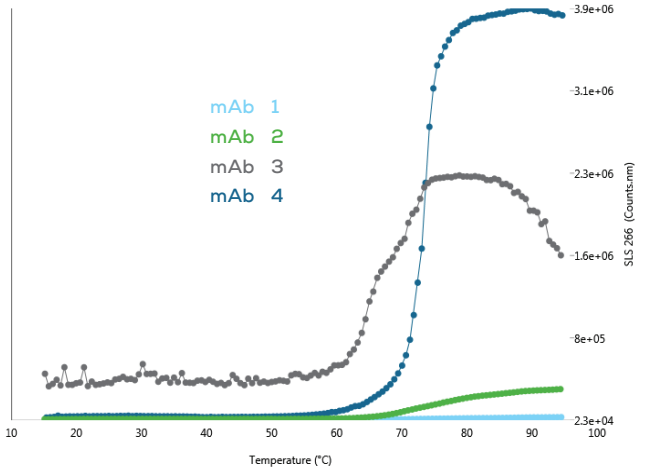


Figure 2: Aggregation curves overlaid for four mAbs (as measured by scattering intensity at 266 nm) over a thermal ramp.

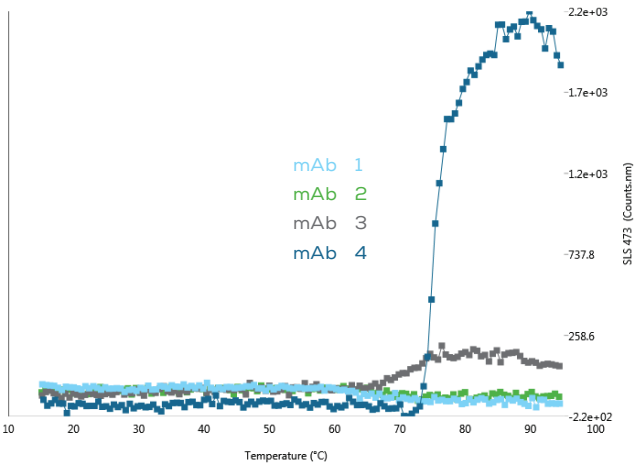


Figure 3: Aggregation curves overlaid for four mAbs (as measured by scattering intensity at 473 nm) over a thermal ramp.

light at 266 or 473 nm, and the sizes and polydispersity of each sample were calculated from the DLS correlation function.

Results

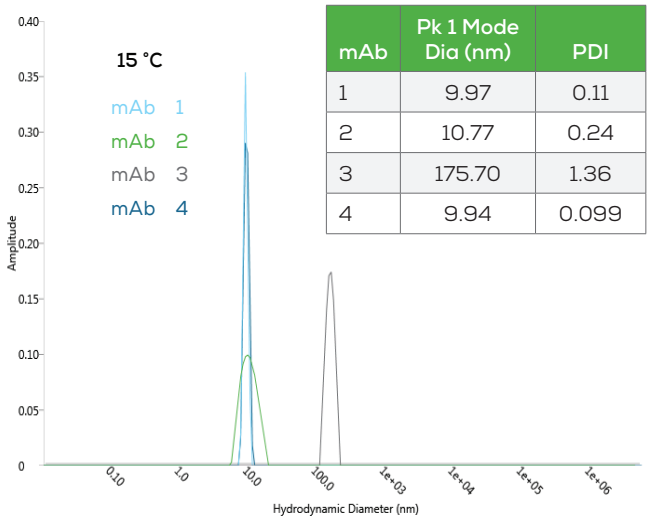
T_m

It can be difficult to determine the significance of T_m measurements if samples yield similar values. After being subjected to a thermal ramp, the pair of antibodies mAb 1 and mAb 3 showed similar melting temperatures, of 63 and 64 °C, respectively (Figure 1). Likewise, mAb 2 and mAb 4 exhibited similar melting temperatures, of 67 and 68 °C, respectively. If this was the only stability information collected about this set of proteins, one might conclude that each pair had similar thermal stability, and focus continued characterization and optimization efforts on mAb 2 and mAb 4.

T_{agg}

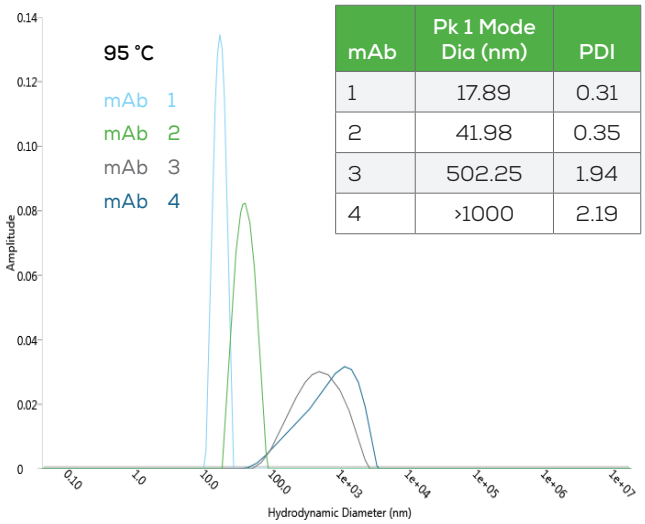
Obtaining SLS information in the same experiment can help provide more information about protein stability. Uncle measures SLS at 266 and 473 nm, giving additional clarity on the onset of aggregation and a sense of particle size. 266 nm intensity is usually picked up first, as it should be more sensitive to changes in sample intensity and should discern smaller particles. 473 nm measurements are useful for detection of larger aggregates, and to discern differences between samples if intensity has saturated at the lower wavelength. In this case, all monoclonal antibodies exhibited significantly different behavior when subjected to a thermal ramp. At 266 nm, the antibody mAb 1 showed almost no aggregation throughout the whole thermal ramp (Figure 2) while mAb 2 exhibited a low level of scattered light intensity after the thermal ramp, with a slight and gradual increase starting at around 62 °C. The monoclonal mAb 3 had a distinct onset of aggregation at 61 °C, reached a maximum scattering intensity and then had a decrease in signal intensity between 80–95 °C. This suggests that precipitation of aggregated material has begun to occur. Additionally, mAb 3 had a higher initial SLS intensity as compared to all other proteins tested (Figure 2). The antibody mAb 4 showed a sudden and sharp increase of intensity at 69 °C.

SLS measurements at 473 nm painted a similar picture (Figure 3). Aggregation was not observed for mAb 1 or mAb 2, which is consistent with the 266 nm measurements, and the lower sensitivity of the 473 nm read. The antibodies mAb 3 and mAb 4 had aggregation onsets at 67 and 73 °C, respectively.



mAb	Pk 1 Mode Dia (nm)	PDI
1	9.97	0.11
2	10.77	0.24
3	175.70	1.36
4	9.94	0.099

Figure 4: Initial DLS measurements for four mAbs. The graph shows the intensity distribution for each sample, and the inset table displays the calculated diameter and polydispersity index.



mAb	Pk 1 Mode Dia (nm)	PDI
1	17.89	0.31
2	41.98	0.35
3	502.25	1.94
4	>1000	2.19

Figure 5: DLS measurements for four mAbs at the end of the thermal ramp to 95 °C. The graph shows the intensity distribution for each sample, and the inset table displays the calculated diameter and polydispersity index.

It can be difficult to make strong conclusions about the absolute amount and nature of aggregates based on the scattering intensity when comparing proteins to one another. If we were to observe the SLS measurements in isolation, we would conclude that mAb 1 and mAb 2 are the more stable antibodies as they seem to have low levels of aggregation or no aggregates at all. A T_{agg} call is based on the change in light scattering. These numbers suggest that mAb 4 is more stable. If used in conjunction with the T_m data, one might conclude that mAb 2 or mAb 4 are the more stable antibodies, as they also have higher T_m measurements.

Initial and final DLS

Uncle has the capability to obtain quantitative sizing and polydispersity measurements at the beginning and end of a thermal ramp experiment. Without sacrificing any more sample, and with a marginal increase in experimental time, sizing and polydispersity information can be added to the experimental data set. With this data, it is apparent that mAb 1, mAb 2 and mAb 4 were all of the expected diameter for a monomeric monoclonal antibody at the beginning of the experiment, and that mAb 3 contained significant aggregate content prior to any thermal ramp (Figure 4). With a polydispersity index (PDI) of less than 0.25, mAb 1, mAb 2 and mAb 4 can all be considered monodisperse, while the high PDI for mAb 3 confirms the polydispersity of that sample. This provides a more quantitative way to confirm what was seen at the beginning of the thermal ramp in Figure 1, and allows us to eliminate mAb 3 in this particular formulation from further consideration.

Likewise, collecting DLS measurements at the end of the thermal ramp can also provide useful information for ranking these molecules (Figure 5). After ramping to 95 °C, the mAb 4 sample shows a large increase in diameter, whereas mAb 1 and mAb 2 both show much more modest increases in size. Once again, the PDI values also indicate that mAb 1 and mAb 2 are more homogenous and monodisperse than mAb 3 and mAb 4. These measurements thus serve to confirm the SLS data (Figures 2 and 3), as well as allowing one to estimate the molecular weights of the species that have formed in each sample after a thermal ramp.

	mAb 1	mAb 2	mAb 3	mAb 4
T_m (°C)	63	67	64	68
T_{agg} (°C) at 266 nm	62	62	61	69
T_{agg} (°C) at 473 nm	n.d.	n.d.	67	73
Initial SLS at 266 nm (counts x10^4)	2.3	3.3	46	4.8
Max SLS at 266 nm (counts x10^4)	5.2	32	230	390
Initial SLS at 473 nm (counts)	< 0	< 0	< 0	< 0
Max SLS at 473 nm (counts)	< 0	< 0	176	2175
Initial diameter (nm)	9.97	10.77	175.70	9.94
Final diameter (nm)	17. 9	42	502	>1000
Initial PDI	0.11	0.24	1.36	0.099
Final PDI	0.31	0.35	1.94	2.19

Table 1: Summary of stability, aggregation and sizing data for the four antibodies tested in this Uncle experiment.

Upon examination of all of the data collected from this single experiment (Table 1), the decision might be made to select mAb 1 over mAb 3, and mAb 2 over mAb 4. Both members of each pair have similar melting temperatures, and at first glance their T_{agg} values would not distinguish them dramatically. However, supplementing the SLS intensity values with the DLS sizing and polydispersity information allows one to conclude that mAb 1 and mAb 2 are more "well-behaved" and do not form large heterogeneous aggregates during thermal stress, unlike mAb 3 and mAb 4. Therefore they may be better and more robust candidates for moving forward to the next step in a development process.

Conclusion

In this single experiment performed on Uncle, multiple types of data were collected from four different monoclonal antibodies, enabling the evaluation of their stability and aggregation profiles. T_m and T_{agg} values were bolstered by quantitative sizing and polydispersity data from DLS measurements. As illustrated in this example, this can be useful for confirming T_m and T_{agg} data, validating sample quality and providing more detailed information about the size and nature of aggregates. The platform thus provides an easy-to-use tool to give researchers a leg up for selecting winners when comparing similar constructs or formulations.

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