Uncle stays hot for proteins with isothermal apps

Introduction

Testing the isothermal stability of proteins at high-throughput as early as candidate screening is the fastest way to see which ones would fail long-term storage trials. Unfolding and aggregation temperatures are key to ranking protein formulations, but so is seeing how well the samples tolerate stewing at a constant temperature for hours or days. To get all that info from tiny volumes and low concentrations, you need a multi-purpose tool with applications tailor-made for biologics.

Uncle boasts 3 detection methods: full-spectrum fluorescence, static light scattering (SLS), and dynamic light scattering (DLS) to seamlessly profile protein stability (Figure 1). Temperature control (15–95 °C) adds flexibility to your experimental design – heat things all the way up in a thermal ramp or hold things constant for isothermal tests. Samples are sealed in 9 µL quartz cuvettes to minimize evaporation and up to 48 can be screened in a single experiment.

With full-spectrum fluorescence detection (250–720 nm), protein intrinsic fluorescence and dyes like SYPRO™ Orange can be scoped to assess protein unfolding. Simultaneously, SLS tracks the formation of large and small aggregates while DLS takes care of sizing and size distributions. All 3 methods are combined in the T_m & T_{agg} application with a thermal ramp to test the stability of protein analytes in under 2 hours. Using information from a thermal ramp to set up an isothermal experiment is a smart test for accelerated stability and complements the stability picture for your protein.

If you want to set up even longer-term experiments but keep Uncle free to do other stuff at the same time, stash your samples in an incubator and pop them into Uncle to grab the data with the Out-of-the-box Stability apps. Whether it's for an hour, a day, or a week, Uncle has the flexibility to analyze any protein and point out which ones are acting up, whenever you want.

Figure 1: Uncle is the first all-in-one biologics stability platform.

In this application note, we illustrate the thermal stability of a monoclonal antibody and RNase A in 3 different formulations with the T_m & T_{agg}, T_m using SYPRO, Isothermal, and Out-of-the-box Stability applications on Uncle.

Methods

Stock solutions of monoclonal antibody 1 (mAb 1) were diluted from 26 mg/mL to 1 mg/mL in 25 mM His-HCl buffer, pH 7.4, with 0, 50, or 250 mM NaCl.

Ribonuclease (RNase) A (Sigma-Aldrich) was reconstituted from lyophilized powder to 10 mg/mL with water, aliquoted, and frozen at -20 °C. Aliquots were thawed at room temperature and diluted to 1 mg/mL in the previously listed formulations. The diluted RNase A was passed through 0.22 µm syringe filters.

Nine microliters of each sample were loaded into cuvettes of a Uni (array of sixteen 9 µL quartz cuvettes, sealed by silicone gaskets) in duplicate or triplicate for each of the following experiments. Each of the Uncle experiments was repeated and representative data is shown.

T_m & T_{agg} by intrinsic fluorescence

Protein unfolding and aggregation experiments were performed with the T_m & T_{agg} application on Uncle. Samples were ramped in Uncle from 15–95 °C at a rate of 1 °C/minute and excited at 266 nm while simultaneously monitoring fluorescence emission and SLS. Uncle Analysis software determined the T_m from the barycentric mean (BCM) of the fluorescence intensity curves from 300–430 nm and the T_{agg} from the intensity of light scattered at 266 nm (Figure 2).

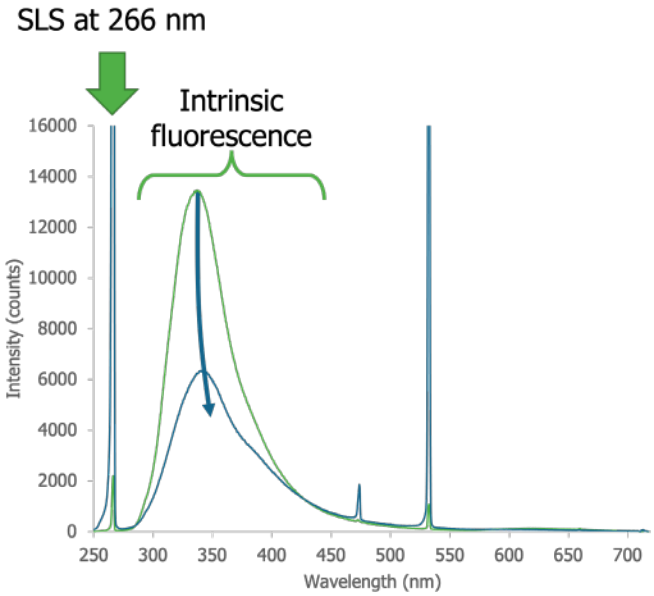

Figure 2: Uncle uses a 266 nm laser to excite intrinsic protein fluorescence and to measure SLS. As proteins unfold, their intrinsic fluorescence typically decreases and shifts to longer wavelengths. This shift is tracked by monitoring the spectral central of mass, or BCM. As proteins aggregate, SLS intensity increases.

T_m using SYPRO

As a second method to assess protein unfolding, the T_m using SYPRO application on Uncle was used. 5000x SYPRO Orange (ThermoFisher) was diluted to 100x in water then added to protein solutions to a final concentration of 10x. Samples were heated in Uncle from 15–95 °C at a rate of 1 °C/minute and excited at 473 nm while simultaneously monitoring fluorescence emission and SLS. Uncle Analysis software determined the T_m from the area under the fluorescence intensity curves from 510–680 nm and the T_{agg} from the intensity of light scattered at 473 nm (Figure 3).

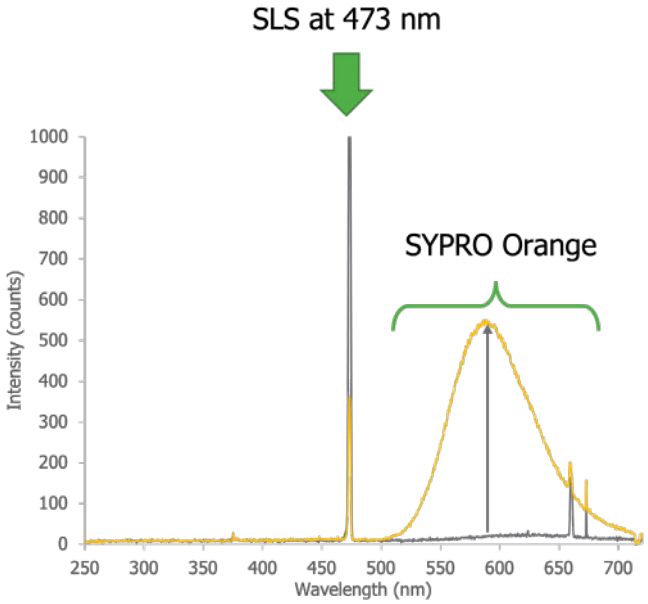

Figure 3: Uncle uses a 473 nm laser to excite SYPRO Orange fluorescence and to measure SLS. As proteins unfold, hydrophobic regions of the protein become available to interactions with the SYPRO Orange dye, increasing fluorescence.

Isothermal experiments

Samples were incubated for 16 hours at 60 °C, below the T_ms of mAb 1 and RNase A, using the Isothermal application on Uncle, and excited at 266 and 473 nm while monitoring fluorescence emission and SLS every minute.

Out-of-the-box experiments

Long-term stability studies were carried out using the Out-of-the-box Stability application on Uncle. Samples were loaded into Unis and held at 60 °C in an incubator. The average hydrodynamic diameter was measured by DLS (with 4 acquisitions of 5 seconds each) once per day for 3 days.

Results

T_m & T_{agg}, T_m using SYPRO and Isothermal stability of mAb 1

Thermal stability of mAb formulations can be ranked based on unfolding or aggregation. Uncle directly tracks both behaviors, simultaneously, by monitoring the protein's full fluorescence emission spectrum and light scattering. The T_m of mAb 1 in 0 or 50 mM NaCl were nearly identical at 70.4 and 70.2 °C, respectively (Figure 4). At 250 mM NaCl,

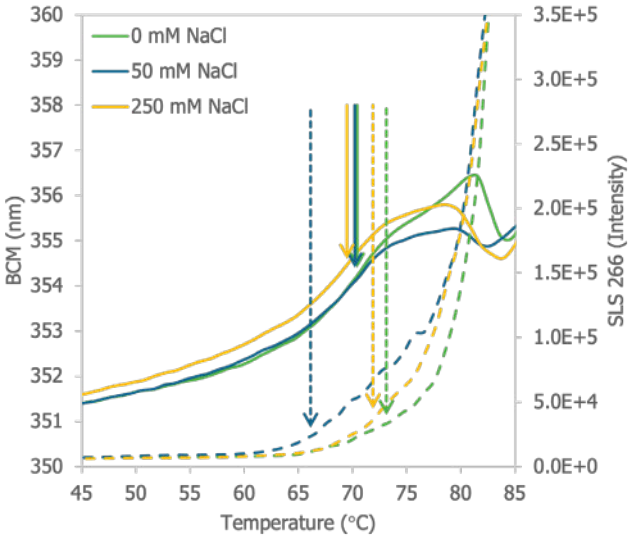

Figure 4: BCM (solid lines) and SLS at 266 nm (dashed lines) from a T_m & T_{agg} experiment of mAb 1 in 25 mM His-HCl buffer, pH 7.4 with 0 mM (green), 50 mM (blue) and 250 mM (yellow) NaCl. Dashed arrows indicate T_{agg} and solid arrows indicate T_m.

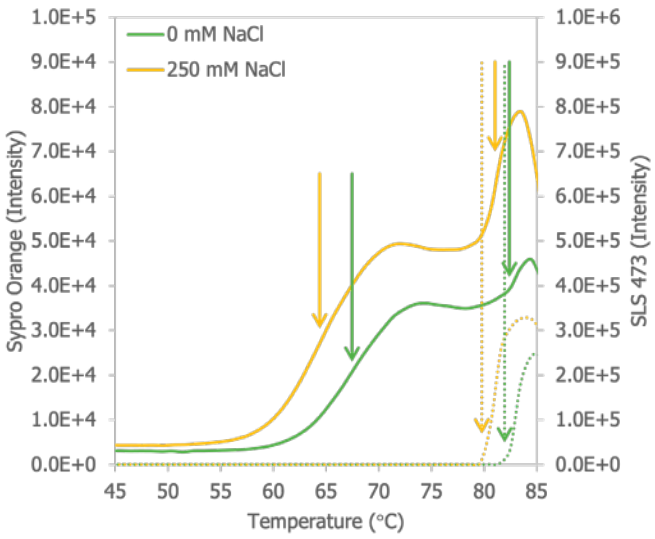

Figure 5: SYPRO Orange fluorescence (solid lines) and SLS at 473 nm (dotted lines) from a T_m using SYPRO experiment of mAb 1 in 25 mM His-HCl buffer, pH 7.4 with 0 mM (green) and 250 mM (yellow) NaCl. Solid arrows indicate T_ms and dotted arrows indicate T_{agg}s.

the T_m of the antibody dropped to 69.5 °C, indicating a slightly less stable condition.

SLS at 266 nm showed aggregation of mAb 1 at 66.1 °C with 50 mM NaCl, but similar levels of aggregation were not detected until 71.9 °C in 250 mM NaCl and 73.1 °C in salt-free buffer (Figure 4). NaCl can stabilize or destabilize proteins and induce or inhibit aggregation, depending on concentration and the nature of the protein. Based on protein unfolding alone (T_m values), the low-salt and no-salt conditions appear to be similar for this mAb. However, examining aggregation at the same time (T_{agg}) we get a much clearer stability picture as there are significant differences in low-salt and no-salt.

We can also use differential scanning fluorimetry (DSF) with extrinsic dyes like SYPRO Orange in Uncle to probe protein stability: in this experiment, two T_ms were detected for mAb 1 (Figure 5). T_m1 was 64.4 °C in the 250 mM NaCl buffer and T_m2 was 81.0 °C. In the no-salt condition, T_ms shifted higher: T_m1 was 67.5 °C and T_m2 was 82.4 °C. Looking at aggregation by SLS, the T_{agg} values in the same experiment closely matched the T_m2 values in both conditions, suggesting that this second "melting" event was due to SYPRO Orange detecting the formation of large protein aggregates, not protein unfolding.

Stability studies involving fluorescent reporter dyes are more difficult to interpret. SYPRO Orange indiscriminately binds to hydrophobic patches, which increases its fluorescence emission. Monitoring reporter fluorescence data alone, it is impossible to distinguish an increase in signal stemming from unfolding from one caused by aggregation. By monitoring both fluorescence (T_m) and SLS (T_{agg}) simultaneously, Uncle can differentiate the two causes of increased fluorescence emission by the reporter dye. Another complication to keep in mind is that an added dye can potentially alter the behavior of proteins (and the T_m) by stabilizing or destabilizing the native state. This is the case for mAb 1, too, as the T_ms with SYPRO Orange are 3-5 °C lower than those determined from protein fluorescence (Table 1). Uncle can be relied upon to uncover this effect by comparing results of both methods.

Thermal ramps are a fast and powerful tool to determine mAb stability but holding samples for a longer time at steady temperatures can yield a more detailed picture of unfolding and aggregation as they occur. For mAb 1, 60 °C was chosen as a temperature for an isothermal experiment since

NaCl	T_m by BCM	T_{agg} 266	T_m1 by Sypro	T_{agg} 473	T_m2 Sypro
0 mM	70.4 °C	73.1 °C	67.5 °C	81.9 °C	82.4 °C
50 mM	70.2 °C	66.1 °C	66.2 °C	80.2 °C	81.3 °C
250 mM	69.5 °C	71.9 °C	64.4 °C	79.8 °C	81.0 °C

Table 1: Summary of protein stability parameters for mAb 1 in 25 mM His-HCl buffer, pH 7.4, with indicated NaCl concentrations (see Figures 4 & 5).

it was well below the protein stability parameters determined using intrinsic fluorescence, SYPRO Orange, or SLS (Table 1).

Aggregates started forming in the 50 mM NaCl formulation within the first hour of the experiment, based on SLS at 266 nm (Figure 6). The increase of SLS at 473 nm set on later in the experiment but still only took a few hours. As SLS intensity is a function of wavelength and aggregate size, the shorter wavelength of 266 nm is more sensitive and picks up on smaller aggregates. The 473 nm signal indicates larger aggregates and adds to the dynamic range of the system. In these formulations, the salt-free condition delayed aggregation much more than either of the salt-containing conditions.

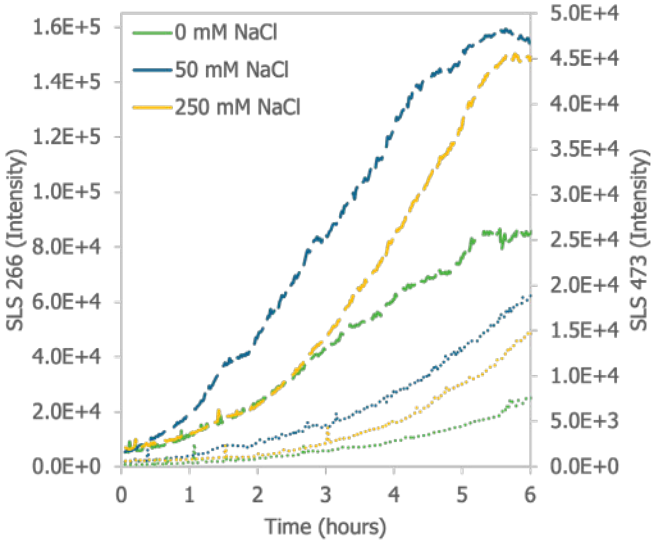

Figure 6: SLS at 266 nm (dashed lines) and 473 nm (dotted lines) during a 60 °C Isothermal experiment of mAb 1 in 25 mM His-HCl buffer, pH 7.4 with 0 mM (green), 50 mM (blue) and 250 mM (yellow) NaCl.

Rank-ordering protein formulations based on short-term stability screens saves time and sample by limiting the number of formulations that move on to longer term storage and efficacy studies. Based on mAb 1 unfolding and aggregation during thermal ramp and isothermal experiments, the salt-free 25 mM His-HCl buffer, pH 7.4, was consistently the most stable of the tested conditions under the widest variety of conditions, while the 50 mM NaCl condition was the least stable. The good correlation between the thermal ramp and the isothermal experiment shows the predictive value of these shorter-term experiments for this particular analyte.

T_m using SYPRO and Isothermal stability of RNase A

Reagent-free methods are preferable for probing protein stability since reporter dyes and tags can influence protein conformation, as described for mAb 1 above. However, intrinsic fluorescence measurements are not possible with all proteins. RNase A is one example, entirely lacking tryptophan so it has exceptionally low intrinsic fluorescence. For these kinds of difficult-to-characterize proteins, Uncle's full-spectrum fluorescence detection can be used in conjunction with extrinsic dyes like SYPRO Orange to meet whatever stability characterizing needs you may have.

In the T_m using SYPRO app on Uncle, RNase A had a single T_m in each of the 3 tested formulations, all at approximately 61 °C (Figure 7A). No increase in SLS signal was visible during the thermal ramp, indicating the protein did not aggregate significantly in any of the formulations. When RNase A was held at 60 °C for 16 hours and monitored for aggregation using SLS, the 0 and 50 mM showed significant aggregation after about 10 hours (Figure 7B).

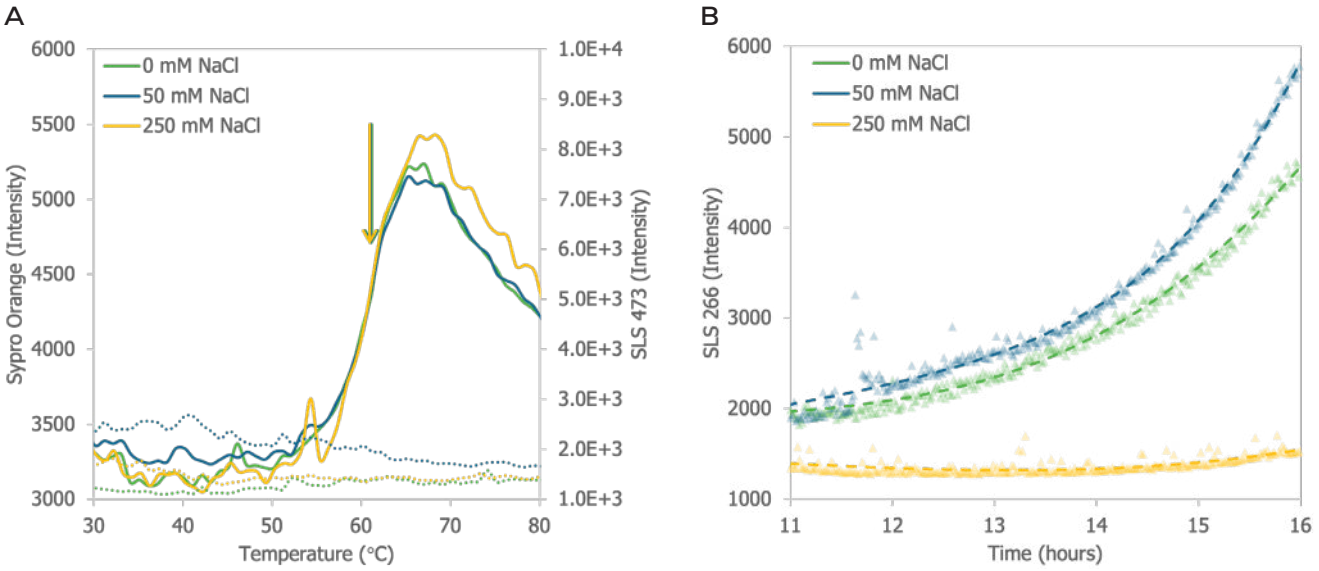

Figure 7: SYPRO Orange fluorescence intensity (solid lines) and SLS at 473 nm (dotted lines) during a T_m using SYPRO experiment of RNase A with solid arrows indicating T_ms (**A**). SLS at 266 nm (triangles) during a 60 °C Isothermal experiment for 16 hours (**B**). Only the last 5 hours are shown.

Rank-ordering RNase A in the different salt concentrations based on data from a short thermal ramp alone would have been impossible. However, with the additional insights provided by the longer isothermal experiments, it was possible to conclude that the high salt condition was more stable for longer periods.

Out-of-the-box stability

Most longer-term protein stability studies rely on complex and time-consuming methods like HPLC, SEC, or mass spectrometry. Uncle has a better way. Unis containing RNase A in the same 3 salt-containing formulations as before were held at 60 °C for 3 days in an incubator and checked daily for aggregates by DLS in Uncle. The high salt condition exhibited no change in the hydrodynamic size of the protein under these stress conditions while the protein in the other formulations showed a significant increase of the Z-Average Diameter by the end of day 2 (Figure 8). The DLS test confirms the behavior of RNase A observed in the Isothermal test above.

For RNase A, in contrast to mAb 1 described previously, isothermal experiments were necessary to determine the influence of the formulation condition on protein stability as the thermal ramp experiment did not show any differences between the three conditions tested.

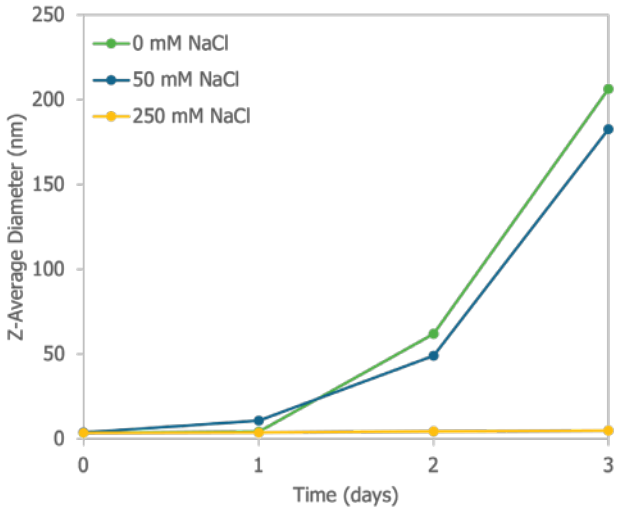

Figure 8: Z-Average Diameter by DLS at 60 °C incubation outside Uncle for 3 days, measured daily, of RNase A in 25 mM His-HCl buffer, pH 7.4, with 0 mM (green), 50 mM (blue) and 250 mM (yellow) NaCl.

Conclusion

Identifying the best formulation that protects your protein from the heat is faster with the flexible, high-throughput stability tools in Uncle. Melting temperatures can be determined in 2 hours with intrinsic or dye-based fluorescence, keeping Uncle ready for any protein. Thermal ramp experiments can show your protein's tendency to aggregate, while Uncle's Isothermal applications complete

temperature testing by monitoring your analyte at a set temperature for hours or days. The Out-of-the-box app allows you to run more experiments while also collecting long-term data. Uncle helps you explore which proteins and formulations can take the heat in the most efficient way possible.

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