Dye Harder with UNcle: Measure Thermal Stability with SYPRO

Introduction

Forced degradation methods that rely on temperature stress to disrupt the three-dimensional protein structure are well-established for the development of stable biologics and formulation optimization. To probe stability, label-free methods are preferable, as the presence of extrinsic dyes or tags can affect the protein conformation. However, there are instances where intrinsic fluorescence measurements are not possible, in which case differential scanning fluorimetry (DSF) can be used to screen for protein stability. With UNcle, either label-free thermal stability measurements or DSF can be performed quickly and easily, so researchers can select the best method as projects evolve and experimental needs change.

UNcle is an all-in-one stability platform that enables 11 different applications with one instrument (Figure 1). Fluorescence, static light scattering (SLS) and dynamic light scattering (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. Samples are loaded into UNis, low volume, multi-well quartz cuvette chambers. 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: UNcle: a one-stop stability platform.

Methods

SYPRO® Orange (Thermo Fisher #S6651) was supplied at 5000X concentration in 100% DMSO and diluted to 40X in the appropriate formulation buffer (PBS, pH 7.4 or Succinate, pH 5). Lysozyme was prepared at 2 mg/mL in PBS and mixed with an equal volume of dye to yield a solution with 1 mg/mL final protein concentration and 20X SYPRO. Nine microliters of this mixture was loaded into a UNi and run with the "T_m using SYPRO" application on UNcle. Samples were subjected to a thermal ramp from 15–95 °C, with a ramp rate of 0.5 °C/minute and excitation at 473 nm. Full spectra were collected from 250–720 nm, and UNcle software used the area under the curve between 510–680 nm to calculate the inflection points of the transition curves.

A monoclonal antibody was formulated in either PBS, pH 7.4, or in 5 mM succinate, pH 5 and mixed with SYPRO as described above. The same monoclonal antibody in succinate buffer was also prepared with SYPRO at final protein concentrations of 0.05, 0.1, 1, 10, and 100 mg/mL. Nine microliters of each sample were loaded in quadruplicate into UNis and run on UNcle as before, but with a thermal ramp from 20–95 °C.

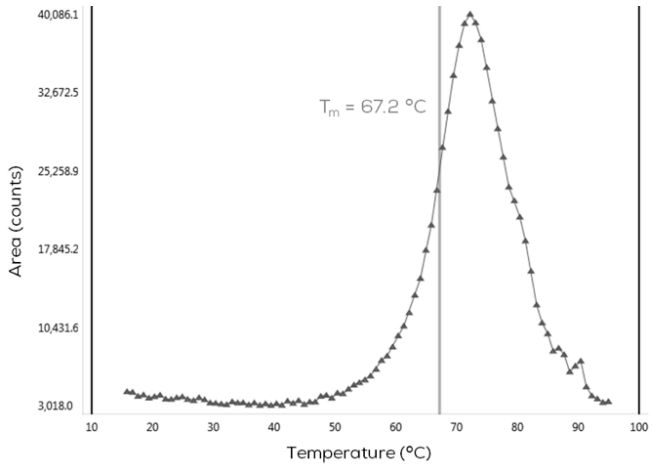

Figure 2: The thermal melting profile of a globular protein, lysozyme, in the presence of SYPRO. The software detected one transition at 67.2 °C.

Results

Lysozyme is a small globular protein that can be represented by a two-state model as it goes from its native conformation to a denatured state upon exposure to heat stress. When the folded protein is mixed with SYPRO, the dye is quenched by the aqueous buffer and the fluorescence intensity is low. As the temperature increases and the protein begins to unfold, the dye interacts with the newly exposed hydrophobic surfaces and yields a dramatic increase in fluorescence intensity. At higher temperatures, the signal decreases upon dissociation and aggregation of the denatured protein.

There is one clear transition for lysozyme in the presence of SYPRO dye. UNcle Analysis software used the first derivative of the area under the curve to calculate a T_m at 67.2 °C, which is in good agreement with previously published results by other methods (Figure 2).

In addition to automatic analysis, UNcle Analysis software allows for raw spectral data to be visualized and exported. As shown in Figure 3, by selecting spectra at two specific temperatures, the intensity change in the region of the graph between 510 and 680 nm is apparent. At 37 °C there appears to be little to no intensity, whereas at 75 °C SYPRO intensity is at its maximum level.

In contrast to a small globular protein such as lysozyme, multi-domain proteins such as antibodies often undergo more than one unfolding transition. A monoclonal antibody was mixed with SYPRO dye and run on UNcle, yielding a three-state melting curve (Figure 4). The software calculated two transitions at 62.7 °C and 76.3 °C.

Both of these proteins showed a significant change in fluorescence intensity from the beginning of the thermal ramp to the maximum level. This high signal-to-noise level is important for good data quality. If high initial fluorescence intensity is observed, it can be indicative of partially unfolded protein in the sample, or additives such as detergents in the formulation buffer that are incompatible with this method.

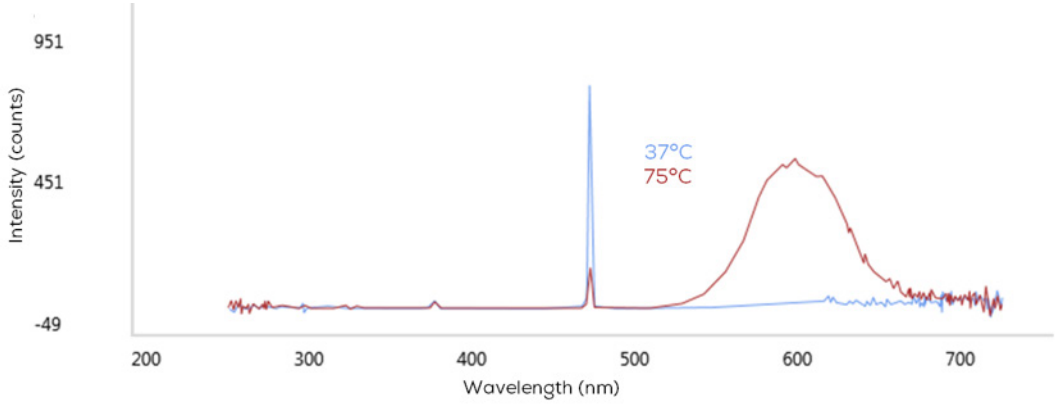

Figure 3: Raw spectra of lysozyme at two selected temperatures in the ramp, showing the dramatic change in fluorescence intensity as the protein unfolds.

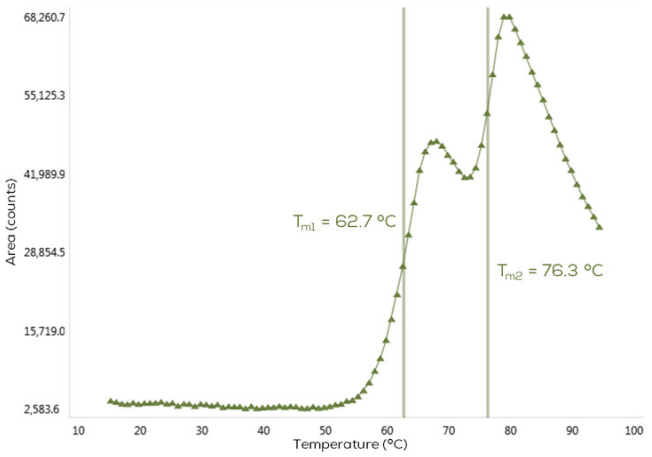

Figure 4: The thermal melting profile of a monoclonal antibody in succinate buffer, pH 5, in the presence of SYPRO. The software detected two transitions at 62.7 and 76.3 °C.

With SLS detection at 473 nm, UNcle can also detect protein aggregation in the same experiment. This can provide a more complete picture of what is happening during the thermal ramp. The same monoclonal antibody was formulated in PBS, which resulted in a similar three-state curve. In this buffer, however, the antibody underwent transitions at 66.1 °C and 75.6 °C. Interestingly, the onset of aggregation (T_{agg}) at 75.5 °C coincides with the second T_m. After a sharp increase in intensity, indicating the presence of aggregated particles, both the fluorescence and SLS signals returned to lower levels at around 80 °C. One explanation for this decrease is that larger particles have precipitated out of solution and hence are no longer scattering light, and the SYPRO dye has dissociated from the aggregated protein.

In contrast, the antibody in succinate buffer, pH 5 did not show any significant levels of aggregation at high temperatures as detected by SLS at 473 nm (Figure 6). There did appear to be some low level of aggregation of the protein at ambient temperatures that disappeared above 45 °C.

For a given protein, the ratio of protein to dye can be optimized to ensure good-quality data. For this monoclonal antibody, we tested a range of protein concentrations spanning several orders of magnitude, while keeping the final concentration of SYPRO constant. As expected, the fluorescence intensity is strongly dependent on protein concentration, as there are fewer binding sites for the dye with fewer protein molecules in the sample. The T_m values calculated from those curves, however, were fairly consistent over that wide range (Figure 7 and Table 1). The average values across this wide concentration range were 63.3 °C for T_{m1} and 78.1 °C for T_{m2}. At a very high protein concentration (100 mg/mL), the fluorescence intensity values were lower than at 10 mg/mL, and a second melting transition was not detected. There are at least two possible explanations for this. First, if protein molecules are packed too closely together, it could preclude their association with the dye. In that

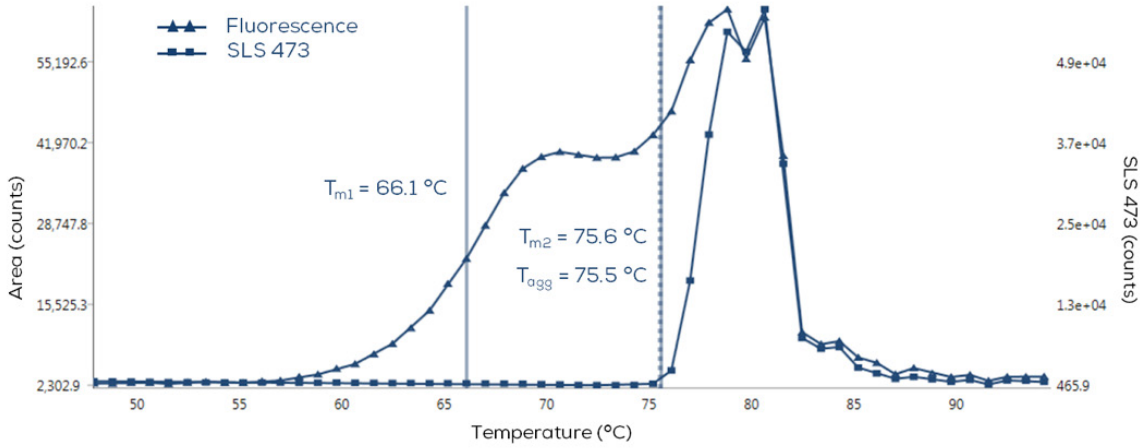

Figure 5: Simultaneous T_m and T_{agg} measurements of a monoclonal antibody in PBS mixed with SYPRO dye.

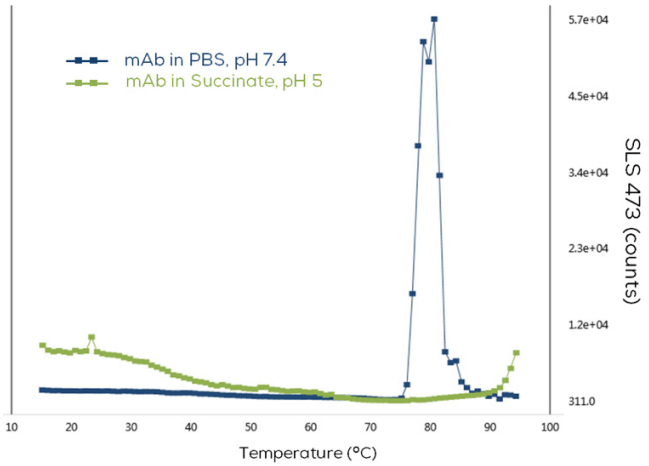

Figure 6: SLS measurements at 473 nm for the same mAb in two different buffers, showing a difference in aggregation over a thermal ramp.

mAb concentration (mg/mL)	T_{m1} (°C)	T_{m2} (°C)
0.05	63.8±0.08	77.4*±0.28
0.1	63.8±0.05	78.2±0.16
1.0	64±0.06	78.1±0.13
10	62.6±0.21	78.6±0.14
100	62.4±0.25	n.d.

Table 1: Average T_m values and standard deviations for 5 concentrations of a monoclonal antibody as calculated by UNcle from 4 replicates of each sample. *T_{m2} was automatically detected in only 2 of 4 replicates.

case, diluting the sample would be the simplest way to obtain reliable stability information for the protein of interest. The other possibility is that the dye:protein ratio might be too low, in which case trying a higher concentration of dye could allow for sufficient signal to make the desired measurements at high protein concentrations.

Conclusion

In this note we describe how SYPRO dye can be used to study protein stability on UNcle for different types of proteins, and at a wide range of protein concentrations. When label-free measurements are not feasible, for example, a lack of aromatic tryptophans within the secondary structure,

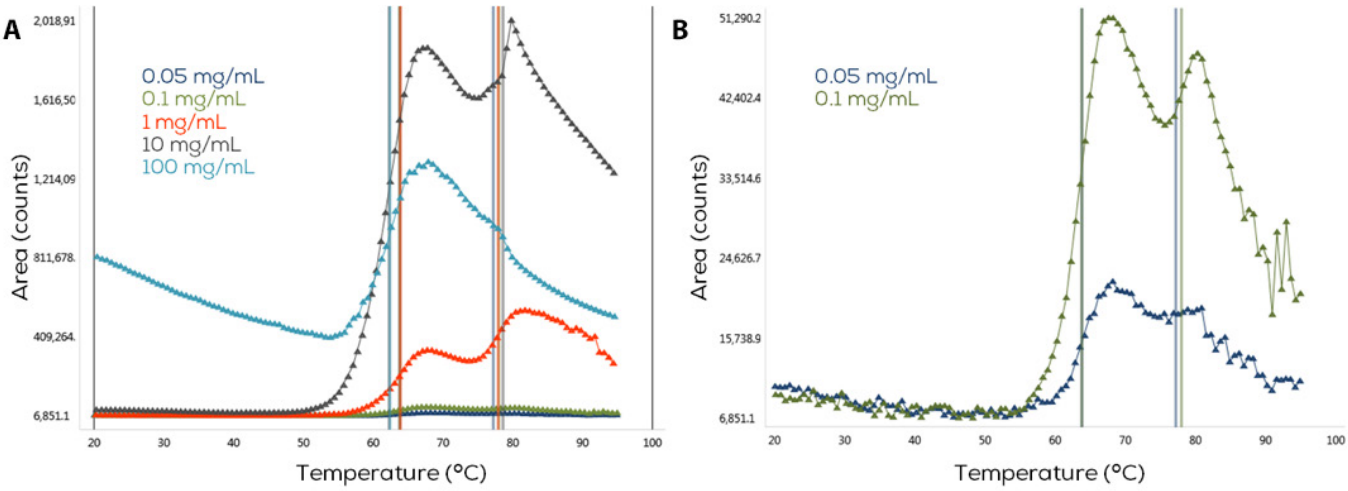

Figure 7: **A:** T_m with SYPRO for a monoclonal antibody at concentrations from 0.05-100 mg/mL. **B:** Expanded view of 0.05 and 0.1 mg/mL for clarity.

this method can be a fast and easy way to screen through many protein constructs or formulations in a short amount of time. With both SYPRO and label-free methods available on the same instrument, researchers have flexibility to switch between the two as project needs change or evolve.

References

1. Lavinder JJ, Hari SB, Sullivan BJ, and Magliery TJ. High-throughput thermal scanning: a general, rapid dye-binding thermal shift screen for protein engineering. *J Am Chem Soc* 131(2009): 3794–3795.

2. Niesen FH, Berglund H, and Vedadi M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nature Protocols* 2(2007): 2212–2221.

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