See it all with Uncle's full-spectrum analysis

Introduction

Measuring thermal stability by pairing heat-induced denaturation with fluorescence detection is a well-established method to optimize stability and formulation for biologics. While in most cases it's preferable to use label-free quantification of protein unfolding, there are instances where intrinsic fluorescence measurements are either not possible or could be complemented by dye- or tag-based techniques. With Uncle, label-free and dye-based thermal stability measurements can be performed simultaneously, so researchers can understand their protein's complex story with powerful analysis of full-spectrum fluorescence.

Uncle is a one-stop platform for protein stability that uses three detection methods: full-spectrum fluorescence, static light scattering (SLS), and dynamic light scattering (DLS) to fully profile protein stability (Figure 1). Temperature control (15–95 °C) and sealed sample chambers provide greater flexibility in how that profiling can be performed. Multiple measurements such as fluorescence, aggregation, sizing, and polydispersity can be performed in just one experiment, allowing you to obtain orthogonal information on protein stability on the same sample. Samples are loaded into low volume, multi-well quartz cuvette chambers requiring only 9 µL of sample. Uncle can measure up to 48 samples simultaneously, enabling greater throughput when characterizing your biologics.

Complex proteins in complex environments require powerful analysis techniques where many other methods fall short. Uncle's unique pairing of customizable analysis with full-spectrum fluorescence detection can remove unwanted noise from underlying protein data, or focus in on fluorescent dyes for deeper information. This application note describes how the full-spectrum fluorescence capabilities of Uncle can be used for characterization of your biologic by intrinsic fluorescence, dye-based fluorescence, and SLS.

Figure 1: Uncle: a one-stop stability platform

Methods

5000x SYPRO Orange was diluted to 400x in 100% dimethyl sulfoxide (DMSO). Polyclonal bovine IgG was diluted to 2.2 mg/mL in phosphate-buffered saline, pH 7.4 (PBS). Solutions of 2 mg/mL IgG and 40x SYPRO Orange or 2 mg/mL IgG with equivalent volume of DMSO were made in PBS.

7-Diethylamino-3-[4-(iodoacetamido)phenyl] -4-methylcoumarin (CPM) was diluted to 20 mg/mL in 100% DMSO. Bovine beta-lactoglobulin A (BLG) was diluted to 1 mg/mL in PBS. Solutions of 40 µM BLG and 80 µM CPM or 40 µM BLG with equivalent volume of DMSO were made in PBS.

8-Anilino-1-naphthalenesulfonic acid (ANS) was diluted to 10 mg/mL in 100% DMSO. Ribonuclease A (RNase A) was diluted to 6 mg/mL in water, then diluted to 0.5 mg/mL in water, 10 mM phosphate buffer, pH 7 or 100 mM phosphate buffer, pH 7.

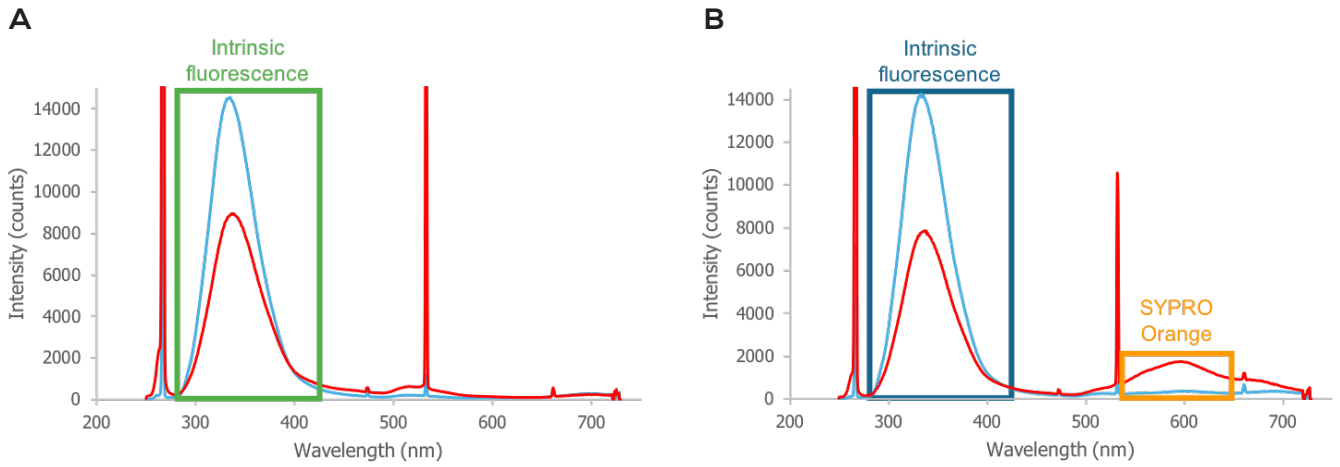

Figure 2: Fluorescent spectra at 15 °C (blue lines) and 67 °C (red lines) of bovine IgG with DMSO **(A)** or 40x SYPRO Orange **(B)**.

ANS solution was added to a final concentration of 80 µM.

For all experiments, 9 µL of each sample were run in triplicate with the T_m & T_{agg} with Optional DLS application on Uncle. Full spectra were collected from 250–720 nm, and a thermal ramp from 25–95 °C with a ramp rate of 0.6 °C/minute was used. IgG and RNase A samples were excited at 266 and 473 nm while BLG samples were excited at 266 nm. Appropriate analysis methods were selected for each sample as indicated in the figures.

Results

Probe antibodies with SYPRO Orange

Uncle collects spectra over the course of a single thermal ramp to quantify the thermal denaturation of a protein. Fluorescent spectra from IgG during heating show sharp peaks corresponding to the wavelengths of Uncle's lasers at 266, 473, and 660 nm and the resonance signal of the ultraviolet laser at 532 nm (Figure 2A).

When the IgG was heated in the presence of SYPRO Orange, the dye interacted with newly exposed hydrophobic surfaces as the protein unfolded, yielding a dramatic increase in fluorescence intensity with an emission maximum at approximately 590 nm (Figure 2B).[1] Uncle software can determine unfolding behavior from both intrinsic and SYPRO Orange fluorescence in a single experiment, and can use SLS to identify differences in aggregation behavior in the presence or absence of SYPRO Orange.

Uncle software uses the barycentric mean (BCM) method for analyzing the intrinsic protein fluorescence of IgG (from 300–430 nm) with and without SYPRO Orange (Figure 3A) to characterize protein unfolding. Alternatively, it can use changes in the area under the SYPRO Orange fluorescence curve from 536–650 nm (Figure 3B). T_m values determined by BCM of the two conditions were similar, indicating the dye likely did not affect the equilibrium state of protein unfolding.

SLS of IgG with and without SYPRO Orange indicates the dye likely delays the formation of small protein aggregates, perhaps by blocking the interaction of the newly-exposed hydrophobic residues of protein molecules (Figure 4). This result, combined with the slightly lower T_m at 70 °C determined by SYPRO Orange fluorescence, suggests that the dye's fluorescence behavior is impacted by both the unfolding behavior of the protein and the aggregation that begins prior to 70 °C.[2]

Uncle uses full-spectrum fluorescence and SLS detection methods to characterize protein unfolding and aggregation, with or without SYPRO Orange. Uncle's advanced fluorescence analysis allows for more complex and nuanced characterization of stability of a wide range of protein types and several fluorescent dyes.

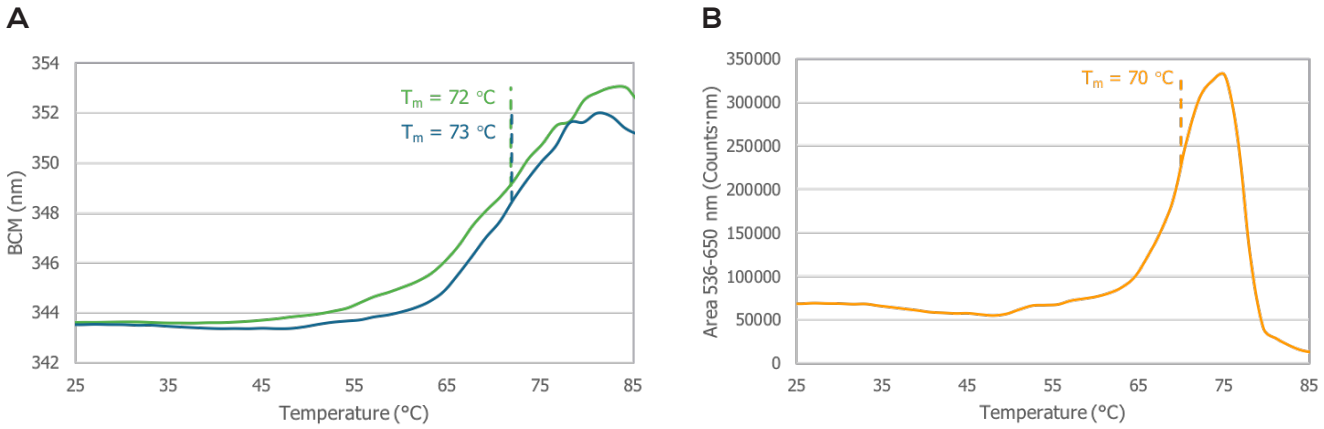

Figure 3: Protein melting curves by BCM analysis (300–430 nm) of IgG with SYPRO Orange (blue) or DMSO (green) **(A)**. Melting curve by SYPRO Orange fluorescence area under the curve analysis (536–650 nm) **(B)**. Dashed lines depict T_m locations.

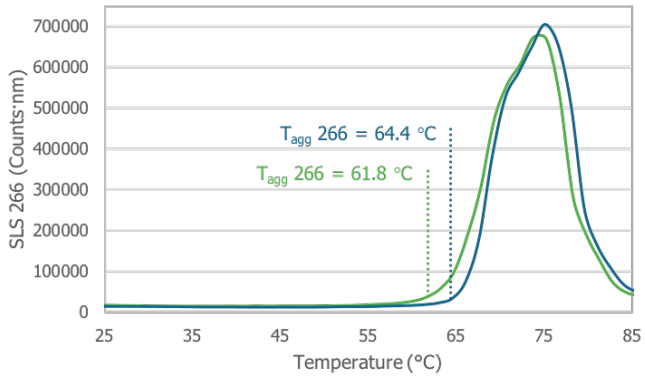

Figure 4: SLS 266 curves of polyclonal bovine IgG with SYPRO Orange (blue) or DMSO (green). Dotted lines depict T_{agg} 266 locations.

Analyze weakly fluorescent proteins with ANS

RNase A is a small, single-domain protein of 124 amino acids that contains no tryptophan, making it difficult to characterize by intrinsic fluorescence. Uncle can use extrinsic fluorescent dyes to overcome these challenges and understand even the toughest proteins.

Like SYPRO Orange, ANS binds to hydrophobic regions of a protein that become exposed during unfolding.[3] Unbound dye has a weak fluorescence emission peak at 545 nm. Once bound, the protein-ANS complex has much stronger fluorescence emission with a broad peak at 470 nm.[4]

The increased fluorescence signal and the blue shift of the emission peak of the protein-ANS complex versus unbound ANS makes it easy to use Uncle's BCM analysis to determine melting curves for RNase A (Figure 5A). T_ms were assigned at the local minima of the first derivative of the BCM curve (Figure 5B). Increasing the concentration of phosphate in the buffer increases the measured T_m values, which agrees with previously published stability results obtained by different methods.[5] Uncle's advanced fluorescence analysis opens the door to using additional tools to study protein stability, regardless of the strength of a protein's intrinsic fluorescence.

Evaluate membrane-bound and lipophilic proteins with CPM

Membrane-bound proteins are notoriously difficult to purify and characterize. They tend to be extremely unstable unless folded into liposomes or stabilized by detergents, both of which often scatter or absorb light in hard-to-quantify ways. However, fluorescent dyes like CPM can be used to determine stability of these proteins.[6] CPM is a thiol-reactive fluorescent dye that emits more strongly when it reacts with free cysteine residues in a protein, typically exposed during unfolding.

BLG is a major component protein in cow and sheep's milk. It serves as a useful model protein for designing stability studies of membrane-bound proteins. Since BLG contains a single free cysteine and 2 tryptophan residues, Uncle can use both intrinsic fluorescence and CPM fluorescence to probe BLG's thermal stability.

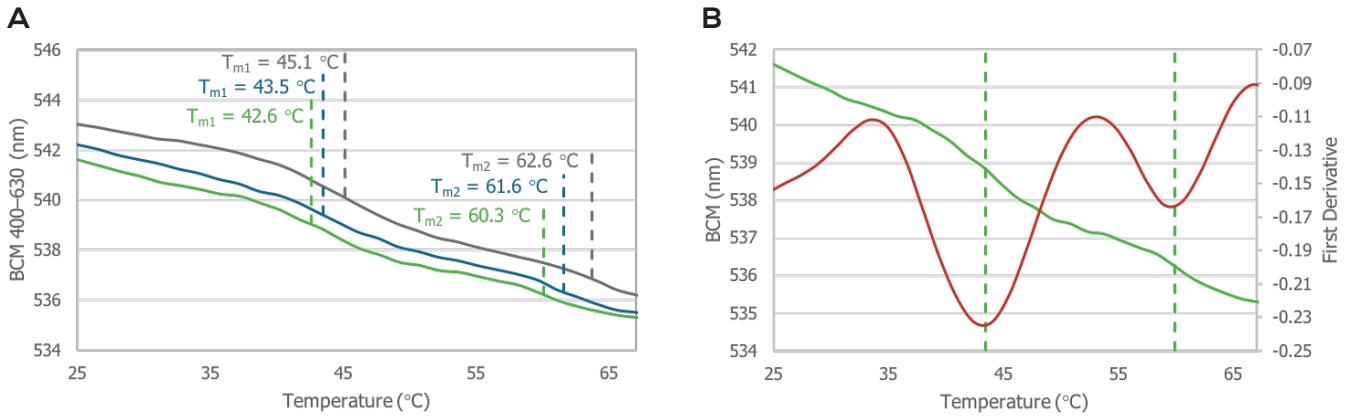

Figure 5: Melting curves (solid lines) and T_ms (dashed lines) of RNase A with 80 µM ANS in water (green lines), 10 mM phosphate buffer at pH 7 (blue lines), or 100 mM phosphate buffer at pH 7 (gray lines) based on change in BCM between 400 and 630 nm **(A)**. Melting curves are representative of 3 replicates and T_ms are the mean of 3 replicates. Melting curve (solid green line), T_ms (dashed green line), and first derivative (red line) of RNase A with 80 µM ANS in water **(B)**.

BLG labeled with CPM can be compared to BLG without dye by observing the full-spectrum fluorescence at 45 °C and 65 °C (Figure 6). Intrinsic protein fluorescence intensity was lower for BLG with CPM than for unlabeled BLG. Since CPM-thiol conjugates have an excitation maximum at approximately 384 nm, they absorb some of the light from fluorescing tryptophan residues. There is a clear increase in CPM fluorescence intensity between 400 and 650 nm as the temperature increases, due to the reaction between the CPM dye and the exposed free cysteine residue in BLG during unfolding.

Uncle determined the T_m of BLG in PBS to be approximately 60 °C by both intrinsic and CPM fluorescence methods (data not shown).

Conclusion

Uncle combines fluorescence, static, and dynamic light scattering measurements with customizable analysis of full fluorescence spectra to enable unique advanced sample analysis. Uncle readily characterizes thermal stability of membrane-bound or soluble proteins with any dye that can be excited by a 266 or 473 nm laser. Protein stability metrics such as T_m and T_{agg} can be collected in a single experiment and can be used to fully characterize a protein. With a wide range of potential dyes that probe stability in a host of proteins with complex and unique structures, Uncle provides a large toolbox for ensuring your protein is well-characterized.

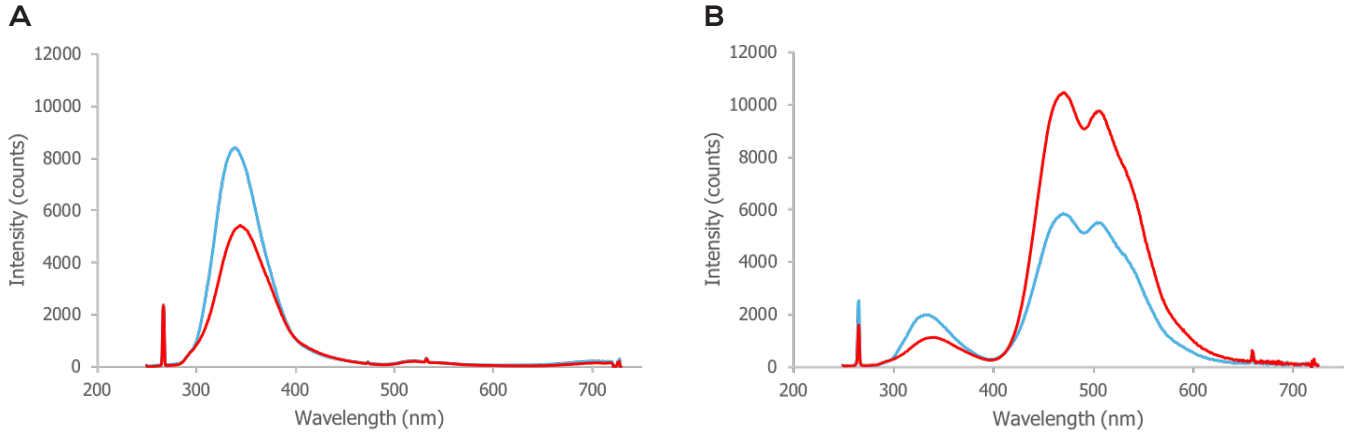

Figure 6: Fluorescent spectra at 45 °C (blue lines) and 65 °C (red lines) of 0.8 mg/mL bovine β-lactoglobulin in 1xPBS with DMSO **(A)** or 80 µM CPM **(B)**.

References

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