Take a snapshot of your protein quality with Uncle

Introduction

Evaluating the quality of samples prior to downstream analysis is an integral part of protein characterization workflows. There are many reasons to quickly assess the quality of a protein sample, such as comparing batches of purified material, changing formulation conditions, or checking the integrity of frozen samples after thawing.

Even after initial protein stability assessments are performed, there may be instances in which sources of physical or chemical stress, such as oxidation or agitation by sample mixing, can lead to protein unfolding or aggregation prior to downstream analysis. Considering all the ways in which protein quality can be affected by long-term storage or handling, performing a fast quality check can save time and ensure sample integrity. Uncle can reliably evaluate the stability of your biologics by using two different measurement modes:

- **Fluorescence and SLS**: Monitor the full-spectrum fluorescence of your protein to look at conformational changes, and SLS to detect the presence of small and large aggregates.
- **DLS**: Track changes in the size and polydispersity of your protein sample.

Uncle is a one-stop protein stability platform that uses fluorescence, SLS and DLS detection to enable 12 different applications (Figure 1). Multiple measurements such as thermal melting, aggregation, and sizing can be performed with the same samples in just one experiment, allowing you to obtain more information on your biologics faster than before. Uncle uses only 9 µL of sample and can measure up to 48 samples simultaneously, enabling greater flexibility for characterizing your proteins.

Figure 1: Uncle: a one-stop protein stability platform.

Methods

Sample preparation

Two therapeutic antibodies, mAb1 and mAb2, were prepared in 20 mM sodium acetate buffer at pH 5.0 and diluted to reach a final concentration of 0.5 mg/mL. The monoclonal antibody reference material distributed by the National Institute of Standards and Technology (NIST), NISTmAb, was supplied at 10 mg/mL in 25 mM Histidine at pH 6.0 and diluted to reach a final concentration of 0.5 mg/mL. The exact concentrations were measured by UV/Vis absorption on Lunatic (Unchained Labs) using 2 µL of sample. Aliquots from the mAb2 stock were stored at either 4°C or 25°C and measured up to 7 days.

Forced degradation

Aliquots from the mAb solutions were subjected to controlled heating at 75 °C for 1 hour. The samples were spun down and re-measured on Lunatic to identify any changes to the soluble protein concentration. For repeated freeze/thaw cycles, an aliquot of mAb1 was prepared in 25 mM Histidine buffer at pH 6.0 and diluted to reach a final concentration of 0.5 mg/mL. The aliquot was frozen at -80°C and thawed six times.

Uncle analysis

Samples were spun in a microcentrifuge at 10,000 x g for 3 minutes prior to analysis. Nine µL of each sample was loaded in a Uni in duplicate, and run with the Isothermal application, using 2 repeats measured at 25°C to measure intrinsic fluorescence and SLS at 266 nm and 473 nm wavelengths. The barycentric mean (BCM) of fluorescence intensity was selected as the analysis method in Uncle Analysis software for all fluorescence measurements. The Sizing & Polydispersity application was subsequently run on the same samples, with 4 DLS acquisitions of 5 seconds each.

Results

Controlled heating resulted in a large increase in the BCM fluorescence of mAb1, indicating a significant amount of unfolded protein in the sample (Figure 2, A). A large increase in SLS intensity at 266 nm for the heat-treated mAb1 signified the presence of small aggregates in the sample, whereas no significant change in SLS counts for the 473 nm laser indicated the absence of larger particles (Figure 2, B). On the other hand, NISTmAb did not exhibit any significant increase in the BCM fluorescence after thermal stress (Figure 2, A). These results were accompanied by minimal changes in SLS (data not shown), indicating NISTmAb did not significantly unfold or aggregate after thermal treatment. To further verify the protein quality of NISTmAb, a thermal ramp experiment was performed from 15 to 95 °C with a ramp rate of 0.3 °C/min. For both untreated and heat-treated NISTmAb samples, three transitions were measured and aligned closely to the reported transitions measured by DSC at 69, 83, and 94 °C (data not shown).[1] This data indicate that snapshot fluorescence and SLS measurements were able to capture the integrity of NISTmAb, before and after the applied heat stress.

After being subjected to thermal stress, mAb1 also showed a large increase in the hydrodynamic diameter (Figure 3). Consistent with the SLS data for mAb1, the presence of moderate-sized aggregates, approximately 70 nm in average diameter, was detected by DLS on Uncle. The absence of larger-sized aggregates

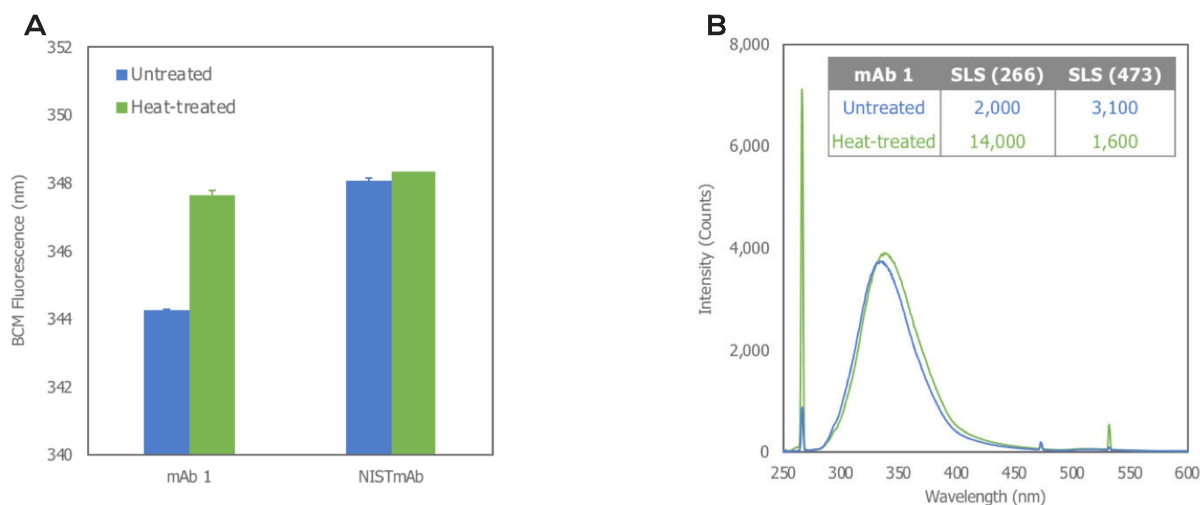

Figure 2: **A**: Intrinsic fluorescence of mAb1 and NISTmAb, with or without a heat treatment. **B**: Fluorescence emission spectrum of mAb1. The SLS intensity counts at 266 and 473 nm are shown in the inset table.

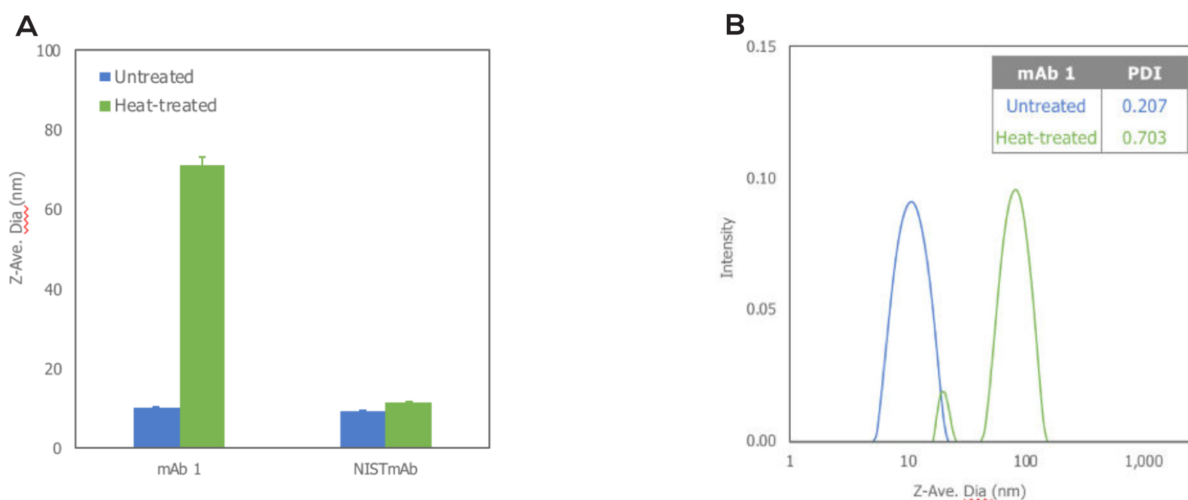

Figure 3: **A**: The Z-Average hydrodynamic diameter for untreated and heat-treated mAb1 and NISTmAb. **B**: Intensity distributions of untreated and heat-treated mAb1. The polydispersity (PDI) values are reported in the inset table.

is supported by both the SLS data measured at 473 nm and DLS. Additionally, an increase in the polydispersity index was measured for mAb1 (Figure 3, B), indicating a mixture of heterogeneous-sized populations after thermal treatment. The heat-treated NISTmAb sample showed almost no increase in the hydrodynamic diameter. Further inspection of the NISTmAb DLS data revealed the presence of a small population (<0.1% by mass) of large-sized particles several hundreds of nm in diameter. Collectively, these results indicate that little to no aggregation or unfolding occurred for NISTmAb when subjected to heat stress.

Monitoring changes in protein concentration

The presence of unfolded protein molecules in solution may inevitably lead to aggregation and irreversible precipitation, thereby reducing the functional yield of protein in a sample. To identify whether thermal stress led to a loss of protein yield of mAb1 and NISTmAb, 2 µL of each sample was measured in duplicate on Lunatic, using the A280 Classic application to quantify the protein concentration. Heat-treated samples were measured and compared against untreated samples, resulting in protein concentration ratios of 0.64 and 0.91 for mAb1 and NISTmAb, respectively. The concentration ratio of mAb1 is significantly less than 1, suggesting a loss of protein in the heat-treated samples likely caused by precipitation. The concentration of the heat-treated NISTmAb was slightly lower than the native sample, indicating that some protein may have precipitated from solution after applied heat stress.

Repeated freeze/thaw cycles

It is well-known that multiple rounds of freeze/thaw cycles can impart severe stress on a protein. During the freezing process, the formation of ice crystals can lead to phase separation of protein molecules from the aqueous phase. This phase separation in turn leads to the formation of concentration gradients which can accelerate protein unfolding or aggregation.

The stability of mAb1 to repeated freeze/thaw cycles was monitored by fluorescence, SLS, and DLS (Figure 4). The DLS shows an increase in size of mAb1 up to approximately 20 nm in diameter by the sixth freeze/thaw cycle. This increase in the Z-Average hydrodynamic diameter was unaccompanied by unfolding, as seen by the steady BCM fluorescence measurements. These results indicate that mAb1 may be undergoing native-state aggregation after repeated freeze/thawing.

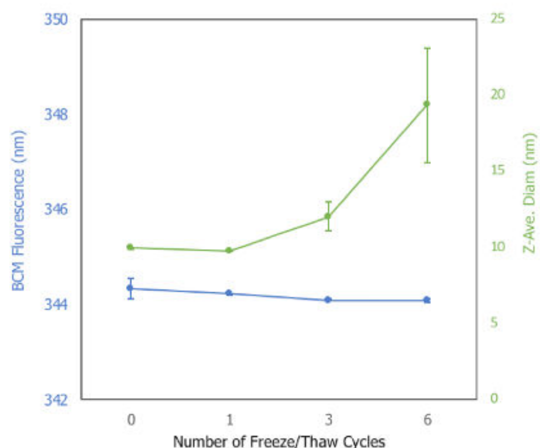

Figure 4: The BCM fluorescence intensity and DLS of mAb1 in 25 mM Histidine, pH 6.0 following repeated freeze/thaw cycles. Error bars denote the standard deviation of duplicate measurements.

Track your protein quality

Maintaining the appropriate storage temperature of protein samples is critical to ensure sample quality. Measuring baseline levels of fluorescence, SLS, and DLS of fresh protein and then evaluating a protein stock under different conditions over time can help parse out the bad conditions from the good. The BCM fluorescence intensity, SLS and DLS were measured for aliquots of a therapeutic antibody, mAb2, stored at 4 °C or 25 °C for a period of 7 days. A slight increase in the BCM fluorescence was observed for the 25 °C condition, indicating that some protein unfolding occurred over the 7-day period, whereas no unfolding was observed for the 4 °C condition (Figure 5). Further, the Z-Average hydrodynamic diameter for both conditions remained fairly steady at ~10 nm, which is the expected size for a monoclonal antibody.

Uncle enables quick checks of protein stability by combining full-spectrum fluorescence, SLS and DLS modes into one platform (Table 1). The fluorescence emission on Uncle is monitored from 250 to 720 nm, allowing the user to choose a suitable method for data analysis. This flexibility is especially important for proteins that deviate from typical unfolding patterns, such as red-shifting and/or a signal decrease of the fluorescence peak.

Conclusion

For fast and reliable QC checks of protein stability, Uncle combines fluorescence, static and dynamic light scattering methods to measure protein unfolding and aggregation. As described in this application note, this information provides wide applicability for checking protein quality quickly. With just minutes of time and 9 μL of sample, Uncle can give you the peace of mind to confidently move forward with your biologic.

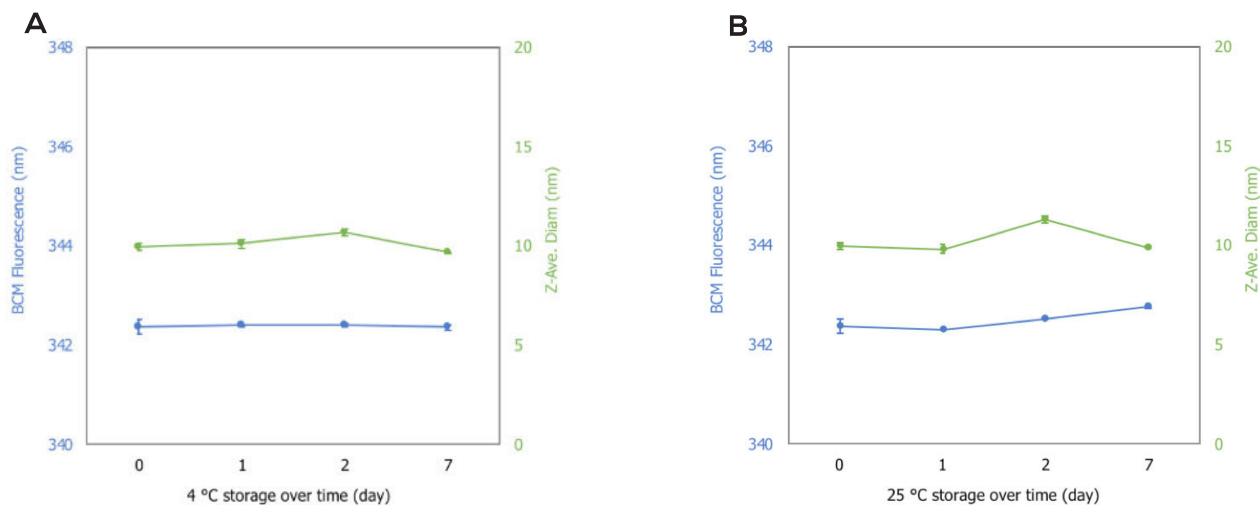

Figure 5: The BCM fluorescence intensity and DLS of mAb2 in 20 mM sodium acetate buffer, pH 5.0, monitored for two storage conditions, 4°C and 25°C, over 7 days. Error bars denote the standard deviation of duplicate measurements.

Application	Measurements	Samples	Time	Samples/min
Isothermal	Fluorescence and SLS	48	7.5 min*	6
Sizing & Polydispersity	DLS	48	23 min	2

Table 1: Capture the quality of your biologic with a snapshot of fluorescence, SLS and DLS measurements for the same set of samples. *Estimated time on Uncle Client for 2 repeat measurements.

References

1. *State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. Biopharmaceutical Characterization: The NISTmAb Case Study.* Ed. John E. Schiel, Darryl L. Davis, Oleg V. Borisov. Vol. 1201. American Chemical Society: 2015.

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