APPLICATION NOTE

AN5008: High-throughput freeze-thaw stability studies with the DynaPro® Plate Reader

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Summary

Freeze-thaw stability studies are key to the development of a wide range of products. We present a method for measuring freeze-thaw effects in a single multi-well plate using the DynaPro® Plate Reader. Simultaneous static and dynamic light scattering measurements in the Plate Reader provide direct quantitation of size and molar mass across each freeze-thaw cycle, allowing researchers to assess the onset of unfolding, aggregation, or other changes in structure and stability. This nondestructive approach eliminates the need for multiple aliquots and ensures that the same sample is evaluated after each cycle. The measurements are rapid, robust, inexpensive and easy to perform, making the DynaPro Plate Reader an excellent choice for streamlining freeze-thaw tests.

Introduction

Perishable products, including biopharmaceuticals, are often stored or shipped at sub-freezing temperatures to prevent degradation and extend their shelf life. Freezing and thawing a substance may, however, compromise its desired physical properties by inducing concentration gradients, cold denaturation or mechanical stress that can lead to aggregation, decreased potency or other undesirable effects. Consequently, freeze-thaw stability studies are a requirement for the successful development, distribution and storage of a wide range of products from cosmetics and foods[1] to fine chemicals, pharmaceuticals, and vaccines.[2,3] Nevertheless, while evaluating freeze-thaw stability can be a requirement for bringing a robust product to market, doing so can be labor-intensive, cumbersome and expensive, especially when samples must be pipetted from the plate to other containers such as microcuvettes for analysis.

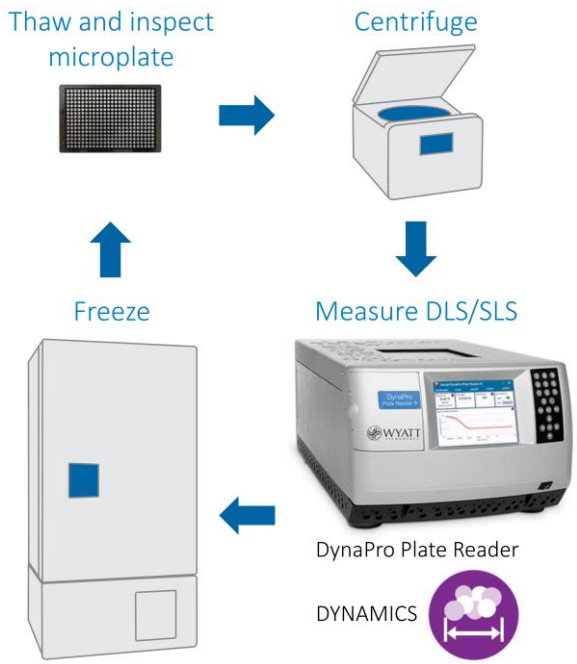

Figure 1. Measuring freeze-thaw stability directly in standard microwell plates by high-throughput DLS/SLS.

The DynaPro Plate Reader enables quantitation of freeze-thaw effects from start to finish in a single microwell plate (Figure 1). Its combined dynamic light scattering (DLS) and static light scattering (SLS) capabilities are ideally suited for quantifying sample stability. Specifically, DLS and SLS are used to monitor multiple stability indicators, several of which would be relevant to any given analyte, such as particle size (R_h), molar mass (M_w), aggregation propensity, intermolecular interactions (quantified as k_D and A_2) and particle concentration (e.g., the physical titer of lipid nanoparticles or viral vectors). The instrument uses minimal volume–just 30 µL per well –and the

samples remain in the same plate throughout the experiment. This method has minimal consumables costs, as low as 15 cents per sample.

In this application note, we demonstrate how the DynaPro Plate Reader can be used to perform freeze-thaw stability studies by high-throughput DLS/SLS. Freezing, thawing, and subsequent analyses are all performed in situ. Consequently, no liquid handling is needed after the initial plate loading — maximizing throughput and avoiding cross-contamination. The method measures the same samples over the entire course of the assay, as opposed to measurements of different aliquots from each freeze-thaw cycle.

Materials and Methods

Samples and instrumentation

Bovine serum albumin (Fisher Scientific) was prepared at 2 mg/mL in phosphate buffered saline (PBS) at pH 7.4, and carbonic anhydrase (Sigma-Aldrich) was prepared at 4 mg/mL in the same PBS. All samples and buffer blanks were filtered to 0.02 µm using Whatman Anotop syringe-tip filters (Cytiva) and loaded into a 384-well microplate (Aurora Microplates). 30 µL of each sample were loaded per well, with 25 replicates per protein.

To enable SLS measurements of molar mass, the plate was calibrated with lot-certified 40 kDa dextran (Wyatt Technology) prepared at concentrations of 2 mg/mL to 10 mg/mL in ddH_2O. The plate was then sealed with polyolefin sealing tape (Thermo Fisher) and centrifuged for 1 min at 1000 ×*g* to remove any bubbles. The sealing tape remained in place for all measurements and throughout all freeze-thaw cycles.

Freeze-thaw stability measurements

All measurements were performed with a DynaPro Plate Reader at 25 °C using DYNAMICS® software. An initial measurement (0^{th} cycle) was performed before the first incubation in the freezer. Prior to freezing, the plate was sealed in a zip-top bag to reduce condensation on the exterior of the plate.

For each freeze-thaw cycle, the plate was loaded in an ultra-low temperature freezer (Fisher Scientific) and frozen at -80 °C for a minimum of 16 hours. The plate was then thawed for at least 30 minutes at 37 °C to accelerate thawing without removing it from the plastic bag. The plate bottom was cleaned with filtered compressed air to remove dust prior to loading the plate into the instrument. Following centrifugation for 1 min at 1000 ×*g,* the plate bottom was again cleaned with filtered compressed air and loaded into the DynaPro Plate Reader for the next round of measurements. Measurements comprised autocorrelation functions, hydrodynamic radius (R_h), polydispersity index (PDI), size distributions and weight-average molar mass (M_w). This procedure was repeated for a total of six freeze-thaw cycles.

Results and Discussion

To demonstrate the method, we use two well-characterized proteins: bovine serum albumin (BSA), and carbonic anhydrase from bovine erythrocytes (CA). Both proteins are known to be predominantly monomeric under our starting conditions, with hydrodynamic radii of 3.5 nm and 2.7 nm[4] and molar masses of 66.4 kDa and 30 kDa,[4] respectively.

Monitoring the two proteins across six freeze-thaw cycles, we find that BSA is stable and unaffected by the freeze-thaw process while CA is unstable and undergoes aggregation.

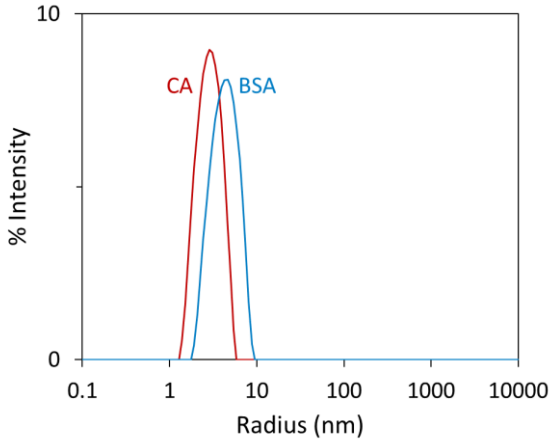

Figure 2. Size distributions of the BSA (blue) and CA (red) samples prior to freezing.

Sample characterization prior to freezing

As freshly prepared samples, both BSA and CA exist predominantly as monomers. Specifically, R_h values by DLS are 3.96 ± 0.09 nm for BSA and 2.70 ± 0.04 nm for CA, and the size distribution is monomodal, i.e., contains only a single peak (Figure 2). The PDI of 0.12 ± 0.03 for BSA

and 0.10 ± 0.06 for CA indicate that both samples are quite monodisperse.

M_w values measured by SLS are also consistent with mostly monomeric protein at 71.3 ± 5.5 kDa for BSA, and 26.6 ± 1.3 kDa for CA. M_w of BSA is somewhat greater than expected for monomeric BSA, as a small population of dimers, trimers, and higher order oligomers are present under the experimental conditions.

BSA: Stable to freeze-thaw stress

Freezing and thawing do not cause BSA to aggregate. The autocorrelation function (ACF) remains unchanged after successive freeze-thaw cycles, supporting the conclusion that the sample is unchanged by the process (Figure 3, top).

Analysis of the DLS data shows neither an increase in the mean hydrodynamic radius or polydispersity (an increase is indicative of oligomerization) nor the generation of large aggregates that could be identified in a size distribution obtained by regularization analysis (Figure 3, bottom). At the end of the sixth freeze-thaw cycle, R_h = 3.85 ± 0.06 nm, which is within 3% of the radius measured before freezing (Figure 4). M_w also remains effectively unchanged at 77.4 ± 3.9 kDa.

Taken together, these results indicate that BSA is a stable protein that can withstand repeated freezing and thawing without changing its size distribution or molar mass.

CA: Unstable to freeze-thaw stress

In contrast to BSA, CA aggregates over the course of the experiment, providing an example of an unstable sample. Prior to freezing, CA is predominantly monomeric with no large aggregates present in the solution (Figure 2). Already in the first freeze-thaw cycle, however, we can detect signs of aggregation in the CA samples. A small amount of large species are found to form in 20 of the 25 replicates, introducing additional decays in the ACF (Figure 5, top). Their PDI increases 4-fold to 0.41 ± 0.19, indicating that the samples have become more polydisperse. M_w also increases by more than 20% in the first freeze-thaw cycle to 32.3 ± 4.7 kDa, with its relative standard deviation increasing from 4.8 % to 14.5 %. These results are all telltale signs of aggregation, reflecting that the heterogeneity of the CA particle size distribution increases within and between replicates upon freeze-thaw in this formulation.

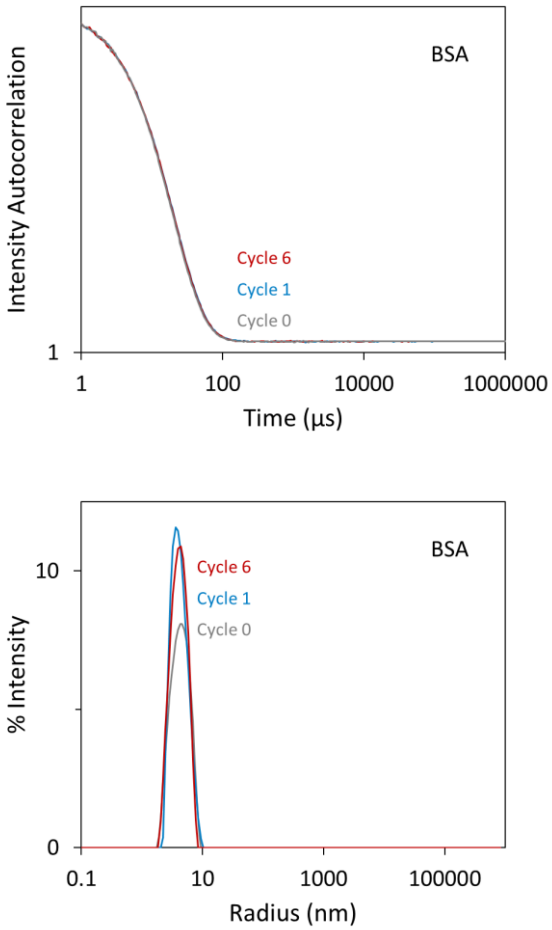

Figure 3. Comparison of BSA autocorrelation functions (top) and their corresponding size distributions (bottom). Before freezing (cycle 0, grey); after freeze-thaw cycle 1 (blue); and after the sixth, final freeze-thaw cycle (red). BSA remains predominantly monomeric and no aggregates form during the freeze-thaw process.

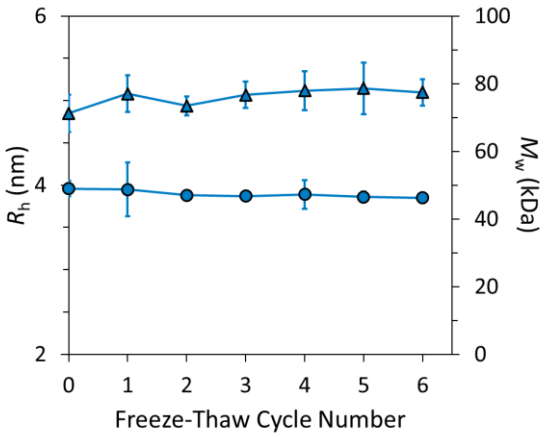

Figure 4. Freeze-thaw results for a stable sample, BSA. R_h (circles) and M_w (triangles) do not change significantly over six freeze-thaw cycles. The error bars represent the standard deviation of 25 replicate samples.

Over the course of the six cycles, all 25 CA replicates become multimodal mixtures of monomers and multimeric aggregates. In cycle 1, for instance, regularization analysis reveals that aggregates in the 10-500 nm size range have formed in 75 % of the replicates, with an average R_h of 244 ± 135 nm and contributing to (8.7 ± 6.0) % of the measured intensity (representative data in Figure 5, bottom). After six cycles, 90 % of the replicates contain aggregates in the 10-500 nm size range, accounting for (23.9 ± 14.0) % intensity. All except one replicate also contain a subpopulation of even larger aggregates that are > 1000 nm in size in cycle 1, accounting for (16.8 ± 13.7) % of the measured intensity. In cycle 6, the intensity contribution from this population is (21.7 ± 15.6) %. M_w appears to increase further over the subsequent freeze-thaw cycles to (49.1 ± 10.0) kDa at the experiment endpoint, suggesting that the molar mass or concentration of large species in the samples has increased in the additional five cycles (Figure 6).

We can also use the regularization analysis to examine how the relative intensity of the initially predominant species changes upon freeze-thaw (Figure 7). In addition to relative intensity, DYNAMICS software can also estimate the mass and number percentages of each of the populations, including the larger aggregates.

For example, reviewing the corresponding mass percentages for the CA results in Figure 7, we find that, while the aggregates are very large in size, they account for much less than 1% of the mass. This result illustrates how DLS is an excellent tool to detect even early stages of aggregation, where the aggregate amounts are minute.

We also note that in SEC-MALS analysis the largest aggregates may be filtered out or disrupted due to shear by the column. Hence, batch DLS measurements nicely complement aggregate analysis by SEC-MALS.

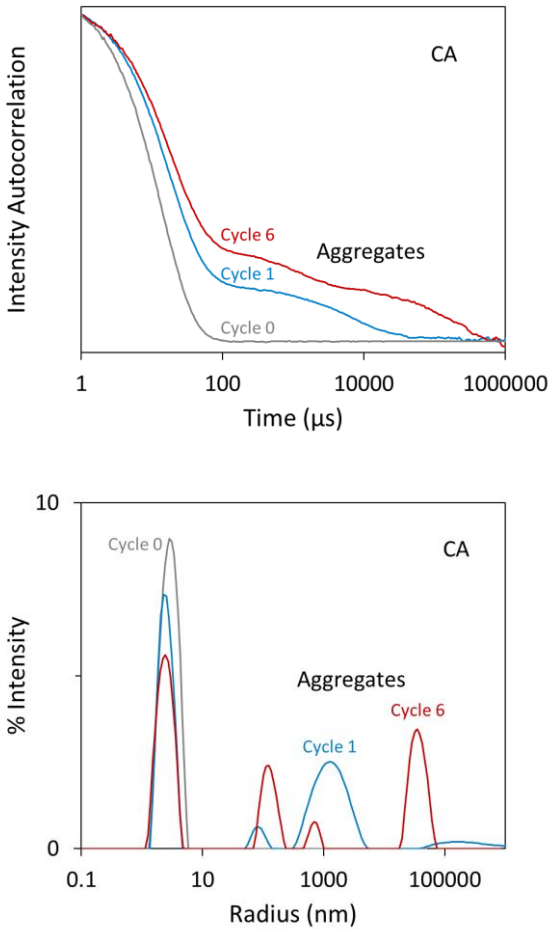

Figure 5. CA stability is compromised after the first freeze-thaw step, as shown in the autocorrelation function (top) and size distribution (bottom). Large aggregates form after a single freeze-thaw cycle (blue). The aggregation is even more pronounced in the final cycle (cycle 6; red).

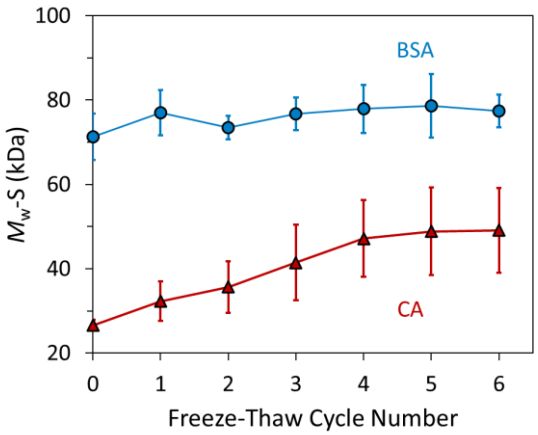

Figure 6. Differentiating between stable and unstable samples by monitoring molar mass. Shown are the average and standard deviation of 25 replicates. BSA (blue) shows no significant change in M_w across six freeze-thaw cycles. The apparent molecular weight of CA (red) starts to increase due to the formation of aggregates beginning with the first freeze-thaw cycle.

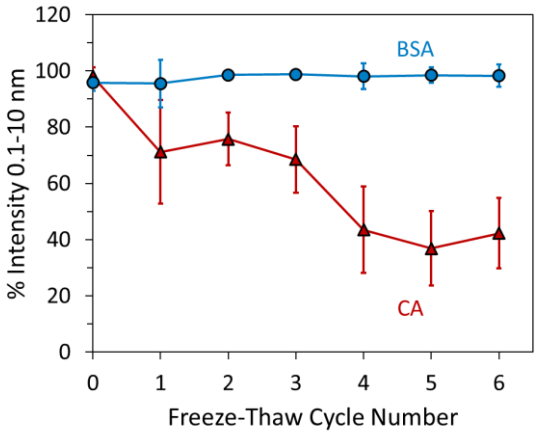

Figure 7. Monitoring the progressive aggregation via the relative light scattering intensity of the sub-10-nm species. This relative contribution decreases as aggregates form in the CA samples, but remains unchanged for the stable BSA samples. The error bars represent the standard deviation of 25 replicate samples.

Conclusions

Here we have demonstrated the benefits of using the DynaPro Plate Reader for high-throughput freeze-thaw stability studies, evaluating the stability of two common proteins, BSA and CA. Repeatedly freezing, thawing, and measuring the samples directly in a standard microwell plate, we find that while BSA is unaffected by the freeze-thaw process, CA aggregates in the first cycle and continues to do so in subsequent cycles. The measurements take less than a minute per sample and the only hands-on time required after loading the plate is for moving the plate between the freezer, centrifuge, and Plate Reader.

While we employed proteins in this proof-of-principle study, the freeze-thaw workflow described here can be used for a broad range of samples. Specifically, the method permits quantification of particles between 0.5 nm to 1000 nm, including AAVs,[5] other viral vectors and lipid nanoparticles. Conveniently, the plates used in this study can be disinfected and sealed inside a hood to prevent exposure to pathogens and are compatible with temperatures down to -80 °C. As the measurements are nondestructive, orthogonal analyses can be performed on the same samples—the performance of the microplate and sealing tape is not compromised by the freeze-thaw procedure.

Data analysis also requires minimal hands-on time. DYNAMICS software provides customizable templates for sample definitions and data analysis, automated data quality indicators providing actionable advice, and numerous quick and easy ways to discriminate between stable and unstable samples. The DynaPro Plate Reader is the only instrument to offer this functionality in combination with precise temperature control (4-85 °C) and automation, making it a uniquely capable tool for faster, more cost-effective freeze-thaw stability measurements.

Acknowledgements

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References

1. Rayner, M. et al. Biomass-based Particles for the Formulation of Pickering Type Emulsions in Food and Topical Applications. *Colloids Surf. A: Physicochem. Eng. Asp.* **458**, 48-62 (2014).
2. Crommelin, D.J.A. et al. Addressing the Cold Reality of mRNA Vaccine Stability. *J. Pharm. Sci.* **110**, 997-1001 (2021).
3. Kahn, M.M. et al. Stability Challenges Not Addressed by Harmonized Guidance – AAPS Workshop of the Stability Focus Group, April 3rd- 4th, 2017 in Rockville, MD. *AAPS Open.* **4**, 2 (2018).
4. Krishnamurthy, V.M. et al. Carbonic Anhydrase as a Model for Biophysical and Physical-Organic Studies of Proteins and Protein–Ligand Binding. *Chem Rev.* **108**(3), 946-1051 (2008).

5. Bee, J.S. et al. Impact of Time Out of Intended Storage and Freeze-thaw Rates on the Stability of Adeno-associated Virus 8 and 9. *Pharm. Biotechnol.* **111**, 1346-1353 (2022).

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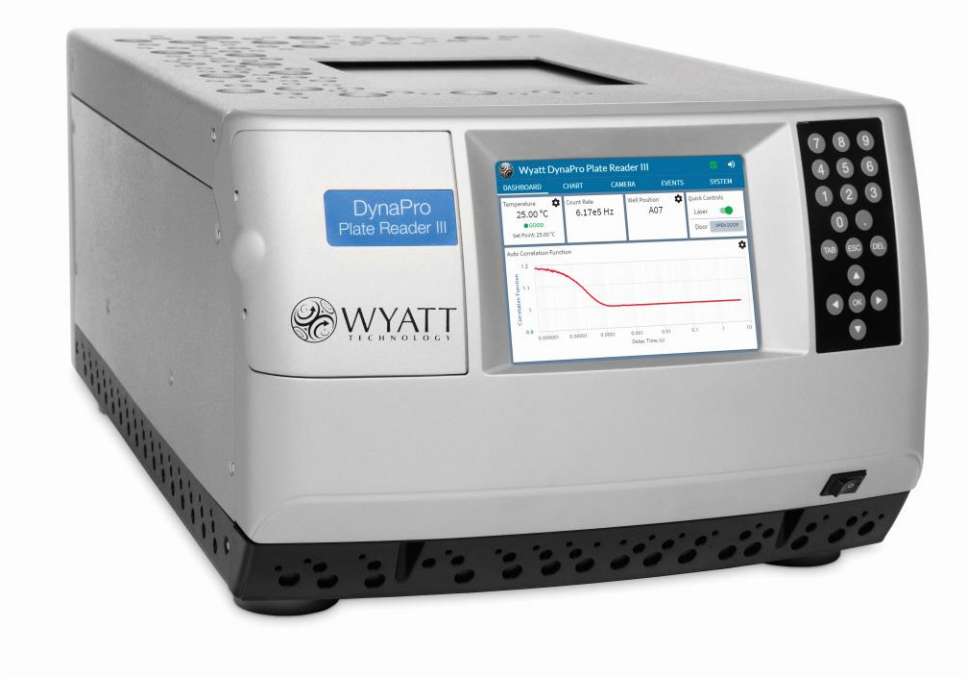

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