Protein Characterization Turned Up to 11

A Flexible Platform for Protein Stability Measurements

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There are many different tools, both old and new, for characterizing proteins. For ranking and comparing the stability of biologics and formulations, the most established tools rely on temperature stress to disrupt the 3D protein structure. These accelerated thermal ramp measurements are good surrogates for room-temperature stability and for extrapolating to downstream processes.

With trends in biopharmaceuticals moving toward high-concentration solutions, thorough characterization is more important than ever for reducing the risk of aggregation and precipitation of the final product. If biologics tend toward self-interaction, high viscosity of the concentrated protein solution is another risk. Determinations of stability and aggregation propensity earlier and on more candidate samples or formulations can save time later.

The UNcle platform from Unchained Labs combines three different detection modes on a single instrument. Fluorescence, static light scattering (SLS), and dynamic light scattering (DLS) are used to characterize protein stability. Temperature can be precisely controlled from 15–95°C, and low (9 µL) volumes of up to 48 samples can be run at once, so that many of the key stability measurements can be made earlier. This can also serve to streamline and simplify data collection and analysis.

Thermal Melting and Aggregation Onset
To determine the melting temperature of a protein, UNcle measures the intrinsic fluorescence of tryptophan and tyrosine residues as the protein undergoes conformational changes. In most cases there is a change in the fluorescence intensity or a shift in the peak as the protein unfolds due to thermal stress. In Figure 1, a monoclonal therapeutic antibody (mAb) was prepared at 0.5 mg/mL and run with a thermal ramp from 15°C to 90°C with a ramp rate of 0.3°C/minute. Two transitions were detected by the analysis software, with thermal melting points (T_m’s) at 67.8°C and 76.5°C.

Aggregation is often linked to unfolding of proteins. Rather than performing separate experiments to detect aggregation in a different instrument, UNcle simultaneously measures SLS at two different wavelengths during the same thermal ramp. An increase in the signal at 266 nm is usually picked up first, as it should be more sensitive to changes in scattering intensity and discerns smaller particles. Scattering intensity measurements at 473 nm are useful for detection of larger aggregates, and for differentiating samples when the intensity has saturated at the lower wavelength. In this case the mAb showed a sudden onset of aggregation (T_agg) at 78.2°C, very soon after the second melting transition.

Obtaining both of these measurements at the same time makes it easier to rank constructs or formulations, or find excipients that prevent or delay the onset of aggregation.

**Sizing and Polydispersity Adds More**
In addition to the $T_m$ and $T_{agg}$ values, DLS measurements can provide valuable information about the size and polydispersity of the molecules. Without adding any additional sample or transfer steps, these measurements can be collected on UNcle at the beginning and end of a thermal ramp. This data can serve as an assessment of sample quality, and to identify molecules that are not monodisperse before they even undergo thermal stress. Alternatively, DLS can provide more clarification for samples that display unexpected behaviors in thermal ramp measurements.

In Figure 2, the size and polydispersity of four different mAbs were measured on UNcle at 0.5 mg/mL. After storage at 4°C, three of the molecules, mAb 1, 2, and 4, yielded measurements of the expected size, with a diameter of 10–11 nm. Their polydispersity index (PDI) values were also below 0.25, which indicates a monodisperse population. In contrast, mAb 3 was measured to have a diameter of 175 nm and a PDI of almost 1.4, suggesting significant aggregate content. Obtaining this information early allows a researcher to eliminate this particular formulation for this mAb from further consideration.

### Simultaneous kD and B22 Measurements

After ranking and refining samples to a set of highly stable molecules or formulations with measurements like those described above, an additional step is to reduce the risk of candidate aggregation under high-concentration conditions. Weak, nonspecific interactions play important roles in solubility and aggregation in a protein solution. The diffusion interaction parameter ($k_D$) and the second virial coefficient ($B_{22}$) are both used for predicting the colloidal stability and aggregation propensity of proteins. These measurements may be used to confirm stability and minimize the risk of the biologic interacting with itself.
Negative $k_D$ and $B_{22}$ values are interpreted to mean that the protein favors self-association over complete solvation. Positive values indicate repulsive interactions between protein molecules, or more favorable protein-solvent interactions. Zero or near-zero values indicate neutral interactions. Both parameters are obtained simultaneously on UNcle with one sample set. Each experiment involves preparing a sample (a specific protein in a given formulation) in a concentration series, covering a range of concentrations spanning a factor of 3–5, with a maximum concentration of 25 mg/mL. For $k_D$ values, UNcle software uses the hydrodynamic radius measured by DLS for each sample across the concentration series to calculate diffusion coefficients. To obtain the $B_{22}$ values, the derived light scattering intensity of each sample is calibrated by the scattering intensity of a known intensity reference.

In the example shown in Figure 3, lysozyme was formulated in a buffer with either 100 mM NaCl or 400 mM NaCl. The solution in 400 mM NaCl became visibly cloudy when stored at 4°C. The cloudy appearance was reversible; the solution clarified upon warming or dialysis into a lower salt buffer. As expected, lysozyme exhibited a negative $k_D$ value of $-6.0$ mL/g in the 400 mM NaCl solution, and a positive $k_D$ value of $3.8$ mL/g in the 100 mM NaCl solution. The $B_{22}$ values (not shown) show the same trends, and were well-aligned with measurements made by others using different techniques.

Conclusions

UNcle combines three detection methods to enable more stability measurements on a single platform. This provides more flexibility by enabling combinations of measurements that can be performed simultaneously or in tandem. Some applications don't involve a temperature ramp, but rather hold a constant temperature for an incubation period, during which samples can be continuously monitored.
For example, isothermal stability experiments can be carried out for hours or even days, just below the melting temperature of the proteins, to allow for a detailed picture of unfolding and aggregation events. Similarly, chemical denaturation experiments can be performed with UNcle, where quantitative $\Delta G$ values are obtained for proteins at ambient temperatures. A DLS-based viscosity application allows for screening of 48 samples at once, which adds viscosity measurements to an earlier stage in the development process.

In all, eleven different applications can be performed with one instrument, greatly reducing laboratory space requirements and sample volume requirements. UNcle creates further efficiency by removing the need to maintain multiple single-measurement instruments and the necessity to train scientists on multiple instruments. These efficiencies provide researchers a leg up for quickly comparing constructs or formulations and selecting winning biologics.