Figure out aggregation early: simultaneous, independent measurements of $B_{22}$ and $k_D$ on Uncle

Introduction

With the trend in biopharmaceuticals to move towards high concentration therapeutics, developing a well-formulated biologic is more important than ever. After ranking and refining down to a set of highly stable molecules, or honing down to good formulations with measurements like $T_m$ and $T_{agg}$, the next critical step is reducing the risk of candidates aggregating at high concentrations. The diffusion interaction parameter ($k_D$) and the second virial coefficient ($B_{22}$) are both well-established parameters for predicting the colloidal stability and the aggregation propensity of proteins. These measurements may be used to confirm stability and minimize the risk of your molecule interacting with itself. With just minutes needed to collect the data, and experiments done at lower concentrations, do it earlier in your process and save time and sample.

Using the model protein lysozyme in the presence of high or low salt, this application note describes how the built-in $B_{22}$ and $k_D$ Uncle application makes independent and simultaneous measurements. These parameters require multiple protein concentrations to be tested for each buffer. Uncle is an ideal platform for this application, as it can measure up to 48 samples simultaneously, and has the ability to do both static light scattering (SLS) and dynamic light scattering (DLS) measurements. This ensures you can obtain both parameters efficiently with a minimal amount of protein, and find conditions that minimize aggregation of your high-concentration biologics down the road.

Uncle is an all-in-one stability platform that enables 12 different applications with one instrument. Fluorescence, SLS and 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. And since you can run low volumes of 48 samples at a time, you can thoroughly characterize more biologics and formulations earlier than before.

Methods

Lysozyme was prepared at 25 mg/mL in 10 mM sodium acetate buffer, pH 5.2 with either 100 or 400 mM NaCl. Samples were filtered through a 0.2 µm syringe filter. Dilutions were made in each buffer to 20, 15, 10 and 5 mg/mL, and samples were centrifuged in a bench top centrifuge for 4 minutes at 14,000 rpm. Nine µL of each dilution were loaded in triplicate in a Uni, with one well containing each buffer alone for blank subtraction. The $k_D/B_{22}$ application was run on Uncle with four DLS acquisitions of 5 seconds each.

Uncle Analysis Software uses the hydrodynamic diameter measured for each sample, in conjunction with solvent information, to calculate the diffusion coefficients. The diffusion coefficients as a function of protein concentration are used to calculate $k_D$. The following table shows the calculated $k_D$ values for each condition:

<table>
<thead>
<tr>
<th>Formulation</th>
<th>$k_D$ (mL/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM NaCl</td>
<td>3.8</td>
</tr>
<tr>
<td>400 mM NaCl</td>
<td>-6.0</td>
</tr>
</tbody>
</table>

Figure 1: Diffusion coefficient as a function of lysozyme concentration at two different salt concentrations, and the calculated $k_D$ values for each condition.
To measure $B_{22}$, toluene is used as a reference intensity standard. HPLC-grade toluene was loaded in eight Uni wells, and the average scattering intensity from the DLS acquisition was used. Uncle Analysis Software used the blank-subtracted sample light scattering intensity in conjunction with the reference intensity to calculate $R_\theta$ values as a function of protein concentration, and then calculated $B_{22}$. Protein concentrations were not independently verified which may result in a small systematic error in these specific $k_D$ and $B_{22}$ results.

**Results**

Weak, non-specific interactions play important roles in solubility and aggregation in a protein solution. Both $k_D$ and $B_{22}$ measure these types of interactions to help identify formulations that prevent aggregation. Negative $k_D$ and $B_{22}$ values are interpreted to mean that the protein favors self-association over complete solvation. Positive $k_D$ and $B_{22}$ values indicate repulsive interactions between protein molecules, or more favorable protein-solvent interactions. Zero or near-zero $k_D$ and $B_{22}$ values indicate neutral interactions. The magnitude of the positive and negative values indicates relative attractive or repulsive interactions.

The $k_D$ and $B_{22}$ parameters are obtained simultaneously on Uncle with one sample set and two independent measurement values. Researchers are provided both parameters without any additional experimental time or sample, but can choose to follow only one parameter if preferred. Each experiment involves preparing a sample (a specific protein in a given formulation) in a concentration series and generating a Debye plot. For obtaining linear fits, we advise using a maximum concentration no higher than 25 mg/mL of protein, and to cover a range of concentrations spanning a factor of 3–5. To obtain the $k_D$ value, the hydrodynamic diameter of each sample is measured by Uncle and converted to a diffusion coefficient. To obtain the $B_{22}$ values, the derived light scattering intensity of each sample (corrected with the appropriate laser attenuation factor) is calibrated by the scattering intensity of toluene, a known intensity standard.

Lysozyme solutions become cloudy if formulated in a high NaCl buffer and stored at 4 °C. This cloudy appearance is reversible: the solution clarifies upon warming or dialysis into a buffer with less NaCl. The clarification implies that irreversible protein denaturation did not occur, but is instead a native state attractive association. As expected, lysozyme exhibited a negative $k_D$ value in a 400 mM NaCl solution, and a positive $k_D$ value in a 100 mM NaCl solution (Figure 1). $B_{22}$ values followed the same trend, and are well-aligned with measurements made by others using different techniques (Figure 2).

The results obtained from Uncle are consistent with literature values (Table 1). The positive/negative interactions are consistent and the magnitude of the measurements are similar.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>$k_D$ (Uncle)</th>
<th>$k_D$ (published)</th>
<th>$B_{22}$ (Uncle)</th>
<th>$B_{22}$ (published)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM NaCl</td>
<td>3.8 mL/g</td>
<td>4.2 mL/g</td>
<td>1.7E-4 mol mL/g^2</td>
<td>3.9E-4 mol mL/g^2</td>
</tr>
<tr>
<td>400 mM NaCl</td>
<td>-6.0 mL/g</td>
<td>-5.7 mL/g</td>
<td>-1.6E-4 mol mL/g^2</td>
<td>-2.0E-4 mol mL/g^2</td>
</tr>
</tbody>
</table>

Table 1: Comparison of Uncle $k_D$ and $B_{22}$ results with literature values.
Conclusion

In this single experiment performed on Uncle, two different parameters, $k_D$ and $B_{22}$, were measured for lysozyme in high and low salt. Following previously reported results and matching visually observable changes in the protein solution, the presence of high salt results in protein aggregation, and negative values for both $k_D$ and $B_{22}$ were obtained. Conversely, a lower salt buffer prevented aggregation and yielded positive values for both parameters. This experiment with built-in analysis in Uncle thus provides an easy and quick tool for selecting winning constructs or formulations that reduce protein aggregation risk.

References