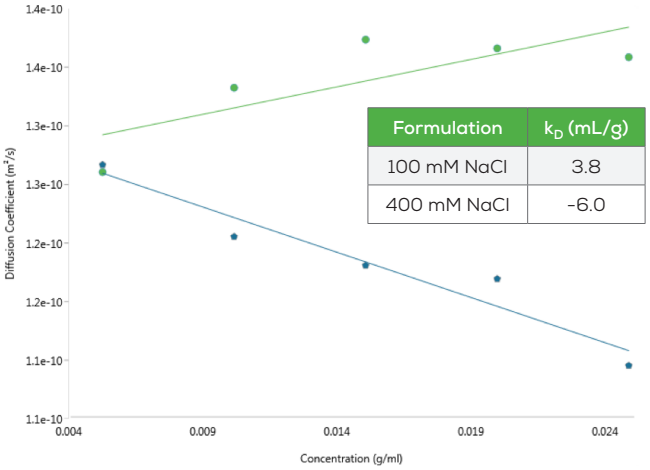
Figure out aggregation early: simultaneous, independent measurements of B_{22} and k_D on Uncle

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.



Formulation	k_D (mL/g)
100 mM NaCl	3.8
400 mM NaCl	-6.0

Figure 1: Diffusion coefficient as a function of lysozyme concentration at two different salt concentrations, and the calculated k_D values for each condition.

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.

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_θ 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.

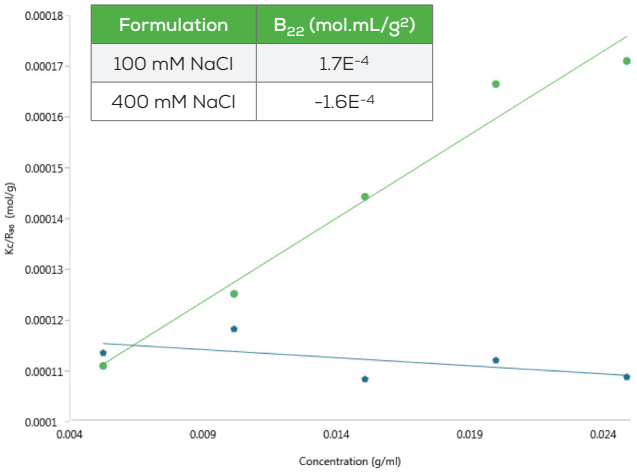


Figure 2: Scattering intensity as a function of lysozyme concentration at two different salt concentrations, and the calculated B_{22} values for each condition.

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.

Formulation	k_D (Uncle)	k_D (published)	B_{22} (Uncle)	B_{22} (published)
100 mM NaCl	3.8 mL/g	4.2 mL/g	1.7E^{-4} mol.mL/g^2	3.9E^{-4} mol.mL/g^2
400 mM NaCl	-6.0 mL/g	-5.7 mL/g	-1.6E^{-4} mol.mL/g^2	-2.0E^{-4} mol.mL/g^2

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

1. Shi S, et al. Method qualification and application of diffusion interaction parameter and virial coefficient. International *Journal of Biological Macromolecules* 62(2013):487–493.

Unchained Labs
6870 Koll Center Parkway
Pleasanton, CA 94566
Phone: 1.925.587.9800
Toll-free: 1.800.815.6384
Email: info@unchainedlabs.com

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