Spot aggregation early with B_{22} and k_D on Stunner

Introduction

Biopharmaceuticals are trending towards high-concentration therapeutics, but with higher concentrations also comes an increased risk of protein aggregation. The right formulation can reduce that risk, making high-throughput screening of biologics formulations more vital than ever.

The diffusion interaction parameter (k_D) and the second virial coefficient (B_{22}) are well-established parameters for predicting the colloidal stability and the aggregation propensity of proteins. These parameters can be determined independently of each other using Dynamic Light Scattering (DLS) and Static Light Scattering (SLS). Stunner's one-of-a-kind combination of protein quantification and light scattering makes measuring k_D and B_{22} fast and easy.

Stunner takes your protein characterization to the next level by combining high-speed UV/Vis spectral analysis with DLS. Using micro-volume Stunner plates, Stunner measures both the concentration and quality of 96 samples in just 1 hour. The Stunner plate's microfluidic circuit uses just 2 µL of sample with no risk of contamination or evaporation. The dual fixed path length, cuvette-based absorbance read-out means you can measure highly concentrated samples without introducing error by making dilutions. Pipet your samples right into the Stunner plate or hook it up to your favorite automation system if you need higher throughput. Tack on 21 CFR Part 11 compliance if you need it. You choose how to sync up measuring your sample quantity and quality.

This application note describes how you can compare the aggregation propensity of different protein formulations with the B_{22} and k_D application. You can even take it a step further and measure the viscosity of your buffers on Stunner with a custom application in the flexible Homebrew toolkit.

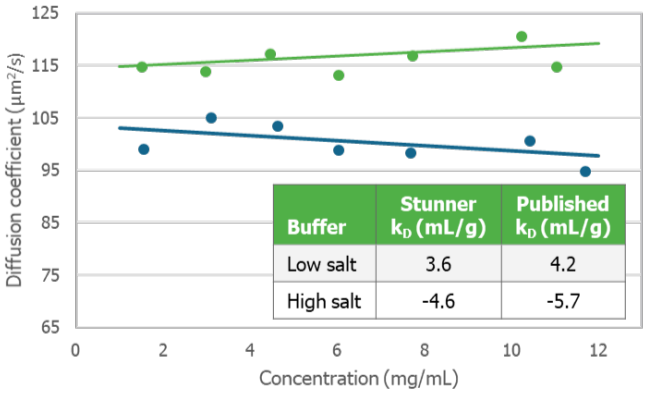
Figure 1: Stunner: a unique combination of simultaneous protein quantification and sizing.

Methods

Hen egg-white lysozyme (HEWL) was diluted into 10 mM acetate buffer, pH 4.6 with either 100 or 400 mM NaCl at concentrations from 1–11 mg/mL. NIST monoclonal antibody (NIST mAb) reference material, RM 8671, was diluted into 25 mM His/His-HCl buffer, pH 6 to concentrations between 0.25 and 6 mg/mL. All samples were filtered through a 0.1 µm filter and samples of HEWL were subsequently filtered through a 0.02 µm filter. 2 µL of each sample were loaded in triplicate, in the case of HEWL, or with 9 replicates, in the case of NIST mAb, in a Stunner plate. Then the B_{22} and k_D application was selected using 4 DLS acquisitions of 5 seconds each, with buffer alone serving as blanks for each dilution series. PEG40 was used as a reference standard for light scattering intensity.

30 nm NIST standard polystyrene beads were added to the buffers and water, and 2 µL of the bead-containing and the bead-free samples were loaded in quadruplicate in a Stunner plate. A custom Homebrew application was used to determine the viscosity of the buffers.

Stunner Analysis used the measured viscosity of the buffers to determine the diffusion coefficients of each sample. Stunner Analysis software uses the average scattering intensity of the reference standard along with the measured light scattering intensity from each sample to calculate the R_θ value for each protein concentration. The linear fits of the diffusion coefficients and R_θ plotted against the measured protein concentration at 280 nm are then used to calculate k_D and B_{22} values, respectively. Stunner Analysis uses an algorithm to identify outliers in both data sets and uses different weighting of the data points to achieve the best linear fit for the results.



Buffer	Stunner k_D (mL/g)	Published k_D (mL/g)
Low salt	3.6	4.2
High salt	-4.6	-5.7

Figure 2: Diffusion coefficient as a function of measured protein concentration of HEWL in low salt buffer (green) and high salt buffer (blue). The inset table shows the Stunner and published k_D values of each formulation.

Results

Predict aggregation with B_{22} & k_D

Weak, non-specific interactions between molecules are important factors in the solubility and aggregation propensity of proteins in solution. These interactions can be quantified independently with k_D and B_{22}. While DLS is often used to determine the size and polydispersity of protein particles in solution, it also measures the diffusion coefficient and the light scattering intensity of protein samples. Correlating these 2 values with protein concentration in a dilution series yields k_D and B_{22}, respectively.

Positive k_D values can be interpreted as repulsive interactions between protein molecules and typically indicate lower likelihood of aggregation. Negative k_D values indicate that the protein readily self-associates under the measured conditions and may aggregate. The magnitude of the value can be used as a relative measure of the strength of the interaction. Large magnitude k_D values indicate strong interactions while very small magnitude k_D values indicate neutral interactions.

Stunner determined a positive k_D value of HEWL in low salt conditions and a negative k_D value in high salt conditions (Figure 2). HEWL solutions in high salt buffer stored at 4 °C became cloudy but clarified when warmed to room temperature. These results indicate a native state attractive association between the protein molecules in high salt buffer, but net repulsive forces in low salt buffer. Sodium chloride tends to interact preferentially with the hydrophilic surface of proteins which minimizes the charge repulsion between protein molecules.[1] Results obtained by Stunner align well with measurements made by others using different techniques.[2]

The second virial coefficient (B_{22}), or self-interaction parameter, is a thermodynamic measure of protein self-associations in solution. Using the same samples in a single experiment, Stunner independently measures both B_{22} and k_D values along with exact protein concentrations to get more information about the aggregation propensity of your samples. A positive B_{22} value indicates net-repulsive forces between protein molecules and lower likelihood of aggregation, while a negative B_{22} value indicates net attractive interactions between proteins. A zero or near-zero B_{22} value indicates net neutral interactions.

Refining formulations is particularly important for therapeutic monoclonal antibodies to reduce the risk of aggregation at high concentrations. NIST mAb is a useful model of a well-controlled monoclonal antibody and can be used to verify analytical assessments. Stunner determined B_{22} and k_D values for NIST mAb in 25 mM His/His-HCl buffer, pH 6 (Figure 3). Both B_{22} and k_D for this optimized formulation of NIST mAb were positive and agreed well in magnitude to published values.[3]

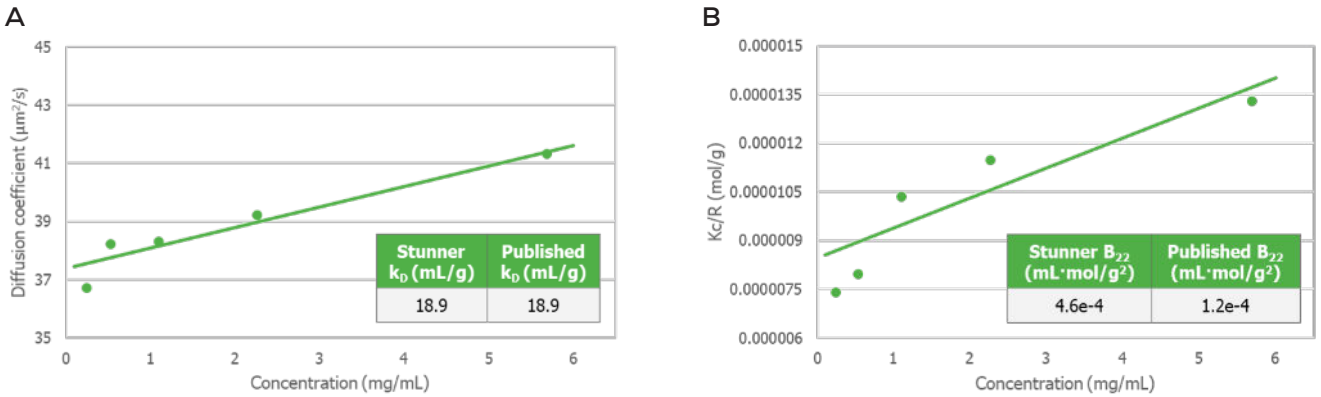


Figure 3: Diffusion coefficient (**A**) and scattering intensity (**B**) as a function of measured protein concentration of NIST mAb in 25 mM His/His-HCl, pH 6. The inset table shows the Stunner and published k_D and B_{22} values.

Measuring viscosity with Homebrew

The Homebrew toolkit provides the flexibility to create new applications with user-defined equations and customizable settings that incorporate wavelength absorbances, sizing and polydispersity results, diffusion coefficient results, and more.

DLS measures the changes in light scattering over time to quantify the motion of particles in a solution. If the viscosity and refractive index of the solution is known, this measurement can be used to determine the diffusion coefficient, size, and polydispersity of the particles. If, on the other hand, the particle is of a standard size, the same methods can be used to determine the viscosity of an unknown solution.

The viscosities of 100 mM NaCl HEWL buffer, 400 mM NaCl HEWL buffer, and NIST mAb buffer were measured by adding 30 nm NIST standard beads to the buffers and using a Homebrew application in Stunner (Table 1). Increasing the concentration of sodium chloride increased the viscosity of the HEWL buffer. All three buffers had higher viscosities than pure water (1.00 cP at 20 °C). These values were entered into the Stunner software when setting up the k_D and B_{22} experiments described above (Figures 2 & 3), to yield more accurate results.

Advanced DLS Analysis

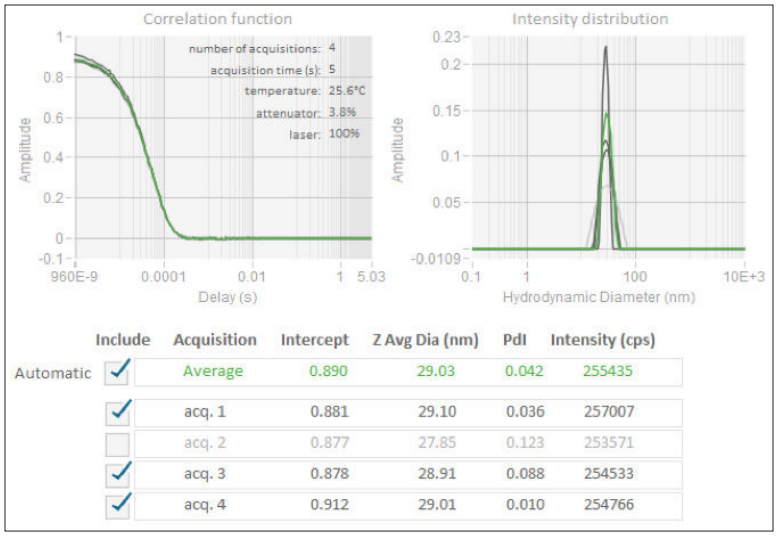
Get more details on individual DLS acquisitions with Stunner's Advanced DLS view (Figure 4). Example individual correlation functions and intensity distributions (grey lines) from polystyrene beads in water are overlaid with the average correlation function and intensity distribution (green lines). Correlation functions and intensity distributions of acquisitions included in the average are displayed in dark grey, while acquisitions excluded by Stunner's automatic outlier removal are shown in light grey. Manual outlier selection is also supported by the software, so users can choose which acquisitions to include. The intercept of the correlation functions, Z avg. diameter, polydispersity index, and scattering intensity are all displayed for each acquisition and the average at the bottom of the screen. This view allows you to examine all the DLS data Stunner acquires and gives confidence in your results while giving you more control over which data is used.

Buffer	Viscosity by Stunner (cP)
100 mM NaCl HEWL buffer	1.16
400 mM NaCl HEWL buffer	1.25
NIST mAb buffer	1.49

Table 1: Viscosities of buffers used for k_D/B_{22} experiments as measured by Stunner using NIST standard beads at 20 °C and a Homebrew application.

Conclusion

Stunner obtains two parameters, B_{22} and k_D independently and simultaneously from a single dilution series of a protein. Combined with the formidable capabilities of Homebrew, multiple formulation buffers or protein constructs can be



	Include	Acquisition	Intercept	Z Avg Dia (nm)	PdI	Intensity (cps)
Automatic	✓	Average	0.890	29.03	0.042	255435
	✓	acq. 1	0.881	29.10	0.036	257007
		acq. 2	0.877	27.85	0.123	253571
	✓	acq. 3	0.878	28.91	0.088	254533
	✓	acq. 4	0.912	29.01	0.010	254766

Figure 4: Stunner Analysis software gets you detailed views of every DLS acquisition for every sample and lets you choose between automatic or manual outlier removal.

assessed for their colloidal stability and aggregation risk on a single instrument. Stunner combines UV/Vis spectral analysis and DLS for quantifying concentration and stability measurements on one platform. With B_{22} and k_D values that agree closely with published values for multiple proteins, you can have confidence in the accuracy of Stunner's results. Unique to Stunner is its capability to measure protein concentration and track particle quality in a high-throughput, dilution-free and reproducible manner, using just 2 µL of sample.

References

1. Protein-excipient interactions: mechanisms and biophysical characterization applied to protein formulation development, DB Volkin, et al., Advanced Drug Delivery Reviews, 2011; 63:1118–1159.

2. Diffusion and sedimentation interaction parameters for measuring the second virial coefficient and their utility as predictors of protein aggregation. A Saluja, et al., Biophysical Journal, 2010; 99(8):2657–65.

3. NISTmAb common technical document case study, J Schiel, et al., presented in Workshop at the 6th International Symposium on Higher Order Structure of Protein Therapeutics (HOS 2017).

UNCHAINED LABS

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