

## Webinar Q&A Report

# Using Chemical Denaturants and Light Scattering to Determine Aggregation Propensity of Biopharmaceuticals

### **What concentration range do you recommend for measuring protein-protein interactions, and over how many individual concentrations?**

At least 8 measurements, covering 1 to 20 mg/mL, is a good starting set. You may experience non-linear effects below 1 mg/mL and above 15 mg/mL, but within this range the dependence of diffusion coefficient or light scattering intensity on concentration should be mostly linear.

### **Is $k_D$ as reliable as $A_2$ for assessing protein-protein interactions?**

Yes, so long as corrections are made where necessary. As shown during the presentation,  $k_D$  is highly susceptible to density fluctuations/clusters arising from small-molecule co-solutes which need to be accounted for to ensure your measurements are representative. It also a good idea to periodically compare  $k_D$  measurements with the corresponding  $A_2$  measurements from time to time to verify that they track as expected.

### **Can you measure protein-protein interactions at high protein concentrations, for example 50 to 100 mg/mL?**

By definition,  $A_2$  and  $k_D$  are first-order effects and measurements should only made in the dilute regime. Due to the increasing effect of hydrodynamics forces, diffusion coefficient measurements at higher protein concentrations should not be interpreted in terms of protein-protein interactions (PPI) since the crowding effect inhibits their natural diffusion rate. On the other hand, SLS measurement can be made and interpreted in terms of PPI at high protein concentrations. In addition to first-order  $A_2$  interactions, higher-order effects also come into play. Assessments of the structure factor  $S_q$  and Kirkwood-Buff integral  $G_{22}$  will be required and are often more significant than  $A_2$ .

You can make these measurements manually in the DynaPro NanoStar with just a few  $\mu\text{L}$  per measurement. If the viscosity is not too high (a few cP) the measurements can also be made using Calypso and a DAWN MALS instrument in an automated fashion, but with higher sample volumes.

### **What is the sample volume required when using the Calypso instrument?**

For a typical  $A_2$  measurement, 3-4 mL of solution are required at the maximum concentration. More complex measurements may require additional material.

**Is it safe to use a SEC-MALS system and column with denaturant in the mobile phase?**

Yes, if you take some precautions. At the higher denaturant concentrations, you should mind the increase in viscosity and you may need to slow down the flow rate. After running the experiment, it is important to flush with copious amounts of fresh ultrapure water to remove all the salts. You may need to change the purge frit and inline filter a little more often (every couple of weeks rather than every 3 months) and it is wise to use a vial needle wash in water as well as injecting a few water samples at the end of the run to keep the needle clean. It is also good practice to back flush a SEC column every so often to increase its longevity.

**Where do you see incorporating the use of denaturants for stability assessment during formulation development?**

This study provides evidence that denaturants can be used to differentiate aggregation propensity for candidate molecules. The denaturant method, carried out either in a DynaPro NanoStar or more productively in a DynaPro Plate reader, could then be used alongside measurements of the thermal onset of aggregation to narrow down candidate selection before formulation development takes place.

**These protein-interaction measurements can also be done in the DynaPro Plate Reader, correct?**

Yes, on the DynaPro Plate Reader III both  $k_D$  (from DLS) and  $A_2$  (from SLS) can be measured.

**How do error bars differ for  $A_2$  and  $k_D$  for the Calypso vs a cuvette-based method?**

A Calypso system offers several benefits relative to cuvette-based methods: it provides highly accurate dilutions that are not subject to pipetting errors, it determines the protein concentration directly, and it makes the measurements in a stable flow cell. For  $k_D$  measurements, flow cell environment has fewer benefits relative to a cuvette than for  $A_2$  measurements. Hence the primary impact on  $k_D$  uncertainty arises from the absence of manual pipetting, so if you are an accurate and undistracted pipettor then there may be no difference. For  $A_2$  measurements, the Calypso tends to give more accurate and precise results, owing to the robust optical system which is roughly 100x more sensitive than a cuvette for SLS.

**Can you explain once more how the SLS measurements at the different GuHCl concentrations tell you if the molecule would aggregate from the native or unfolded state?**

The SLS measurements were made on the DynaPro NanoStar at 20 g/L to indicate net repulsive or attractive interactions. These were compared to the unfolding behaviour under the same denaturant conditions. At the midpoint of unfolding, there would be 50% of the protein in the native state and 50% in the unfolded state. If you find you have a peak in net attractive interactions under denaturation which corresponds with the midpoint of unfolding, you could expect that the protein aggregates from the unfolded state. However, if you find that net attractive interactions occur well before the midpoint of

unfolding and then dissipate when approaching that midpoint, you could expect the protein to aggregate from the native state.

### **How can you extend this study for virus characterization - $A_2$ , $K_d$ , $\Gamma_{23}$ , $T_{agg}$ ?**

I do think there is merit in measuring  $\Gamma_{23}$  (the interaction between virus and co-solvent) or even  $B_{23}$  measurements (the interaction between the virus and another small protein) in order to understand which excipients or proteins are best suited to offer stability-enhancing properties.

For AAV's there are a number of common serotypes used, but I suspect that in-depth analysis of how excipients interact with the three capsid proteins, as individual units, has not been made and would be useful. For example, whilst one solvent condition may benefit one of the surface proteins, it may not be the case for all three.

I also believe there is not only scope for enhancing stability of the final drug product, but indeed downstream processes where yields are often suboptimal and different solvent conditions would benefit different processes. For example, there is place to study the interactions of the virus with excipients that could serve as cryoprotectants for freeze/thaw, "shock absorbers" that protect against shear stresses, or short-term-aggregation suppressors (whether protecting against ionic and/or hydrophobic effects) during high-concentration-filtration processes. The DynaPro Plate Reader would be especially valuable for these measurements since the plate can be removed, frozen and thawed or heated for a day or two, and then remeasured without touching the sample. Collaborations with academic groups might be beneficial to help understand the underpinning interactions/mechanisms.

### **How to decide the correlation time limits when analysing a DLS measurement to calculate $k_D$ , when the solution has co-solute clusters?**

DYNAMICS software allows you to set the cutoff limits between which the autocorrelation curve is fit to determine size. If the solution has co-solute clusters, first measure the co-solvent alone and determine in DYNAMICS the point where the autocorrelation decays to baseline. Then apply this point as the lower cutoff limit when analysing the protein sample. Suitable lower cutoffs I have found were 15  $\mu$ s for Arg HCl, 7.5  $\mu$ s for guanidine HCl, and about 10  $\mu$ s for urea.

### **What is the correct $dn/dc$ for highly glycosylated glycoproteins?**

For a heavily glycosylated protein, you need to use ASTRA's protein conjugate analysis which combines data from UV and RI to determine the amount of glycans and protein in the glycoconjugate and calculate the overall  $dn/dc$ . Input to this analysis includes the extinction coefficients of the pure protein (can be calculated from the sequence) and of the glycans (usually zero) and their respective  $dn/dc$  values. If you are a Wyatt customer, please check technical note TN1006.

## Contact Information

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