Live Webinar Q&A Sheet:

Using chemical denaturants and light scattering to determine aggregation propensity of biopharmaceuticals

The recorded webinar may be viewed from the Biotherapeutics webinars page. These questions were submitted by live viewers. Additional information on DynaPro® Plate Reader, DYNAMICS®, DAWN®, Calypso®, SEC-MALS and CG-MALS may be found in the Wyatt website Resources section under Application Notes, and Bibliography, as well as on the corresponding Product page and Solutions page of our web site.

Please contact info@wyatt.com with any additional questions.

Stability assessment

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

A: 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.

Q: *Do you think you could predict aggregation rate in the absence of denaturant? What would be your next steps?*

A: At present, no, since subtracting out the effects of the denaturants is quite difficult. Whilst denaturation increases the relative concentration of partially unfolded/ intermediate states, thus increasing non-native aggregation, it also attenuates the forces between these intermediate states and consequently inhibits non-native aggregation.

To understand this relationship further, much smaller increments in denaturant concentration could be tested rather than the 0.5 M increments used in this study. Equally the study should be conducted at lower temperatures, such as 25 °C for a week as well as 12 hours at 40 °C to understand the impact of temperature. The impact of denaturation should ideally be probed carefully with more than one protein at a single formulation condition see if there are any underlying similarities.

Q: *Are there any ways to improve the standard models for the relationship between temperature and degradation rate?*

A: Yes, absolutely. The primary limitation to understanding the relationship between temperature and degradation rate is simply the number of data points acquired. Testing at 5 °C, 25 °C and 40 °C as a standard approach is insufficient. Ideally you would want to measure at least six different temperatures within that range to get a reasonably representative indication of the relationship. I recommend holding samples at specific temperatures for the duration of the study and also performing monomer-loss kinetics using SEC-MALS.

Q: *These unfolded and aggregated forms appear soluble. How would you analyze insoluble forms?*

A: All protein aggregates should become soluble a high enough degree of denaturation. The stability of insoluble aggregates can be probed by finding the concentration of denaturant required to solubilize them.

Q: *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?*

A: 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.

Q: *How can you extend this study for virus characterization - A_2, K_d, Γ_{23}, T_{agg}?*

A: I do think there is merit in measuring Γ_{23} (the interaction between virus and excipient) 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.

Q. *What factors do you think made the difference in stability of mAbs? Is pI important?*

A. Structural stability is the number one indicator of mAb stability, so thermal ramp and/or ΔG experiments are paramount. Beyond this, PPIs are very important, so yes, the relationship between pI and pH of your solution is important as well as total ionic strength (Debye screening length). You must also bear in mind that there is a fine balance between maximizing colloidal and structural stability. Whilst lowering pH relative to pI is colloidally beneficial, it will also have a detrimental effect on structural stability, as many aspects of protein structure are driven by ionic interactions that can become screened.

Interestingly, I did notice a correlation between the experimental UV extinction coefficient and mAb stability. The UV extinction coefficient is determined by the solvent exposure of mostly aromatic amino acids, predominately tryptophan and tyrosine, which for a very stable protein should in theory be buried deep within the hydrophobic core of the protein. MAb 1, being very stable, had a UV extinction coefficient of 1.435, whereas mAb 2, being very unstable, had a UV extinction coefficient of 1.755 suggesting that the hydrophobic components maybe more solvent-exposed and the protein thus more prone to non-native aggregation. This of course assumes that the mAbs are comparable in terms of their amino acid composition.

Q. *If your protein's native state is a dimer would denaturation such that it dissociates to the monomer still determine stability?*

A. I think the first question worth asking is, what is the driving force behind your protein forming a dimer? Is it concentration dependent or temperature dependent? Is it a specific interaction or are there electrostatic or hydrophobic forces at play? You may find you can shift the dimer into a monomer by subtly changing solvent conditions. Even a small amount of urea can disrupt hydrophobic interactions without causing the protein to unfold. Once this is established you could then find novel ways to probe stability of both the dimer and monomer separately.

Measuring protein-protein interactions

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

A: 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.

Q: *Is k_D as reliable as A_2 for assessing protein-protein interactions?*

A: 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.

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

A: 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 Sq 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 µL 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.

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

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

Q: *Does the A_2-k_D relationship always stand true?*

A: Yes, for a given protein, in the dilute protein regime and corrected for solvent conditions, we find that the relationship described by Muschol and Rosenburger stands true.

Q: *How do you feel your SLS analysis of native versus non-native PPI compares to what has been proposed utilizing concentration-dependent ΔG?*

A: Although I did perform analysis of concentration-dependant ΔG using a HUNK instrument from Unchained Labs, the data acquired at a number of concentrations were of poor quality with several failed runs. Whilst I could obtain a general unfolding curve at a fixed concentration of 12.5 µg/mL, I was unable to ascertain the concentration dependence using this instrument.

Q. *Do you have any idea about the measurement of interactions between protein fibrils and incoming monomer?*

A. I have no personal experience of these types of measurements, but in essence you want to treat your protein fibrils and protein monomer as separate components and measure the cross interactions between them, i.e. measure a decreasing concentration of monomer and at the same time increase the concentration of protein fibrils. This approach would require that the interaction is not too strong, so that you can reach an equilibrium between a low fibril concentration and large monomer concentration. Another possibility is to use light scattering to measure the interaction kinetics, which is probably easier than equilibria, though it does not yield a K_d value.

Calypso & CG-MALS

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

A: A Calypso CG-MALS 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.

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

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

Excipients and denaturants

Q: What to do preferential interactions tell you about the effect of excipients on the stability of the protein?

A: In general, excipients that are preferentially bound cause the counter effect of excluding water molecules from the protein surface. As the hydrophobic core is a large component of protein structure, the exclusion of water often destabilizes that structure. The opposite is true for excipients that are preferentially excluded, as this would increase the concentration of water molecules around the protein surface and thus increase protein structural stability.

Q: Why does arginine HCl appear to be excluded from the protein?

A: At the measured concentration of 0.25 M, arginine was excluded from the lysozyme protein surface. However, Arg HCl effects differ quite significantly depending on the protein and its concentration level. Arg HCl typically has the greatest effect above 0.5 M and is a highly effective additive with respect to protein refolding.

Q. Was the effect of the charge of guanidine HCl taken into account when the data were compared to urea, which is not a charged molecule.

A. Although urea is not a charged molecule and does not contribute to ionic strength, it does actually change the activity of water and thus increases the pH of any buffer condition. It is therefore quite difficult to control the total ionic strength of the buffer conditions with any degree of precision as this effect is not that well documented. The use of guanidine HCl was preferred since the total ionic strength could be controlled, and it also happened to be a stronger denaturant and thus less concentrated solutions were required.

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

A: 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.

Q: Why did you use guanidine HCl instead of urea?

A: Initial studies were actually performed with urea, but there are several reasons for using guanidine HCl. Firstly, controlling the ionic strength of urea solutions is actually quite difficult, as it increases the pH of the solution by changing the activity of water. When using guanidine HCl, you can easily calculate the total ionic strength and adjust the final condition by adding some

NaCl or another salt. Guanidine HCl is also a much more effective denaturant and thus lower concentrations could be used to probe interactions and unfolding behavior.

Light scattering basics

Q. *What is the source of nonlinear measurements at low and high concentrations?*

A. Non-linear measurements are typically seen in low-ionic-strength solvents, even at low protein concentrations, and more so if you do not dialyze the protein to maintain constant solvent chemical potential. Excipient-excipient interactions (which give rise to clusters) especially impact the linearity of dynamic light scattering (DLS) measurements at low protein concentrations.

As you increase protein concentration further, you then run into other effects such as the extended protein-protein interactions that are described by the Kirkwood-Buff integral G_{22}, protein-excipient interactions. Finally, you approach the realm where hydrodynamic forces (H_q) arise from molecular crowding.

Q: *How can I tell if I need to make manual measurements of dn/dc or if the standard value of 0.185 mL/g is sufficient to calculate Mw and A2 measurements?*

A: The *dn/dc* of individual amino acids range from 0.165 to 0.277 mL/g and the overall *dn/dc* of a protein is the weight average of the component amino acid dn/dc values. For the majority of pure proteins in aqueous buffers, *dn/dc* falls in the range 0.181 to 0.188 mL/g, so 0.185 mL/g is a reasonable value to assume.

In order to be more accurate, there are two factors to consider: the analyte and the solvent. If the protein is sufficiently large, i.e. > 300 amino acids, and is dissolved in common aqueous solvents, then 0.185 mL/g will work. Smaller proteins and peptides can differ significantly as the average value can be easily skewed by just a few amino acids.

Where the solvent is concerned, a good rule of thumb is, if the refractive index of the solvent alone differs from that of water (1.333) by more than 1% then I would advise measuring *dn/dc* experimentally. You can measure a solvent's refractive index ('absolute RI') using an Optilab® differential refractometer with the purge valve open. I recommend injecting at least 2 mL of solvent.

Q. *What is the correct dn/dc for highly glycosylated glycoproteins?*

A. 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.

Q. *In classical batch measurements one measures dn/dc using dialysis equilibrium but performs the batch Zimm-style measurements on samples not subjected to dialysis equilibrium. What's the right procedure for SEC-MALS?*

A. Interestingly, SEC-MALS is somewhere between full dialysis and no dialysis, but the dialysis is relative to the mobile phase and not to the formulation buffer as you would want to prevent aggregation during separation. During SEC-MALS any excipient in the sample elutes much more slowly than the protein and you will see it as a 'solvent peak' following the protein, close to the total inclusion volume (the peak could be negative if the mobile phase has more excipient than the injected sample). The protein ends up primarily in the mobile phase regardless of the formulation of the injected sample.

Ideally for chromatography the mobile phase should match as closely as possible to the formulation buffer used but this is rarely the case, as it not efficient to have so many different mobile phase preparations and certain excipients at high concentration may not be amenable to SEC.

Q. *What are acceptable day-to-day variations in dn/dc measurement in a specific buffer?*

A. I would expect < 2% differences in *dn/dc* measurements.

Q. *Would you expect 50% glycerol in the solvent to have any negative effect with MALS?*

A. Due to increasing refractive index of the solvent, the *dn/dc* value of the analyte will decrease and need to be calculated or measured in order to obtain correct molar masses. In addition, 50% glycerol will be very viscous, and you therefore need to reduce the HPLC flow rate to accommodate. More typically, I've seen customers run between 5 to 15% glycerol in SEC-MALS.

Q. *How to decide the correlation time limits when analyzing a DLS measurement to calculate k_D, when the solution has co-solute clusters?*

A. 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 analyzing the protein sample. Suitable lower cutoffs I have found were 15 µs for Arg HCl, 7.5 µs for guanidine HCl, and about 10 µs for urea.

Miscellaneous

Q: *Where do you obtain 0.02um and 0.1um syringe-tip filters?*

A: Whatman Anotop 0.02 µm filters are available in both 10 mm and 25 mm diameter, from numerous suppliers including ThermoFisher, Sigma and others.