



## Live Webinar Q&A Sheet:

### The Versatility of High Throughput Dynamic Light Scattering in Protein Characterization and Formulation Development

The recorded webinar may be viewed from the [DLS](#) webinars page. These questions were submitted by live viewers. Additional information on SEC-MALS, DLS, CG-MALS, and ELS may be found on the Wyatt web [Library](#) under Webinars, Application Notes, Featured Publications and Bibliography, as well as on the corresponding [Product page](#) and [Theory](#) page of our web site.

Please contact [info@wyatt.com](mailto:info@wyatt.com) with any additional questions.

#### Formulation screening strategies

*Q: What is your approach to combining the results of various isothermal, thermal ramp and  $k_D$  measurements to select a final formulation?*

*A:* At the end of a formulation screen, we combine all of the biophysical and biochemical results together into a pseudo-heat map, identifying formulation conditions that improved or worsened the stability of the molecule when stressed to freeze/thaw, agitation and/or temperature stress. Most of the time, if one assay picks up a negative effect of a buffer or stress condition on the molecule, there will be orthogonal data from another method to corroborate the evidence. We use not only light scattering techniques ([DLS](#), SLS/MALS), but also sub-visible and visible particle analysis, differential scanning calorimetry, isothermal chemical denaturation, capillary isoelectric focusing, capillary electrophoresis SDS, SEC and more. We try to collect as much information about possible degradation products that form as a result of solution condition and stress to provide a holistic view of the molecule and those solution conditions that will facilitate a stable product.

*Q: How many buffer conditions do you test for a buffer screening on DLS and what is the dilution that you perform in the plate with the new buffer?*

*A:* The number of formulations is dependent upon the stage of the formulation development program. Early in development when we are determining the “personality” of the molecule, up to 20 buffers may be assessed. As we narrow in on the formulation, we reduce this number to 12, then 3, then finally the top two. In the early stages of development, we are usually using DLS to



look at protein concentrations <10 mg/mL (usually every 1 mg/mL). As we refine the formulation, we start to work closer to the final concentration that could reach over 100 mg/mL.

*Q: Do you have a buffer template that you used for your buffer screen, to share?*

A: No. Each molecule is really unique and of all the proteins that we have worked on over the years, no two formulations have been exactly the same. In the early stages of development, it is important to cover a wide pH range and have multiple buffer species at each pH value. The chemical nature of the buffer is as important as the pH. In addition, be sure to screen excipients that will mitigate known stressors such as freeze/thaw (carbohydrates, polyols) and shear/agitation stress (surfactants). In this manner, you will start to build the “skeleton” of your formulation with the understanding of what excipients that are destabilizing or stabilizing to your molecule. In addition, stick with excipients that are known to be in approved products and are GRAS.

*Q: You mentioned several orthogonal techniques. Do you employ mass spec in your workflow?*

A: Not typically in the formulation development workflow. However, mass spectrometry is a very integral part of our analytical toolbox to examine post-translational modifications, glycosylation, and chemical degradations (oxidation, disulfide bonds, glycation, truncation, side chain modifications, etc).

### Understanding $k_D$

*Q: Any ideas why sucrose formulations exhibit apparently attractive behavior in  $k_D$  analysis by DLS versus repulsive behavior by CG-MALS analysis?*

A: This is a very good question and I think the answer is complex! Let’s first start with the protein concentration difference that is used for each of the methods: DLS/ $k_D$  usually  $\leq 10$  mg/mL, CG-MALS from <1 mg/mL to over 100 mg/mL. As a protein is transitioned from low concentration to high concentration, many complex things are going to happen that are due to the molecule itself *and* the formulation components. As the protein concentration is increased, the free volume available to the protein molecule is reduced. Without any intervening mitigation, the loss of free volume will bring the molecules closer together and provide more opportunities for both attractive and repulsive interactions (dependent upon the surface properties of the molecule). Repulsive interactions will dominate at high protein concentrations due the “hard sphere” phenomenon (two molecules cannot occupy the same space, essentially). But we have to remember that across the concentration gradient, there is also a population “gradient” that will include monomers in solution with Higher Order Oligomers (HOO). The hard sphere repulsion at high concentration dominates the light scattering signal and masks any underlying attractive interactions that are present (needle in a haystack when the protein concentration is really high). This results in an overall positive  $A_2$  by CG-MALS and to “reveal” the HOO that is present, fitting of the data to reversible association models is required. However, at low protein concentrations,



hard sphere repulsions are less dominating and the underlying HOO will be revealed by DLS. So, it comes down to both the physical state of the protein in solution as impacted by the concentration and what physical states dominate the light scattering signal at a given concentration. Now to sucrose...Please refer to Rubio *et al.* (2018) *J. Pharm. Sci.* **107**, 1269 for a better explanation of the current theory than I can give (I will try). Essentially, sucrose is large compared to water and it gets excluded from the protein surface, but not before causing, in a sense, dehydration of the protein surface by replacing the water at the hydration layer. This "sucrose solvation" decreases the gain in free energy that occurs upon close protein contact and increases the effective excluded volume (reducing the conformational space the protein has to occupy). The increase in effective excluded volume increases the strength of apparent steric/repulsive interactions.

*Q: Would you say that  $k_D$  analysis at low concentrations is a good predictor of stability for high-concentration analysis?*

*A:* Based upon the answer to the previous question and due to the fact that  $k_D$  analysis is only valid under dilute solution conditions, it would be better to use CG-MALS by means of a [Calypso®/DAWN®](#) system to predict colloidal stability and HOO formation at high protein concentrations.

*Q: How does viscosity affects interaction/ $k_D$  determination using DLS?*

*A:* The viscosity of the formulation/solvent has to be known to accurately calculate  $R_h$ . This is a value that is input into the software as the experimental parameters are being set. In many cases the viscosity of the protein solution will increase with increasing protein concentration and will play a role in the calculated  $R_h$ . However, it is difficult to separate the viscosity that arises due to protein concentration from the underlying protein-protein interactions that are driving an increase in this value. Best practice is to use the viscosity of the solvent as a constant across the protein concentration range of the experiment.

*Q: Can you do  $k_D$  measurements on Wyatt [Mobius®](#) instrument in cuvettes?*

*A:* Yes, if the  $R_h$  is to be measured alongside the zeta potential.

*Q: Is there an easy way to determine if the  $k_D$  curvature is "real", i.e. sucrose formulation?*

*A:* Yes, perform CG-MALS experimentation, even under the same protein concentration range.

*Q: Why can't you just use the DLS for higher concentration instead of CG-MALS for sucrose formulation?*

*A:* If I would only use DLS for the higher concentration range of the sucrose formulation (or other formulations, as well), I would not be able to detect the underlying higher order oligomers that are within the overall species population dominated by hard sphere repulsions. At high enough protein concentrations, the DLS data will start to plateau as hard sphere repulsions dominate. By



performing CG-MALS, I am able to characterize both the nature of the species in solution and how their relative populations change as a function of protein concentration.

### Thermal ramps

*Q: In the thermal stability measurement, does the temperature ramp rate affect the measured onset temperature? Could different ramp rates explain the differences between onset temperatures measured by DLS and DSC?*

A: Yes, the ramp rate will affect the measured  $T_{\text{onset}}$ . A faster scan rate will artificially shift the  $T_{\text{onset}}$  to higher temperature due to the fact that the rate of temperature increase is faster than the time it takes the molecule to reach an equilibrium at each set point. For a direct comparison of DLS and DSC, we set the scan rate the same at 1°C/minute, a standard rate used for thermal unfolding of proteins.

*Q: Regarding the  $T_{\text{onset}}$  values from DLS, how are you distinguishing lack of sensitivity (compared to DSC) vs. structural or aggregation changes? Or is DLS sensitivity at  $T_{\text{onset}}$  not an issue of concern?*

A: It is a difference in what each technique is measuring and how much change in the molecule has to occur as a result thermal stress for the DLS or the DSC to detect it. For most proteins, the  $T_{\text{onset}}$  as measured by DSC and DLS represents some level of unfolding of the molecule. DSC will detect even local (versus global) unfolding events due to the fact that it measures the energy required to unfold the molecule. Even small changes to the conformation of a protein will result in energy differences that are detected by DSC. On the other hand, DLS will detect unfolding once the size population of the molecule ( $R_h$ ) gets big enough to distinguish it from the native state. In most cases more of the molecule is unfolded at this point of detection by DLS as compared to DSC. Monitoring the intensity or normalized count rate of the scattered light in your DLS experiment will allow for discrimination of unfolding events (similar intensity counts) as opposed to aggregation events (increased intensity counts).

*Q: How do you prevent evaporation during DLS thermal ramp experiment? Please comment on sample evaporation during thermal stress and overcoming this issue - overlay of oil is an approach, any other approaches that were found to be successful?*

A: The DLS plates are sealed with a plastic film to prevent evaporation, and the inside of the instrument lid (overtop the plate) is heated to dissipate condensation of water from the surface



of the sample. Sealing with plastic film is a better option than mineral oil, which may destabilize the molecules at the oil/water interface.

*Q: Do you check the  $T_m$  of your protein and the energy of activation in different buffers?*

A: Yes, both by DSC and DLS by varying pH and the chemical nature of the buffer.

*Q: Is  $T_m$  from CD relate to  $T_m$  from DLS?*

A: It could be...this is only if the unfolding midpoint is detected prior to aggregation. One of the main drawbacks of thermal denaturation methods (apart from DSC) is that you are competing with aggregation as the protein unfolds.  $T_{onset}$  values can usually be measured prior to aggregation, but  $T_m$  values are very difficult to capture using methods such as thermal scanning DLS, CD, FL, dye binding, etc. This is especially the case for mAbs. DSC is the best method for  $T_m$  detection and quantification.

## General

*Q: What is the concentration range of the [DynaPro® Plate Reader III](#)?*

A: Light scattering is proportional to size. For mAbs at 150 kDa, we can usually obtain adequate signal-to-noise at  $\geq 0.2$  mg/mL up to over 100 mg/mL. For smaller proteins 0.5 to 1 mg/mL is usually required to obtain reliable signal at the lower end.

*Q: Would all the tools you have presented be applicable to viral vector products, especially the ones containing an envelope? What approach would be taken for formulation development for such products?*

A: For viral vector products, a number of the tools that we have would be applicable. AAV's would be the most amenable to techniques such as sub-visible particle analysis, DLS, MALS and DSC. Viral vector products, especially enveloped, also benefit from techniques such as AUC and microscopic evaluation. This is a new area of investigation for me, so I will also be looking to the scientific community for guidance for the formulation development process of these vital products.

*Q. How significant is the change in  $R_h$  from 6 to 8 nm in 3 weeks as shown in your stability monitoring graph?*

A. If the  $R_h$  values were random over the 3 week period, basically jumping around between 6-8 nm with no real trend, then I would not view it as significant. It is because we identify an upward trend in the change in  $R_h$  as a function of incubation time that makes the change significant.

*Q: How can we interpret sucrose-based formulation data? Sometimes it shows some artifact peaks. Could I exclude those by using MALS or SLS ?*

A: If your sucrose concentration is high enough, you may see small species showing up in the histogram (integration of the correlation curve). Anything in the 1-2 nm range is usually excipient



related and can be “ignored” if the protein peak is well separated from these nuisance artifacts. For mAbs, the  $R_h$  can range from 5-9ish nm, so you should be able to monitor the protein peak without issue. MALS detection does not suffer from the same interference with sucrose, as the protein signal will dominate.

*Q: How sensitive is this technique with respect to solubility, aggregation, and impurity?*

A: DLS is very sensitive in that it can detect even small changes in  $R_h$  due to protein unfolding and even small amounts of protein aggregation and HOO species. The difficulty comes in the discrete quantitation of the nature of the HOO species and relative populations. This is overcome by using methods such as CG-MALS or AUC.

*Q: Can you estimate soluble concentration of protein in a partially aggregated protein sample?*

A: Yes, by CG-MALS and fitting the data to determine the monomer MW and its relative population versus protein concentration. Insoluble aggregates will be evident by the quality of the light scattering signal and confirmed using sub-visible particle analysis. It is highly recommended to filter protein samples prior to light scattering analysis so that all the species that you are monitoring are soluble. Combining SEC-MALS testing along with batch MALS will allow you to quantitate the level of higher order oligomers (HOO) that are reversible versus irreversible. These values can then be accounted for in the data analysis.

*Q: Is it possible to measure other impurities like DNA, other HCP if yes, how much we can rely on impurity measurement?*

A: DLS is not the ideal technique to measure residual DNA or HCP. qPCR and HCP ELISAs are better suited for these analyses.