



## Live Webinar Q&A Sheet:

### Rational use of dynamic light scattering for the characterization of cytokines, bispecific antibody fusion proteins and binary antibody mixtures

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, RT-MALS and FFF may be found on the Wyatt web Library under [Webinars](#), [Application Notes](#) and [Bibliography](#), as well as on the corresponding [Product page](#) and [Solutions](#) page of our web site.

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

#### DLS General

- Q: What is the sample volume for DLS measurements in the DLS plate reader? How much protein is needed to determine the aggregation temperature of one formulation condition?*
- A: Using the [DynaPro® Plate Reader](#), DLS measurements are possible with as little as 5  $\mu\text{L}$  in 1536-well plates. Measurements in 384 well plates require 20-50  $\mu\text{L}$  per well, depending on the type of plate being used. The total amount of protein depends on the protein size, concentration, and type of multi-well plates. In 1536-well plates, as low as 5  $\mu\text{L}$  of 0.1 mg/mL antibody (150 kDa) can be sufficient. This makes around 0.5-1  $\mu\text{g}$  per condition, which is all that is needed for the aggregation temperature of that condition.
- Q: Do you have to filter your protein samples before DLS measurements?*
- A: Filtering the sample is a good approach to remove particulates (like dust) that can affect the quality of the DLS measurements. Centrifuging the sample at high centrifugal force (10,000  $g$  - 15,000  $g$ ) is a good alternative to filtration. However, filtering or centrifugation are not required. For example, you may filter your sample to ensure a clean starting material prior to performing accelerated stress tests (e.g., freeze-thaw, agitation, etc.). In this case, filtering the final product is not desirable since you want to characterize the particles that may have formed.
- Q: Why is the hydrodynamic radius used as a standard of size measurement instead of hydrodynamic diameter?*
- A: Both the hydrodynamic radius and hydrodynamic diameter are reported by Wyatt's [DYNAMICS® software](#). The Stokes-Einstein equation written in its traditional form relates the translational diffusion coefficient ( $D_t$ ) to the hydrodynamic radius ( $R_h$ ). Since dynamic light scattering directly measures  $D_t$ , we choose to report  $R_h$  as the primary result, but a user may select either value to report, according to their needs.



*Q: When measuring  $R_h$  of different concentrations of protein using DLS, were different viscosity values used?*

A: The Stokes-Einstein relationship, which converts the translational diffusion coefficient ( $D_t$ ) to the hydrodynamic radius, requires the solvent viscosity, not the solution viscosity. The viscosity used for this analysis should not change as a function of protein concentration.

One assumption for calculating  $R_h$  from  $D_t$  is that  $D_t$  is measured at infinite dilution ( $D_0$ ) so that the molecules are not interacting with each other. At high concentrations, intermolecular interactions can cause the protein's  $R_h$  to appear larger (in case of attractive interactions) or smaller (in case of repulsive interactions) than the true  $R_h$ . Performing a  $k_D$  analysis allows you to measure  $D_0$  and determine the hydrodynamic radius at infinite dilution ( $R_{h,0}$ ). It is important to note that attractive interactions may *also* cause an increase in the solution viscosity; however, the solution viscosity should not be used to correct for this apparent change. Rather, the source of the self-interaction should be investigated.

*Q: How do you approach the analysis of complex samples (multiple protein mixtures) and sample preparation for DLS?*

A: Size and stability measurements may represent an average of all the proteins or may be dominated by a single component. If possible, perform measurements with the individual species prior to mixing to see the impact of the mixture on the individual properties.

If many different proteins with different sizes are present in the sample, you can still measure hydrodynamic size with dynamic light scattering, but the data might be difficult to interpret. Likely, the proteins in the mixture will be of similar hydrodynamic size (within a factor of 2), and so DLS will report an average radius with some polydispersity.

As for DLS sample preparation in general, it is important that there are no particulates that affect the quality of the DLS data. Often, centrifugation or filtration of protein mixtures is used before making DLS measurements. If possible, filter each protein before mixing so you don't lose large aggregates that result from mixing.

*Q: How do you calibrate your light scattering methods?*

A: Dynamic light scattering (DLS) measurements do not require calibration in order to determine hydrodynamic radius ( $R_h$ ), diffusion interaction parameter ( $k_D$ ), or the onset temperature for unfolding or aggregation. Including a system suitability standard with a known hydrodynamic radius and molecular weight, like BSA or lysozyme, as part of your experiment design is always a good check to verify that your instrument is working well.



Static light scattering (SLS) requires calibration to relate the scattering intensity to the molar mass and concentration of the molecules in solution. Multi-angle light scattering (MALS) detectors and the DynaPro NanoStar are calibrated with toluene. The DynaPro Plate Reader is calibrated with a certified dextran standard of known molar mass and second osmotic virial coefficient, prepared at very accurate concentrations.

*Q: Can you determine by DLS how many antibody molecules are necessary to complete neutralization of a corona virus particle?*

A: Dynamic light scattering can only be used to determine physical changes and cannot be used to determine biological changes, like neutralization of a virus particle. With DLS, you may see an increase in the overall radius of the virus that is caused by antibodies being bound to the surface, and could estimate from that many antibodies are bound, but this would be an approximation and not a guarantee of biological effect.

### General protein stability

*Q: Can you study isothermal chemical denaturation with DLS?*

A: You can prepare your protein samples in different concentrations of denaturants. If the protein unfolds in a certain denaturant concentration, the  $R_h$  will increase. It is important to measure the viscosity of the different solvent compositions and include it in the calculation of the  $R_h$ . An example of this analysis was published in <http://doi.org/10.1002/jps.23746>.

*Q: Is DLS suitable to investigate chemical changes in a protein?*

A: DLS cannot directly see chemical changes. However, if a chemical change like oxidation or deamidation affects the aggregation state, colloidal stability, or thermal stability of a protein, DLS is a very good method to study this.

*Q: Can you use DLS as a method to study the stability of therapeutic protein formulations during long-term storage?*

A: Absolutely, DLS can fill the analytical gap for detecting aggregates with a size between 100 and 1000 nm. With the [DynaPro Plate Reader](#), an entire set of samples can be stored and measured in the same plate multiple times throughout the long-term storage study without any perturbation or additional sample handling.

*Q: Can you study the effect of freeze-thaw using DLS?*

A: Yes, DLS can be used to study changes in a sample upon freeze-thaw. Some undesirable effects of freeze-thaw can include changes in the amount of small oligomer or aggregate, which would increase the hydrodynamic radius or polydispersity measured by DLS. Freeze-thaw can also



cause changes in stability, which may be measured by changes in  $k_D$  or in temperature-dependent properties, like  $T_{agg}$ .

*Q: Has the use of DLS for assessment of long-term stability been accepted by regulatory agencies such as the FDA or EMEA?*

A: Data from Wyatt's MALS and DLS instruments have been submitted as part of regulatory filings. For information about method validation and other specifications, please contact [support@wyatt.com](mailto:support@wyatt.com).

### Protein colloidal stability

*Q: For  $k_D$  measurements with DLS, which parameters, such as attenuation, equilibration time, etc. are most crucial? How do they affect the diffusion coefficient?*

A: The diffusion interaction parameter,  $k_D$ , measures net attraction or repulsion among molecules. For proteins, these are typically nonspecific interactions that occur at concentrations on the order of 1-10 mg/mL. For accurate, reproducible measurements, we recommend preparing 8-10 concentrations evenly spread out across the range of interest, with the maximum concentration being 10-20 mg/mL. More information can be found on the [Wyatt Support Center](#) in technical note [TN2001](#).

To increase accuracy and precision, you may include a short equilibration time for the entire plate prior to measurement (typically <5 minutes) to ensure the samples are equilibrated at the instrument temperature and to allow any reversible interactions in the solution to come to equilibrium.

As with all DLS measurements, having an optimal amount of light reach the detector is important for accurate diffusion coefficient and hydrodynamic radius measurements, and the Plate Reader will automatically adjust the laser power and attenuator settings to achieve these optimal levels.

*Q: Does NaCl always have a negative effect on the colloidal protein stability?*

A: Not always. NaCl has a negative impact on proteins that are colloidally stable due to strong electrostatic repulsion. However, there are proteins that form oligomers due to electrostatic attraction. In such case, the addition of NaCl will have a beneficial effect.

### Protein thermal stability

*Q: What is the highest temperature necessary in a temperature ramp experiment for good results?*

A: Most proteins aggregate between 40 °C and 80 °C; therefore, heating to 80 °C or 85 °C is usually sufficient. The temperature range of the DynaPro Plate Reader is 4 °C to 85 °C. Especially heat-



resistant proteins and other samples requiring higher temperatures can be analyzed in the [DynaPro NanoStar®](#) cuvette-based DLS instrument, which covers -15 °C to +150 °C.

*Q: Should the onset of aggregation ( $T_{agg}$ ) only be measured with SLS, and not DLS?*

A: The best evidence for the onset of aggregation is an increase in molar mass as a function of temperature, and this change is detected by SLS. However, typically aggregation is also closely associated with an increase in size, measured with DLS. In many cases, the increase in size that accompanies aggregation is much, much larger than the increase in size that results from pure unfolding. For example, we presented a case of lysozyme increasing in size from ~2 nm to >100 nm. In this case, it is safe to infer that the sample is aggregating. Both SLS and DLS may be measured simultaneously in the DynaPro Plate Reader.

*Q: Is it possible to find melting temperature with DLS?*

A: It is possible to determine a melting temperature by a combination of DLS and SLS, provided that the protein unfolds without aggregating. When the protein unfolds, the hydrodynamic radius will increase but the molecular weight will remain constant. This is a clear indication that what you are measuring is a melting temperature.

*Q: Are you using  $T_{agg}$  and  $T_{on}$  interchangeably?*

A: It depends on the context.  $T_{agg}$  usually refers to the temperature at the onset of aggregation.  $T_{on}$  can be used as the onset temperature of aggregation or of unfolding. The DYNAMICS software includes an onset analysis that can be applied to hydrodynamic radius data or molar mass data to determine the temperature at which a significant change starts to happen. Other analyses are also available to quantify trends in measured parameters as a function of temperature, concentration, buffer composition, or other user-defined parameters.

*Q: Do you determine aggregation temperature at a specific concentration? Which one? Do you try several concentrations or do you settle on one?*

A: The aggregation onset temperature can depend on the protein concentration. Usually, higher protein concentrations lead to lower aggregation onset temperatures. This was seen in the lysozyme example at the beginning of the webinar where the onset of aggregation occurred at 48 °C for lysozyme at 100 mg/mL and at 60 °C for lysozyme at 2 mg/mL. Lower aggregation temperatures at higher concentrations may be due, at least in part, to higher collision rates and therefore a slower ramp rate may increase the apparent onset temperature. Whether several concentrations should be tested depends on the specific study and material availability.

*Q: I am also working with antibody therapeutic stability with DLS. I want to make a temperature-ramped study with 10 °C intervals. Should I do triple repeats, or for each 10 °C, should I measure*



*only once? I mean, does the time past attaining the specific temperature cause increased stress and decrease stability, or does it provide equilibrium and makes it better?*

A: The answer depends on the specific time constants of the sample, which may be quite different for equilibration and aggregation rate, and for different conditions. For temperature-dependent studies with the DynaPro Plate Reader, we suggest preparing replicate wells for each condition and measuring them within the same experiment. If there is reason to believe that aggregation kinetics are slow then you can hold at each temperature and remeasure after pre-defined time periods.

However, it is important to recognize that the differences between the samples and conditions are often smaller than 10 °C. If you use steps of 10 °C, you can miss important information. We recommend much smaller intervals between measurements of each well, typically 1-2 °C rather than 10 °C. The DYNAMICS Experiment Designer will determine the appropriate ramp rate to achieve the desired temperature interval for each well. More information about designing and performing temperature ramp experiments can be found on the [Wyatt Support Center](#) in technical note [TN7001](#).

Q: *When analyzing protein aggregation in a stability study, is it recommended to use the cumulants hydrodynamic radius or to use the NNLS regularization to determine each type of size distribution (mass, intensity, and number)?*

A: Cumulants analysis is appropriate if the sample is monomodal, i.e. if only a single peak appears in the regularization graph, with relatively low PDI (polydispersity index). This means that at most only small oligomers such as dimers, trimers and tetramers occur. The appearance of a second peak indicates much larger aggregates. In DYNAMICS you don't have to review each regularization graph one by one – the data table automatically indicates if the sample was found to be multi-modal and the PDI values also appear.

If the sample is polydisperse and you see multiple peaks in the regularization analysis, I would report the size and fraction of the different peaks. The distribution by mass and intensity will look different, but they both carry important information and can be reported. The Intensity distribution is directly calculated by the fit to the DLS data and is most sensitive to a small number of large aggregates. For that reason, it is often most suitable for looking at slight changes that may result from changes in a formulation or from stress conditions. The mass and number distributions are derived from the intensity distribution and require some knowledge about the sample. More information on the distributions determined by DLS can be found on the Support Center in technical note [TN2004](#).



*Q: In the temperature ramp measurements, what would be the highest concentration that can be used?*

*A:* The maximum concentration for DLS depends on the size of the samples. Larger particles, including proteins that self-associate into larger species, require lower concentrations than protein monomers. For well-formulated antibodies, concentrations of 50-100 mg/mL can be no problem in temperature ramps with DLS. One example in the webinar used lysozyme concentrations from 2 mg/mL to 100 mg/mL.

#### **Application-specific**

*Q: What is the isothermal temperature used in denaturation?*

*A:* It depends on the type of stability information that you want to investigate. The denaturation can be performed at 4 °C if you are interested in the stability at refrigerated temperatures, or it can be performed at 25 °C or 40 °C if you are interested in conditions more relevant for accelerated storage stability studies.

*Q: What is the range of pH for IFN $\alpha$ -2a to remain active? It seems pH 3.5 showed the least tendency to aggregate based on  $R_h$  yet this pH is not considered?*

*A:* The conformational stability of IFN $\alpha$ -2a is very low at pH 3.5. Also, the PDI was much higher at pH 3.5. That is why the condition was excluded.

*Q: What is the pH used in your work for comparison study in acidity?*

*A:* The solutions for the lysozyme study at the beginning of the webinar were prepared at pH 4, pH 7, and pH 9.