Live Webinar Q&A Sheet:

Stability Characterization of Vaccines, Gene Vectors and Therapeutic Antibodies with Light Scattering

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 FFF may be found in the Wyatt website Resources section under Webinars, Application Notes, and Bibliography, as well as on the corresponding Product page and Solutions page of our website.

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

DLS Technical

Q: *How do you calibrate your light scattering methods for DLS & ELS measurements?*

A: Calibration is not required to determine hydrodynamic radius (R_h), diffusion interaction parameter (k_D), or the onset temperature for unfolding or aggregation using dynamic light scattering (DLS). However, to ensure your instrument is working well, it is good practice to run standards like bovine serum albumin (BSA) or lysozyme with a known molecular weight and hydrodynamic radius or polystyrene latex (PSL) with a known particle concentration as controls in the experiment. Similarly, calibration is not required to determine mobility using electrophoretic light scattering (ELS). But we recommend validating the system with a Mobius Mobility standard.

Static light scattering (SLS) requires calibration to relate the scattering intensity to the molar mass and concentration of the molecules in solution. The DynaPro® NanoStar® is calibrated with toluene. The DynaPro® Plate Reader is calibrated with a certified dextran standard of known molar mass and second virial coefficient (A_2), prepared at very accurate concentrations.

Q: *Your description of dynamic light scattering sounds a lot like fluorescence correlation (FCS) spectroscopy. Could you clarify the difference between the two?*

A: Fluorescence correlation spectroscopy is a fluorescence version of dynamic light scattering. FCS measures the fluctuation of incoherent light (fluorescence) emitted by the particles, while in DLS, fluctuations in coherent scattered light are measured.

Q: *What is the precision (inter-assay reproducibility) of DLS for RNA-LNP?*

A: In our experience, the DLS inter-assay repeatability is 1 – 2 %.

Q: *We just started using the DynaPro Plate Reader for DLS measurements for our LNP's containing RNA. Which mode would you recommend? Monomodal vs multimodal? Also, we see a size*

difference between these measurements and the ones made on our old (non-Wyatt) instrument, can you think of a reason why?

A: Monomodal and multimodal distributions, which are reported by DYNAMICS® software with the DynaPro Plate Reader, depend on the properties of the sample. DYNAMICS will let you know if the sample is monomodal or multimodal. If it is multimodal you should not use results from the method of cumulants. There are various parameters that may influence the size measurements, especially if the sample is multi-modal, including the autocorrelation range that is selected for fitting, the specific fitting algorithm, and the resolution parameter selected for the regularization analysis. These may differ between your non-Wyatt instrument's software and DYNAMICS settings.

Q: Can light scattering detect small molecules? If so, what is the limit of the molecular weight?

A: Both dynamic and static light scattering can detect small molecules. The DynaPro Plate Reader and the DynaPro NanoStar can measure radii as small as 0.5 nm and 0.2 nm, respectively. The lowest molecular weights measured by static light scattering (SLS) are 1000 Da and 300 Da for the Plate Reader and the NanoStar respectively. However, the concentrations required for detection at the lowest size range are certainly higher than they would be for larger molecules like proteins, nucleic acids or polymers.

Q: In light scattering applications, is the wavelength(s) of the light source important? Are different lasers chosen for different applications?

A: The wavelength is important in light scattering applications. In general, the lower the wavelength, the stronger the light scattering. However, effects of absorption and fluorescence on light scattering are significant at shorter wavelengths. NanoStar uses a laser at 658 nm, which is suitable for most analytes. Plate Reader uses a laser wavelength of 830 nm, which is best suited for a wide range of analytes including fluorescent materials. Optical filters can also be used in the Mobius™ dynamic/electrophoretic light scattering instrument, which operates at 532 nm, to eliminate fluorescence for specific applications.

Q: Will light scattering degrade biological samples during the measurement process?

A: In general, the DynaPro Plate Reader , the NanoStar and Mobius™ instruments offers reliable, reproducible and non-destructive measurements for size, molar mass and (with Mobius) mobility or zeta potential. Unlike UV-based technologies, the wavelengths in these instruments are long and do not lead to photodegradation. For measuring zeta potential of fragile biomolecules, the typical applied voltage in the Mobius is usually between 2 and 3.5 V, which is low enough to prevent damage, as opposed to tens or even hundreds of volts in some other zeta potential detectors.

Q: *How do you make sure excipients do not disturb the DLS measurements?*

A: This depends on the excipient; very small excipients such as salts that are well-dissolved generally do not affect DLS measurements. Sucrose (R_h ~ 0.2 nm) and other sugars at high concentrations affect DLS measurements of small analytes like lysozymes (R_h = 1.9 nm). However, for larger analytes such as IgG (R_h > 5 nm), the effects are negligible.

Sucrose impurities, e.g., cross-linked nanocrystals, are large. It is critical to ensure that these are not present in the sample during analysis. Filtering sucrose and similar excipients prior to use or by purchasing high-quality raw materials that are verified to be pure can minimize excipient impurities influencing DLS measurements.

The most challenging, but common, excipients that affect DLS measurements are micelle-forming surfactants. The micelles often have similar sizes or are larger than most biotherapeutics. Unfortunately, there is no good way of eliminating the signal from micelles when conducting DLS measurements except dilution to below the critical micelle concentration, which may result in an unstable sample.

Q: *In parenteral therapeutic applications, where a colloid is essentially injected into plasma, can we predict the dissolution behavior of the solutes using light scattering? For instance, can we predict aggregation of solutes (the therapeutics) in blood? This, conceptually, could affect therapeutic effect.*

A: You would have to make the measurements in plasma rather than in blood, since blood cells and other larger particles would overwhelm the light scattering detector. In addition, the signal from the solute of interest would have to stand out relative to other plasma components, so unless there is a pretty significant concentration or the therapeutic is a fairly large particle (e.g. 100 – 200 nm) it is likely that its signal will be lost relative to that from lipoproteins, serum albumin and other plasma components. It might be possible to isolate the therapeutic from other solutes and analyze it using FFF-MALS, if their behavior does not change upon dilution.

However, if the scattering signal does stand out in unseparated samples, we can analyze the size and dissolution behavior of particles as a function of concentration using the DynaPro Plate Reader. A similar application of critical micelle concentration or aggregation can be found in this application note. DLS with regularization fitting method can be used to characterize nanoparticles that are at least 3 - 5 times larger in radius than the proteins in plasma. See DYNAMICS Benefits for more information.

Q: *Are the mentioned instruments applicable to non-transparent solution/samples?*

A: Non-transparency may arise from absorption or turbidity. In both cases, if the effect is so strong that the solution is not transparent to the eye, then analysis by DLS will probably not be

successful. A translucent sample might be amenable to analysis, but translucence is usually the result of multiple scattering and may lead to incorrect size measurements. Sufficient dilution of the sample may bring it into measurement range.

Q: *How do you compare intensity percentages between different measurements? What exactly do they stand for/do they represent?*

A: %Intensity refers to the fraction of total scattered light arising from particles of the given size. Because the scattering efficiency is highly dependent on particle radius or mass, as well as refractive index, %Intensity does not translate to the mass fraction of the given size range in the sample. However, the %Intensity distribution is directly determined from fitting the collected data and does not require any assumptions, and therefore is the most fundamental distribution that can be obtained by DLS. DYNAMICS software can convert the %Intensity distribution to %Mass or %Number distribution, but certain assumptions must be made in the conversion, and therefore these distributions tend to be less reliable than %Intensity.

Thermal Properties

Q: *Is melting temperature* (T_m) *from circular dichroism (CD) related to melting temperature from DLS?*

A: In both cases, T_m can only be measured if the unfolding midpoint is detected and distinguishable prior to the onset of aggregation. T_m values are very difficult to capture using methods such as thermal scanning DLS, CD, dye binding, etc. This is especially the case for mAbs. One of the main drawbacks of thermal denaturation methods is that you are competing with aggregation as the protein unfolds. When aggregation occurs at, or slightly after, melting, only T_{onset}—the temperature for onset of changing size, whether due to unfolding or aggregation—can be determined. Because DSC is less susceptible to interference from aggregation, it is considered the gold standard for T_m detection and quantification, though it is not unusual for aggregation to impact DSC as well.

Q: *What is an ideal temperature ramp rate when screening many samples?*

A: The ramp rate will affect the measured T_{onset}. A faster scan rate will artificially shift T_{onset} to a higher temperature since the rate of temperature increase is faster than the time it takes for the molecule to reach equilibrium. We recommend keeping the scan rate at 1 °C/minute or slower (a standard rate used for thermal unfolding of proteins by DSC). The NanoStar is an ideal instrument for testing the effect of scan rate on apparent T_{onset} since it has a wide range of scan rates and uses very little sample in doing so – as little as 2 µL per run. See AN4003: Influence of heating rates on the thermostability characterization of antibodies by DLS and SLS.

Q: *Can you using T_{agg} and T_{onset} interchangeably?*

A: T_{agg} refers to the temperature at the onset of aggregation as indicated by a change in size (DLS) and/or molecular weight (SLS), whereas T_{onset} is the onset temperature of either aggregation or unfolding, indicated solely by a change in size. Sometimes it may be possible to distinguish the onset of unfolding from the onset of aggregation if the ramp rate is low and data are taken at close temperature intervals, and the size axis is sufficiently enlarged to observe the changes in size due to unfolding (which are quite small compared to changes in size due to aggregation). DYNAMICS software includes an onset analysis that can be applied to hydrodynamic radius or molar mass data to determine the temperature at which a significant change starts to happen.

Q: *Could you further explain how to differentiate between protein unfolding and protein aggregation using light scattering?*

A: Please refer to the lysozyme experiment discussed in this webinar. Under the experimental conditions, the lysozyme radius increases from about 2 nm to 2.6 nm as the temperature increases from 40 °C to 85 °C. The midpoint of this transition occurs at 69 °C. However, the measured molar mass remains constant at 14 kDa throughout the entire experiment, indicating that the lysozyme is a monomer across the temperature ramp. Therefore, the change in size is purely due to unfolding and not aggregation.

Diffusion interaction parameter (k_D)

Q: *What is the recommended concentration range and number of samples for k_D analysis, especially when I am limited on sample?*

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 mg/mL and above. For accurate, reproducible measurements, we recommend preparing 8 - 10 concentrations evenly spread out from 0.5 - 1.0 mg/mL up to 10 - 20 mg/mL. Wyatt customers can find more information about k_D measurements on the Wyatt Support Center in technical note TN2001 - Determination of the Diffusion Interaction Parameter kD by DLS.

Q: *How does viscosity affect interaction or k_D determination using DLS?*

A: The viscosity of the solvent is required to calculate R_h. This value is entered into the software as an experimental parameter. However, k_D is determined from the diffusion coefficient, which is measured independently of solvent viscosity. While solvent viscosity might impact the overall (self-) diffusion coefficient, it should not impact the relative change with concentration that is quantified by k_D.

In many cases, the viscosity of the protein solution will increase with increasing protein concentration, but this is related to those interactions which are captured in the k_D value. Therefore, for purposes of k_D, it is best to consider the viscosity due to the protein independently from the viscosity of the solvent.

Opalescence

Q: *Can the DynaPro instruments be used to measure opalescence?*

A: Absolutely! Opalescence is measured with static light scattering, and both the NanoStar and DynaPro Plate Reader can perform this function. We encourage you to check out this open-access publication which utilized the NanoStar: https://doi.org/10.1126/sciadv.abb0372 .

Polydispersity (PDI)

Q: *What is an acceptable polydispersity index (PDI)?*

A: An acceptable PDI depends on many factors and is primarily determined by the application. It is important to keep in mind that sample preparation techniques, such as filtration or centrifugation, and experimental conditions could affect size and PDI measurements.

Q: *For a polydisperse formulation with broad distribution, can we still use DLS? When will it fail to give an accurate result?*

A: Yes, you can certainly use DLS for broadly polydisperse samples. The measurement is performed in batch mode without any separation, and therefore resolution is relatively low, so here DLS is best used for screening purposes. Separation-based methods such as SEC-MALS can be used for in-depth characterization to determine well-resolved and accurate size distributions.

Characterization of AAVs

Q: *Can you address how light scattering is affected by mass? For instance, the masses of "full" vs. "empty" capsids would differ, but their sizes (and surface charges) are theoretically the same.*

A: Static light scattering is affected by molar mass, since the magnitude of scattered light is proportional to the product of molar mass and mass concentration. This property is used to determine capsid content in a SEC-MALS viral vector analysis procedure. Dynamic light scattering is not affected by mass, since it only measures the fluctuations of the scattered intensity that arise from Brownian motion, which is independent of mass. We encourage you to check out our application note AN1617 - AAV critical quality attribute analysis by SEC-MALS to learn more about quantifying AAV capsid content.

Q: *Do the various AAV serotypes affect the ability of DLS to measure the concentration?*

A: No, since these measurements depend only on the refractive indices of the proteins and DNA, which are independent of serotype.

Characterization of proteins

Q: *What concentrations of protein are suitable for measurements on the NanoStar and Plate Reader instruments? How do these concentrations relate to the protein concentrations commonly used in biopharmaceutical applications?*

A: The instruments are specified with a lower limit of measurement for lysozyme (MW = 14.4 kDa) of 0.1 – 0.125 mg/mL. For better accuracy and robustness, you would probably want to measure them at ~ 1 - 2 mg/mL or higher. Since light scattering intensity is proportional to molar mass, mAbs at 150 kDa are best measured at a concentration ≥ 0.2 mg/mL.

You can usually make successful measurements of therapeutic proteins up to, or even above, 100 mg/mL, but in all likelihood concentrations above 1 - 2 mg/mL will not yield the true size due to the impact of protein-protein interactions on light scattering. However, DLS performed at higher concentrations can yield information on protein-protein interactions and their dependence on formulation buffer via k_D analysis, and on changes in formulation viscosity arising from protein-protein interactions.