Live Webinar Q&A Sheet:

Measuring the Opalescence of mAb Solutions with Microscale Nephelometry

The recorded webinar may be viewed from the DLS webinars page. Additional information on DLS, SEC-MALS, RT-MALS, 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.

The responses to the questions below were prepared with the assistance of the webinar speaker, Dr. Jon Kingsbury of Sanofi, to whom Wyatt extends sincere appreciation for his support. Please contact info@wyatt.com with any additional questions.

Turbidity measurement

Q: *How do turbidity/nephelometry results obtained using the NanoStar® DLS/SLS instrument compare with those obtained using a standard turbidity meter?*

A: As described in this webinar, the NanoStar results are quite comparable to those of the commercial turbidimeter (https://doi.org/10.1016/j.xphs.2021.05.005). In another study, scientists from Wyatt and Bristol-Myers Squibb recently published an article comparing measurement of the opalescence of mAb solutions using different instruments, including the commercial nephelometric turbidimeter, Wyatt's DynaPro® NanoStar, and others. Please refer to https://doi.org/10.1016/j.xphs.2021.06.013 for more details.

Although the standard instruments deliver suitable turbidity information, they require milliliter quantities of solution and are limited in determining other valuable product attributes, whereas the NanoStar requires just microliters of solution and further provides information on product size, aggregation and stability.

Q: *Can you please confirm—given the scattering angle required—that nephelometry can be performed using the DynaPro Nanostar?*

A: Yes. NanoStar uses the 90° angle SLS detector for opalescence measurements and thus fulfills the requirement of standard nephelometry.

Q: *Is there a significant difference relative to nephelometry if a UV/Vis spectrophotometer is used to measure absorbance, back-calculating the transmittance to derive a measurement for opalescence and turbidity?*

A: Absorbance (actually, extinction) - based quantification of opalescence is a common practice, wherein the results are reported in units of AU (absorbance units), OD (optical density) or %Transmission. It is possible to calibrate a UV-Vis spectrophotometer against formazine

standards and subsequently report the results in units of FTU (formazine turbidity units). However, the transmitted-light approach suffers from low signal-to-noise ratios and is far less sensitive to early-onset opalescence than the light-scattering approach utilized in the work presented here. In addition, protein absorbance in the wavelength range sometimes used for turbidity measurements (e.g. 340-360 nm) leads to inaccuracy in the determined NTU (https://www.sciencedirect.com/science/article/pii/S0022354921002495).

Q: Can the DynaPro Plate Reader also be used for the turbidity method, or is the NanoStar preferred?

A: The NanoStar is preferred for opalescence measurements since it uses a 90° SLS detector, which aligns with the definition of nephelometry and will provide much better correspondence to NTU standards than other scattering angles. The DynaPro Plate Reader uses a backscattering angle of 167° and therefore, while being desirable in terms of automation and convenience, will not perform well in terms of calibration against NTU standards. Hence, DYNAMICS® software does not support turbidity calibration for the Plate Reader.

Q: Can you distinguish the effects of large particles on opalescence, i.e. if there are aggregates much larger than 1 micron will you know those are the cause of opalescence?

A: Regularization analysis by DLS provides the relative scattering intensities of the different size populations, so comparing the %Intensity from the monomeric protein (which may include oligomers) and from populations with larger sizes will indicate which contributes the most to overall scattering and hence to opalescence. The reversibility of the temperature dependence can also be used to check for large particles (https://doi.org/10.1126/sciadv.abb0372). The magnitude of opalescence due to optical density inhomogeneities varies with temperature and is reversible on heating-cooling cycles whereas scattering from large particles is not.

Q: Is there an accepted way to measure, or equation to use, that can correct the transmitted light (at zero degrees) for the level of turbidity of the sample?

A: In order to correct the transmission for turbidity, you would have to calculate the total amount of light scattered by the sample. In order to calculate the total amount of scattered light from the turbidity measurement at a given angle, you must know the size or size distribution and concentration of the scattering particles as well as their refractive indices. It's possible for simple suspensions of known composition (for example, DLS/SLS will give you the size distribution and concentration if you know the particle refractive index) but would be difficult for complex samples containing unknown particles.

Q: *In the opalescence workflow, how are the samples treated before measurement? Are they centrifuged or filtered?*

A: For opalescence measurements with the DynaPro NanoStar, the samples are simply loaded into a 2 or 45 µL pre-calibrated quartz cuvette. If there are large impurities present, filtration may be necessary.

Turbidity in protein formulation

Q: *In mAb drug products, is there a generally accepted maximum or threshold for opalescence (NTU – nephelometric turbidity units)?*

A: A maximum turbidity value of 12 NTU is often used for the threshold, as it is the midpoint of the opalescent range of 6 to 18 NTU as classified by the European Pharmacopeia for parenteral products. It should be noted, however, that there are examples of approved products with opalescence higher than this value (https://doi.org/10.1016/j.xphs.2021.05.005). As such, there is currently no established threshold NTU value associated with the risk of phase separation or other adverse solution behavior. This needs to be characterized on a case-by-case basis during product development.

Q: *Assuming the underlying mechanisms for both phenomena are strong protein-protein interactions, why are some mAb solutions highly viscous but not opalescent at all, and vice versa?*

A: Viscosity and opalescence in mAb solutions are related to the interplay between attractive and repulsive, as well as short-range and long-range, protein-protein interactions, and your question has been the topic of many studies. A simple to way to think about this is, opalescence arises when proteins clump together into particles or phase-separate, while viscosity is caused by extended protein networks that remain distributed homogeneously throughout the solution. See, for example, https://doi.org/10.1002%2Fjps.21797 and https://doi.org/10.1107/S2053273322099193.

Q: *You mentioned a publication that related k_D at low concentration, to viscosity and turbidity at high concentration. Is k_D a better predictor than the second virial coefficient for this application? How about for solutions with some population of reversible aggregates at lower concentration?*

A: The theoretical differences between k_D and the second virial coefficient A_2 arise from the added contribution of a term related to viscosity in k_D. Both are equally affected by reversible aggregates.

There are also practical considerations, for example SLS measurements of A_2 are more prone to interference from large particles or aggregates, while DLS measurements of k_D are more prone to interference from small excipients like sucrose or arginine, making one or the other better for studying the specific protein formulation.

Q: *My protein is opalescent at 4 degrees C. Does the opalescence at low temperature in general correlate with some functional and structural properties?*

A: Opalescence arises from reversible self-association, which depends on factors such as buffer pH, ionic strength, and temperature. This is "colloidal" behavior and does not necessarily correlate with conformational changes that may relate to function. However, temperature-dependent structural changes leading to increased light scattering, for instance as caused by irreversible aggregation, could also explain your observation. Additional characterization is needed to address nature of the observation. Some example approaches include characterization by size exclusion chromatography and testing the reversibility of the temperature dependence of opalescence.

Q: *Can this method of measuring the opalescence and viscosity in mAb solutions be directly applied to ADC formulations as well?*

A: Yes, the measurement and analysis would be the same as for mAbs or any other macromolecule.

Particle analysis

Q: *Particle concentration is often measured using techniques such as TRPS (tunable resistive pulse sensing) or other particle counting techniques. How do particle concentrations from an SLS/DLS measurement compare to particle counters?*

A: Concentration measurements of relatively homogeneous samples by DLS/SLS are quite comparable to those obtained by particle counters, typically within 30%. The batch (unfractionated) DLS results tend to be less accurate when samples are highly heterogeneous in size or contain mixtures of particles made of different materials. On the other hand, SLS/DLS is sensitive to smaller particles than most counting techniques, measures a robust ensemble (rather than just hundreds or thousands of particles), is quite rapid and never clogs.

Combining light scattering with size-based fractionation (FFF-MALS) using Wyatt's Eclipse™ field-flow fractionation system provides much more accurate particle concentrations for heterogeneous solutions than batch SLS/DLS. FFF-MALS has the added benefit relative to particle counting techniques of being able to isolate fractions of different sizes while providing extended information on each particle size through online spectroscopic or ICP-MS measurements. Plus, it does not clog either!

Q: *Are there data analysis techniques that combine SLS and DLS measurements to estimate particle distributions better than the qualitative results from regularization analysis?*

A: The combination of SLS and DLS does not improve size distribution analysis. Rather, it serves to determine absolute particle concentrations (particles per mL) since both scattering intensity and particle size are required for the calculation.

If high-resolution particle distribution analysis is of interest, it is best to incorporate a size-based separation technique upstream of the light scattering detector. Wyatt's DAWN® multi-angle light scattering detector can be combined with its Eclipse field-flow fractionation system. Such an FFF-MALS setup determines particle size distributions and concentration using multi-angle light scattering (MALS) and/or dynamic light scattering (DLS), which cover complementary size ranges. As an added benefit, combining DLS and MALS provides information on particle shape, and FFF can be used to isolate size-based fractions for further offline analysis.

Q: *Why is the detector response non-linear with particle diameter?*

A: This is dictated by light scattering theory. For a fixed particle concentration (particles per mL), the intensity of static light scattering varies with the sixth power of the diameter, or the square of the volume. Hence even for a fixed total mass of particles, scattered intensity varies with the third power of diameter.

Instrument-specific

Q: *What is the lowest sample temperature that can be measured on the NanoStar? Can you go below 5 degrees C?*

A: The NanoStar can be operated with sample temperatures from -10 to +120 °C.

Q: *Why do you use fixed laser power and attenuation rather than auto-attenuation for the opalescence method?*

A: The NanoStar's auto-attenuation algorithm modifies both the attenuator and the laser power in order to keep the DLS detector (a high-gain avalanche photodiode) from saturation. However, the SLS detector in the NanoStar (a p-i-n photodiode) is not affected by the DLS detector's attenuator, so we need to fix the laser power at a very low level in order to prevent SLS detector saturation by turbid samples.

Q: *Can the NanoStar determine the zeta potential of a sample for colloidal stability analysis?*

A: No, the DynaPro NanoStar does not offer the option of measuring zeta potential. Wyatt's Mobius™ DLS/ELS instrument does so; it measures simultaneously size (via dynamic light scattering) and zeta potential (via electrophoretic light scattering), typically requiring just 30 seconds per measurement and as little as 60 µL of solution. These measurements can be automated by means of an autosampler in case you need to test multiple formulations or production steps.