Live Webinar Q&A Sheet:

Vaccines Illuminated: Biophysical Characterization, PAT, and Quality Control via Light Scattering Techniques

The recorded webinar may be viewed from the Biotherapeutics webinar 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.

Multi-angle light scattering (MALS)

Q: *What is the lower size limit and resolution for FFF-MALS?*

A: Wyatt's FFF-MALS technology combines an Eclipse™ FFF instrument with a DAWN® MALS instrument for effectively separating and characterizing molecules with radii ranging from 1 nm to 1 µm. A single channel and instrument can cover the entire range, though, for optimal performance in a specific range, a membrane of suitable pore size can be selected. FFF can separate two monodisperse populations which differ in molar mass by 40% or in radius by 15%. The resolution in terms of detecting a change in size as fractions elute is very high – on the order of 2-3%.

Q: *Would the presence of any excipients in the sample buffer affect analysis via FFF-MALS?*

A: During FFF-MALS typical excipients elutes much faster than the analytes and do not affect the analysis. Like chromatographic separations, the analyte ends up primarily in the mobile phase regardless of the formulation of the injected sample.

Q: *What's the detection limit of MALS with respect to concentration for vaccine particles?*

A: This depends on a number of factors including particle size and refractive index.

For adeno-associated viruses, with a radius of 13 nm, the lower limit of detection is about 5 x 10^{10} virions/mL in the flow cell. Scientists at the CDC published the limit of quantitation for influenza virions with an average radius of 52 nm as 2.3x10^{6} total viral particles (Bousse, T. et al. Journal of Virological Methods 193, 589–596 (2013)). Assuming an average radius ~40 nm for SARS-CoV-2, we can calculate the limit of quantitation as 2x10^{7} total viral particles.

Q: *Can we use SEC-MALS to determine the equivalency of secondary structures between two different DNA products?*

A: No, MALS is not sensitive to secondary structure.

Q: *What is the reason for the appearance of curved (convex or concave) molecular weight overlays on the chromatogram?*

Assuming the eluting peak is expected to be reasonably homogeneous in molar mass, there are two common causes of curvature in the plot of molar mass versus elution time across the peak.

1. Insufficient or incorrect band-broadening: Since molar mass is determined by the ratio of scattered intensity (measured by the MALS detector) to concentration (measured by a UV or RI detector), the signals from the two detectors must be aligned and corrected for interdetector dispersion, i.e. band broadening. Failure to provide an accurate band broadening correction will lead to upwards or downwards curvature of the molar mass, depending on whether the concentration detector is upstream or downstream of the MALS detector as well as if the dispersion is under-corrected or over-corrected. Application of the band-broadening correction in ASTRA, with parameters determined by measuring a suitable monodisperse standard, eliminates the curvature.

2. Background particles: If the mobile phase contains a steady background of particles, e.g. due to column shedding, then the molar mass that is calculated for each slice is essentially a weight average of the molar masses of the eluting molecule and the background particles. Around the center of the peak the concentration of analyte may be sufficient to overwhelm the particulate signal, leading to a low measured molar mass, whereas at the edges of the peak the particulate signal may dominate, leading to a higher apparent molar mass, and hence upwards curvature of the molar mass plot. The only way to avoid this curvature is to eliminate the background particulates or to narrow the peak selection.

Q: *Is it possible to quantify a mixed signal (% monomer and % HMWs) during co-elution of monomers and HMWs which typically happens during the latter part of a peak during cation exchange chromatography (CEX)?*

A: In general it is not possible to quantify each of two species that elute together. MALS simply computes a weight-average molar mass M_w of whatever is present in the mixture. However, if you assume that the species are known, for example monomer and dimer with known molar masses, then it is possible to extract the %monomer and %high molecular weight from M_w.

The picture above is greatly complicated when the protein concentrations are high, since protein-protein interactions impact the relationship between light scattering intensity, concentration and molar mass. In preparative CEX, not only are the concentrations generally high, but the buffer

conductivity and pH change in the course of elution, leading further variations in the protein-protein interactions which may be quite difficult to track without extensive preliminary characterization of the protein's light-scattering characteristics. Still, there are ways to mitigate these confounding effects. Please contact info@wyatt.com if you would like to explore process analytics using MALS, whether for real-time control or for process development and characterization.

Q: *Can RT-MALS measure AAV particle size and residual DNA levels during anion exchange chromatography (AEX) purification?*

A: RT-MALS can be used together with UV260 and UV280 to determine VgCp, titer and molar masses of AAVs, similar to the measurements made in SEC-MALS. The AAV particle size may also be measurable, though it is close to the lower limit of size quantification by MALS and the result may not be reliable. Residual DNA levels can only be measured properly if they are separated from the AAV, not if they co-elute.

Q: *How does the molecular distribution of heterogeneous samples appear in a MALS measurement? How does one assess the reliability of the measurement?*

A: Since MALS determines the weight average molar mass (M_w) and z-average root-mean-square radius (R_g) of the sample present in the flow cell, it must be combined with a separation method to determine molecular weight and size distributions. Typically the result of such a measurement is displayed as an overlay of molar mass or R_g versus elution time, but ASTRA software can also create distribution plots on linear or log scales indicating differential or cumulative distributions of the weight or number fraction of each molar mass or size.

Reliability can be determined by evaluating the reproducibility and the accuracy of the measurement. The methods themselves are robust and reliable, and the accuracy is specified to be within 5% and reproducibility within 2%. Authors at the NIH have published long-term stability data for a monoclonal antibody, including molar mass data measured by SEC-MALS over a three-year period (Soman, G. *et al. mAbs* **4**, 84–100 (2012).). The measured molar mass of their BSA standard was accurate with reproducibility of 1.3% over 3 years (average and standard deviation of (65 600 ± 888) g/mol). The mAb being studied in this paper was found to be stable for 5 years, and the measured molar mass over 2 years was reproducible within 1.1%.

Composition-gradient Multi-angle Light Scattering (CG-MALS)

Q: *Composition-gradient technology seems to provide data that could rival isothermal titration calorimetry (ITC). Can you comment on this? ITC provides thermodynamic information. But the ability to measure protein-protein interaction (PPI) stoichiometry and affinity here is of interest.*

A: While CG-MALS—which combines a DAWN MALS instrument with a Calypso® composition-gradient system—does not provide thermodynamic information like enthalpy and entropy of an interaction, it does provide both binding affinity and absolute stoichiometry of non-covalent macromolecular complexes such as protein-protein or protein-DNA complexes. Whereas ITC only provides an overall equilibrium dissociation constant K_d for self-association, CG-MALS can determine which oligomers are forming (e.g. dimer, trimer and hexamer) and the equilibrium constants for each. Likewise for multivalent heteroassociations, where one—or both—partners have more than one binding site for the other, CG-MALS can determine not only the stoichiometric ratio and overall binding affinity (which ITC provides) but actually the absolute stoichiometry (number of molecules of each type present in the complex) and K_d for each complex – for example, it differentiates 1:1 complexes from 2:2 complexes. In fact, CG-MALS can analyze simultaneous self- and hetero-association to determine the absolute stoichiometries and affinities of each complex that forms (e.g. A homodimerizes, B forms homotrimers and A and B interact to form AB heterodimers). It is an excellent tool for studying the interactions of a homotrimeric viral surface glycoprotein with an antibody, as shown in the webinar *Analyzing Ebola virus glycoprotein and its interactions with therapeutic antibodies using CG-MALS*.

In addition, CG-MALS does not require concentrating the samples as does ITC. Typically they are prepared at 3 – 5x K_d, while ITC requires concentration up to 50x K_d which may lead to aggregation or precipitation. The total sample requirement of CG-MALS is similar to, or somewhat less than, standard ITC, and run times are shorter.

General: Light scattering detectors and separation techniques

Q: *How does multi-angle light scattering (MALS) compare to dynamic light scattering (DLS) in performing the following:*

Conformational analysis

A: Proteins

In general, conformation can be assessed by comparing molecular weight and size. MALS (with a concentration detector) determines both molecular weight and size, but the typical lower limit for radius is about 10 nm, which excludes size measurements of most proteins. Often online DLS is added to a SEC-MALS system in order to measure the hydrodynamic radius R_h of proteins, since this technique can go down to 0.5 nm in R_h. What's more, proteins of different size will usually

elute at different times from SEC, even if they have the same molecular weight, so they can be analyzed separately. Hence combining online MALS and DLS results in a comprehensive separation and biophysical characterization of proteins for understanding conformation.

'Batch' DLS, measured in a cuvette or microwell plate, determines both size and molecular weight, but if the sample is not monodisperse then you will get weight-average molecular weight and z-average size, both of which are heavily influenced by the high-molecular-weight species (usually aggregates). Hence the assessment of conformation by DLS alone may be skewed. Separating and characterizing the individual species as is done in SEC-MALS is preferable by far.

Polymers

As described in the white paper WP1003: Analyses of Polymer Branching, the branching of a polymer-based vaccine such as polysaccharide or a glycoprotein is most reliably determined from the slope ν of the log-log plot of the rms radius R_g vs molar mass M_w. Since MALS determines both parameters—simultaneously and independently—downstream of a size-based separation technique such as SEC-MALS or FFF-MALS, it is the ideal technique for this purpose, as long as molecules are larger than about 10 nm in radius. For smaller molecules, the hydrodynamic radius R_h as determined by differential viscometry or online DLS may be substituted for R_g. The addition of a ViscoStar® differential viscometer to a MALS system introduces the parallel capability for dVI analysis to determine branching and the Mark-Houwink parameter.

Because polymers tend to by highly heterogeneous, batch DLS—which only determines average values—cannot provide relevant information on polymer conformation.

The shape of particles, protein aggregates or amyloid fibrils

The same principle is also applied to distinguish between amorphous proteins aggregates and amyloid fibrils. Increasing values of the slope ν indicate a change in shape from spherical, to random coil, to linear conformations. DLS alone cannot provide indication of particle or aggregate shape.

Relating the rms radius R_g (determined by MALS) to the hydrodynamic radius R_h (determined by DLS) is helpful for understanding the shape or conformation of viral particles, lipid nanoparticles and aggregates larger than 10 nm in radius. The analysis calculates the shape factor, $\rho = R_g/R_h$, for each eluting fraction. The theoretical shape factor of ellipsoids, often taken to represent macromolecules and their assemblies or aggregates, is well known as a function of the semi-axis ratio. Hence MALS with simultaneous, integrated DLS by means of the WyattQELS™ module, NanoStar or Mobius is an independent and orthogonal method to confirm the shape of an assembly or nanoparticle. We encourage you to visit our conformation page to learn more.

Measuring colloidal stability

A: Colloidal instability arises from various sources such as electrostatic or hydrophobic interactions. These interactions are often quantified as the second virial coefficient (A_2 or B_{22}), determined by static light scattering (SLS or MALS), and as the diffusion interaction parameter (k_D), determined by DLS. The measurements determine scattered intensity or diffusion coefficient over a concentration series. The white paper WP5004: The Diffusion Interaction Parameter (kD) as an Indicator of Colloidal and Thermal Stability provides an overview of how k_D measurements may be utilized to investigate the stability of a monoclonal antibody.

A_2 is a purely thermodynamic property of the solute in a given solvent, while k_D incorporates both thermodynamic and hydrodynamic components. A comprehensive explanation of k_D and A_2, and how to measure them, is available to Wyatt customers in TN9102: Assessing Nonspecific Interactions with Light Scattering: A_2 by SLS and k_D by DLS. Discussions of these parameters may also be found in standard textbooks dealing with polymer physics.

Because these measurements relate to intermolecular interactions, they are generally carried out in static systems without separation, such as microcuvettes, microwell plates or in a stop-flow configuration. SEC-MALS is not commonly used to assess colloidal stability except to characterize irreversible aggregates. CG-MALS, a stop-flow method without separation, may be applied for maximum precision in A2 determination.

Formulation development screening

A: Generally, for high-throughput screening studies of protein-based therapeutics, mRNA-bearing lipid nanoparticles, viral vectors or other biomacromolecules, the DynaPro® Plate Reader is an excellent tool. HT-DLS evaluates candidates for quality and developability, and formulation buffers for optimal stability and minimal aggregation, as described in WP5003: Automated dynamic and static light scattering in microwell plates.

Following a pre-screen step to select the best candidates, SEC-MALS and FFF-MALS provide a more comprehensive analysis of vaccine molecules or particles before and after applying stress. We encourage you to read WP9007: Characterizing vaccines with light scattering which describes the uses of DLS and MALS in conjunction with different separation techniques.

Stability during storage and transportation

A: Freeze-thaw stability study is an important assay that is relevant to the storage and transportation of vaccines. Like the previous case, we recommend HT-DLS for initial screening and MALS coupled with a separation technique for a more comprehensive analysis. An HT assay for measuring stability is discussed in detail in AN5008: High-throughput freeze-thaw stability studies with the DynaPro Plate Reader

Q: *Which technique do you recommend for separating and quantifying a protein sample having a mixture of two monomer conformations?*

A: SEC is the best mode of separation for proteins. In both SEC and FFF separation is based on hydrodynamic volume. The chemical composition does not play any role. Conformers with the same hydrodynamic volumes will elute at the same elution volume no matter of molar mass and/ or chemical composition, but different molecules might have different conformations or densities which would result in at least some separation. MALS can also be used with gradient chromatography such as ion-exchange chromatography or reverse phase chromatography, but the measurement capabilities are limited relative to SEC or FFF.

Q: *Is plate-based DLS temperature sensitive?*

A: Yes, both the measured diffusion coefficient and the computed hydrodynamic radius are directly affected by temperature. All DLS measurements, be they plate-based or cuvette-based, rely on the Stokes-Einstein equation determine the hydrodynamic radius. Visit our DLS theory page to get an in-depth understanding of DLS.

Q: *Can FFF separate full and empty viral particles?*

A: FFF separates by hydrodynamic radius, and the genomic content does not generally impact viral particle size, so it would not be able to resolve empty from full virions. However, you can still use either FFF or SEC in combination with MALS, UV and possibly differential refractometry to analyze capsid content, titer and other attributes of small viral vectors like AAV via the Viral Vector Analysis method provided by ASTRA.

MALS can also be coupled to a technique such as ion-exchange chromatography that does resolve empty and full AAVs, at least partially, to determine the Vg/Cp (full:total) ratio in each eluting fraction. Note that this method does not differentiate partial genomes from mixtures of co-eluting empty and full capsids. ultraDAWN™ may be coupled inline with a prep-scale FPLC system to perform these measurements, in real time, at flow rates up to 150 mL/min, or configured for online sampling for higher flow rates.