Live Webinar Q&A Sheet:

Biophysical characterization and quality control of vaccines and gene vectors with light scattering

The recorded webinar may be viewed from the Biotherapeutics 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 web site.

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

DLS

Q: *How does the DynaPro Plate Reader measure particle concentration?*

A: To determine the particle concentration with a DynaPro® Plate Reader or NanoStar®, DYNAMICS® software uses the particle's volume, the scattered intensity at angle 0, and the refractive indices of the particle and solvent. The particle volume is calculated from the hydrodynamic radius determined by dynamic light scattering (DLS), assuming a spherical shape. The scattered intensity at angle 0 is calculated from the static light scattering (SLS) intensity measured at the detector's scattering angle, assuming the form factor (angular scattering dependence) of a sphere with the measured hydrodynamic radius. The refractive indices, which must be entered by the user, can be obtained from the literature or measured. In this way, the instruments measure the concentration of particles up to 160 nm in radius (Plate Reader) or 175 nm (NanoStar).

Q: *Does AAV serotype affect the DLS concentration measurement? Do vector samples of different AAV serotypes respond differently when changing sample concentration, sample buffer and temperature?*

A: The same constants (refractive index) are used for all of the AAV serotypes in DLS. The physical-chemical behavior of different AAV serotypes may vary as a function sample concentration, buffer composition and temperature, but there is no impact on how concentration is measured or the parameters needed for DLS analysis. Physical-chemical changes can be identified by DLS if they lead to changes in the particles' diffusion coefficients.

Q: *What is the DLS precision (reproducibility & inter-assay) for RNA-LNP and what is the acceptable polydispersity index (PDI)? How does sample prep affect size and PDI measurement?*

A: The DLS inter-assay repeatability is typically 1-2%, and 5% for reproducibility. The acceptable PDI depends on many factors and is primarily determined by safety and efficacy concerns rather than by instrument performance. However, keep in mind that sample preparation such as filtration or

centrifugation, measurement settings, and analysis conditions could all affect size and PDI measurements. DLS is often used to screen the RNA-LNP samples throughout development, or to detect big differences in size and PDI in QC. SEC-MALS or FFF-MALS should be considered for more detailed analysis, with high resolution.

Q: *Would you say it is a "must" to use SEC or FFF-MALS for QC testing of an adenovirus size (D_h = 100-150 nm) or would it also be possible to use a DLS method for size determination of an adenovirus product (as a QC release test method)?*

A: Depending on your specific QC requirements, DLS can indeed be used as a QC release test method, and it is currently being used as such by some of our customers. While we always recommend SEC or FFF-MALS in scenarios where maximal precision and resolution is required, the NanoStar and the DynaPro Plate Reader are excellent tools for rapid and robust batch screening of particle size, molar mass or concentration. For example, DLS is fine for identifying the primary virus size and concentration, but will not reliably detect small quantities of oligomers such as dimers or trimers; SEC/FFF-MALS will do so.

Q: *Are 96-well plates available?*

A: Yes, standard 96-well plates are available for both DLS and SLS measurements with the DynaPro Plate Reader.

Q: *Can the DLS plate reader differentiate membrane vesicles from other nanoparticles, like lipoprotein, lipids etc.?*

A: The DLS technique measures the translational diffusion coefficient of the particles in a sample. Consequently, if two particles of different chemistries have identical diffusion coefficients, they appear the same to DLS.

Q: *Would it be possible to measure the zeta potential using DLS plate reader?*

A: The DynaPro Plate Reader cannot measure the zeta potential. That said, we do offer the Mobius™ for zeta potential measurements. The Mobius is the first and only light scattering instrument that makes reliable, reproducible, and non-destructive electrophoretic mobility measurements of macromolecules as small as 2 nm in radius (as well as micron-size nanoparticles) in most aqueous buffers. It is also Wyatt's best-performing detector for nanoparticle size analysis by means of batch DLS. Please visit our Mobius product website, or contact us at info@wyatt.com for more information.

Q: *Is there agreement between DLS and other particle concentration assays (NTA, EM, FAX...)?*

A: There is typically good agreement between DLS particle concentration and robust measurements by other methods when the particles fall into the ranges of both. However, not all methods can

measure particles in the same size or concentration range. For example, NTA measures particle concentration over diameters of 50 – 800 nm, while Wyatt's DLS instruments measure over diameters of ~ 2 – 350 nm.

Q: *Does DynaPro use forward scatter, and if so, at what angle?*

A: The DynaPro NanoStar cuvette-based DLS instrument measures scattered light at 90°. The DynaPro Plate Reader detector angle is 158° backscatter in aqueous solution.

Q: *Does the Mobius take well plates or are samples loaded do it one by one?*

A: The Mobius does not take well plates, but the Mobius flow cell enables automated sample handling with a standard autosampler. Samples may also be measured in microcuvettes.

MALS

Q: *How is MALS used to get estimates for viral genome titer and Vg/Cp for AAVs?*

A: MALS data are combined with UV and possibly RI data to determine the viral genome titer. The combination of UV and RI or, alternatively, absorption at two UV wavelengths, provides the total concentrations of protein and nucleic acid, and these may be integrated across the peak to calculate the total protein mass and nucleic acid mass. Including MALS data, the molar masses of the protein capsid and the encapsidated DNA or RNA may be calculated. Finally, dividing the total protein mass by the capsid molar mass gives the capsid titer, and dividing the total nucleic acid mass by the encapsidated molar mass gives the viral genome titer. Vg/Cp is defined as the ratio between the viral genome titer and the capsid titer.

Q: *What range of angles and optical wavelengths are sampled in your static light scattering instrument?*

A: The DAWN uses MALS angles from 15 to 165 degrees. The nominal laser wavelength is either 660 nm or 785 nm, depending on the MALS instrument's configuration.

Q: *What are the limits of detection for viruses and lipid nanoparticles?*

A: The limits depend on size and the refractive index of the particle. For smaller viruses such as AAVs, the limits are ~ $5x10^{10}$ particles/mL to $1x10^{15}$ particles/mL. For larger, 100 nm diameter viruses, the limits of detection are approximately $1x10^{6}$ to $2x10^{11}$ particles / mL, respectively. For 200 nm lipid nanoparticles, I would estimate the limits of detection as $1x10^{5}$ and $1x10^{10}$ particles / mL. Recently we have developed an optical hardware kit that may be added to a DAWN in order to extend the upper range roughly 20-fold, at the expense of increasing the lower limit roughly 10-fold.

Q: *How is the built-in ultrasonic cleaning device activated in the DAWN?*

A: The COMET is directly controlled by Wyatt's ASTRA® software package for MALS and can be programmed to automatically clean the flow cell after or even between measurements. Moreover, the device operates in situ while the mobile phase is flowing through the cell. There is no need to disconnect any fluid fittings, change solvents or disassemble any of part of the setup while the mobile phase flushes out the particles.

Q: *Did you ever use IEX-MALS for these types of molecules? It will give you additional information about heterogenicity by charge.*

A: Yes, IEX-MALS – where a MALS instrument is placed in-line with ion-exchange chromatography – has been performed on a variety of molecules, e.g., proteins and AAV samples. That said, SEC-MALS tends to be the method of choice for most of our users, and using SEC-MALS for second dimension measurements on fractions from ion-exchange (IEX) chromatography is a popular alternative to IEX-MALS.

Q: *Can the hydrodynamic radius R_h be measured during SEC-MALS or FFF-MALS?*

A: Yes, R_h may be measured simultaneously with MALS using a DAWN® or miniDAWN® MALS detectors. We offer two solutions for doing so: the internal WyattQELS™ module, or the DynaPro NanoStar DLS instrument connected to the MALS detector via an external optical fiber cable.

Q: *What are the accuracy and precision of the particle concentrations determined by DLS and MALS? How reliable are particle concentration values for LNP samples? How can you determine if the measurement is reliable?*

A: Particle concentrations measured with Wyatt's MALS or DLS methods have high precision and accuracy, and are generally in good agreement with results obtained with orthogonal methods. The precision and sensitivity depend on the specific method. For example, the AAV concentration by SEC-MALS, which uses MALS with UV and RI concentration detectors, is precise to 1 – 2% and accurate to ~ 5%, and even precision in Vg/Cp of AAVs is 3% with a minimum quantifiable change of 5% for SEC-MALS multi-detector analysis. Even a relatively complex technique, the SEC/FFF-MALS multi-detector LNP payload analysis, has been validated to within a couple of percent by size-based fractionation of various LNPs with subsequent offline analysis of the fractions [Jia, X. et al., (2021) *J. Chromatography B*. 1186, 123015]

Particle concentration measurements by MALS or DLS that rely on determining the radius are somewhat less robust, since a 5% deviation in radius can result in a 30% deviation in concentration, and for 50 nm radius polystyrene we have measured a 17% uncertainty. Likewise, an error of 1% in the specified refractive index results in 20-30% error in concentration. For viruses or LNPs, where sample composition can be more complex, I would expect the method to yield reproducibility within 30%. The ASTRA and DYNAMICS software provide multiple utilities for

rapid evaluation of data quality, including estimates of statistical precision and data quality indicators.

FFF

Q: How much sample volume is required for FFF-MALS?

A: With the analytical short channel, we usually inject 2-10 µL of the starting AAV concentration. You can also load more in order to characterize low-abundance species such as aggregates.

Q: What is the dilution factor in the AF4 channel?

A: Since FFF provides a large degree of flexibility during method development, the dilution factor depends on the method in question. It can be for example 10-fold or up to 40-50 fold or greater. That said, dilution can be reduced with the new Eclipse™ Dilution Control Module, and you can even design your method to produce a sample that is more concentrated that the injected sample.

Q: How does the run time of an SEC-MALS experiment compare to that of an FFF-MALS, and can FFF-MALS be run in a high throughput setting?

A: SEC-MALS analyses of AAVs and other biologics generally take 20-30 min. For FFF-MALS, the run time and throughput largely depends on the channel volume and the complexity of the separation method. Typical methods that we employ take less than 30 min but may run up to 60 min. Wyatt's Eclipse FFF-MALS system is compatible with standard HPLC autosamplers, allowing for automation, though clearly not high throughput at 30-60 minutes per run.

Q: How does recovery from FFF compare to SEC-MALS?

A: This is highly dependent on sample, column and membrane chemistry. Sometimes an SEC column cannot be found that elutes a sample, and FFF is the obvious choice, and sometimes interactions with the membrane reduce recovery in FFF. For the most part (but not always), membrane interactions in FFF can be minimized by selecting the right membrane material and buffer. The recovery of some fragile analytes such as lipid nanoparticles will benefit from the lower shear in FFF, especially with the dispersion-inlet channel.

Q: Can you give any examples of the limitations of AF4?

A: Like any other method, AF4 has its limitations. For example, it works for a finite size range, a finite range of pH for aqueous solvents and a limited set of organic solvents. Other than those, the main potential limitations relate to:

- Aggregation: During the focusing step the sample is concentrated close to the membrane. If your sample undergoes concentration-dependent aggregation, depending on the achieved

concentration in the focusing step, this may lead to either reversible or irreversible aggregation. This behavior can be overcome using the dispersion inlet channel (formerly, the frit inlet channel), which does not require focusing.

- Dilution: Once the sample exits from the channel, it is diluted by a factor ranging from 10 to 1000, which may lead to dissociation, escape from encapsulation, and/or poor signal to noise ratio. Dilution can be reduced by up to 10x with the new Eclipse Dilution Control Module.
- Membrane interactions: certain analytes will stick to the FFF membrane, precluding good recovery and separation. We offer two membrane materials, polyether sulfone and regenerated cellulose, and in addition users may prepare custom membranes from other ultrafiltration membrane materials.

Q: *Can the Eclipse be used as a semi-preparative technique for collecting fractions? How much sample volume can be separated by FFF-MALS with a fraction collector?*

A: Yes, the Eclipse can use a range of channels, from a low-volume analytical channel with microgram loading to a larger volume, semi-preparative channel which can handle a few milligrams of material. The system can be integrated with a standard HPLC fraction collector for automated fraction collecting. The actual solution volume, rather than analyte mass, is not important in FFF since excess buffer is removed during the FFF focusing step. The DCM (dilution control module) can effectively concentrate the fractions without compromising resolution and other adverse effects.

Q: *How does DLS data collected in FFF using ASTRA compare to stand-alone DLS?*

A: On the one hand, stand-alone DLS offers a larger range of hydrodynamic radius (0.2 – 1000 nm) compared to on-line DLS (0.5 – 300 nm). Sensitivity of stand-alone will also be higher since FFF generally dilutes the sample. On the other hand, the resolution of size distributions determined by on-line DLS is far greater than stand-alone DLS: whereas stand-alone DLS can only resolve populations that differ by 3x – 5x in radius, with FFF it is possible to resolve differences as low as 15-20%, and subcomponents of any size that can be separated by the FFF method may be characterized individually. In terms of accuracy, particle size standards give very similar results – to within a few percent – in the two methods.

Q: *Can DLS and FFF/SEC-MALS multi-detector analysis characterize lentiviral vectors?*

A: The DLS and MALS methods discussed may also be used to characterize lentiviral vectors. Due to their relatively large size, we typically recommend FFF over SEC when separation is desired. However, the multi-detector methods used to determine LNP payload cannot at this time determine lentiviral genomic content.

Q: *What kind of resolution can you expect for AF4, with respect to the sizes at small and large end since it spans both?*

A: The resolution of FFF is tunable, and determined by the FFF method. FFF, like SEC, separates based on hydrodynamic volume, and can separate macromolecules and nanoparticles from 1 nm - 1000 nm and beyond.

Q: What happens if I have particle size around 1-10 µm?

A: Particles in this size range may be amenable to separation by FFF by applying the so-called steric elution mode, wherein larger particles elute first (as opposed to the normal FFF elution order). Online MALS and DLS are not, however, suitable for determining the sizes of eluting fractions larger than 1 µm.

Q: I read a recent publication considering the frit inlet channel for FFF analysis of LNPs. Is that channel currently favored for LNP analysis?

A: While Mildner et al., recently demonstrated that the frit inlet channel should be used for LNP analysis in order to guarantee high recovery [Mildner et al., (2021) Eur J Pharm Biopharm 163:252-256], we recommend to first try analyzing your LNPs using the short channel. If the results from the short channel are not satisfactory, you may switch to the frit inlet channel (now called the dispersion-inlet channel).

Q: You show an example of aggregate dissociation upon dilution. Can the samples be analyzed without dilution by DLS or FFF to provide a meaningful particle size distribution?

A: Samples can be analyzed by DLS without dilution if the particle concentration is not so high that it leads to detector saturation or multiple scattering. In FFF, dilution is inherent to separation, but the dilution factor is determined by the specific settings and steps used in the FFF method, and can be reduced significantly with the Dilution Control Module.

Q: Can we use FFF-MALS to characterize liposomes? Also, when measuring EE% with MALS, do we need to lyse the liposome, or can we run the intact liposome solution, so we can get the free RNA signal along with the liposome signal?

A: FFF-MALS is a powerful tool for liposome characterization, and can determine both the free RNA and the encapsulated RNA quantity in intact lipid nanoparticles, in the same run. For more details, see e.g., our White Paper WP2608: Lipid Nanoparticle and Liposome Characterization with FFF-MALS-DLS or contact us at info@wyatt.com.

Q: *How do you quantify lipids if there is no UV chromophore?*

A: For particle size and concentration of liposomes or lipid nanoparticles, MALS suffices and a UV concentration signal is not needed. For payload quantification, molecules that do not produce a quantifiable UV signal may be still amenable to characterization by SEC/FFF-MALS multi-detector analysis, as the method leverages simultaneous online MALS, UV, and RI measurements. For

more information about the method, see e.g., Jia, X. et al., (2021) Enabling online determination of the size-dependent RNA content of lipid nanoparticle-based RNA formulations. *J. Chromatography B.* 1186: 123015. https://doi.org/10.1016/j.jchromb.2021.123015.

Q: *Are there limitations in LNP size when using online DLS for AF4 R_g/R_h evaluation? For example, are there issues with particles < 100nm size?*

A: The lower limit for R_g measurements by MALS is 10 nm, and the lower limit for DLS measurements of R_h can be as low as ~0.5 nm. Therefore, R_g/R_h for LNPs with diameter < 100 nm in size may be determined. The upper size limitation for LNP would be a diameter of 600 nm for DLS and 1000 nm for MALS.