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Live Webinar Q&A Sheet:

Successful SEC-MALS Analysis of Proteins and AAVs

The recorded webinar may be viewed from the SEC-MALS webinar page. These questions were submitted by live viewers. Additional information on SEC-MALS, DLS, CG-MALS, and FFF may be found on the Wyatt web Library under Webinars, Application Notes, Featured Publications, 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.

Questions & Answers

Q: *Can you elaborate on how the SEC-MALS calculates the hydrodynamic radius and radius of gyration R_g?*

A: Multi-angle light scattering (MALS) can determine the radius of gyration (R_g) by measuring the angular dependence of the light scattering intensity. Inside the DAWN, we simultaneously measure the scattering intensity at 18 angles, and we can use that angular dependence to measure R_g from 10 nm to 500 nm. The hydrodynamic radius (R_h) is measured separately and independently in the same measurement volume by dynamic light scattering (DLS) using a dedicated DLS detector. The DLS detector can be incorporated inside your instrument with the WyattQELS module or using a fiberoptic connection to a NanoStar or Mobius. More information about the static and dynamic light scattering theory can be found here.

Q: *How does the quantitation of aggregates using the new column compare to methods such as AUC?*

A: The XBridge Premier columns offer high-resolution separation, making them great columns for the main species analysis. Although they can reveal the presence of oligomers and allow quantitation by MALS, some larger aggregates may be removed due to the small pore and particle size as well as the limitation of SEC. Hence, if one wishes to quantify all the aggregates in the sample, other techniques, such as FFF-MALS, DLS, or AUC, that don't remove aggregates provide good orthogonal methods.

Q: *How do you define LOQ and LOD?*

A: For each specification, Wyatt has defined the limit of quantitation (LOQ) as the minimum sample requirement to measure within the acceptable tolerances of the technique. For example, the MALS sensitivity specifications on our website are the minimum requirements to achieve a molar mass measurement with ±5% accuracy and reproducibility under the listed chromatography conditions. The *AAV SOP Guidance Manual* provides detailed information for establishing suitability criteria and describes the LOQ established by Wyatt.

Q: *Can you distinguish between free DNA and DNA inside the particles?*

A: The XBridge Premier GTx column is an AAV and nucleotide column. With sufficient resolution, the free DNA and the loaded particle should separate into distinct peaks with SEC. Additionally, we can identify pure DNA from the

UV, MALS, and RI signals, as the responses on these detectors are different between free and DNA, protein, and AAV.

Q: *Is the XBridge compatible with high pH mobile phases?*

A: The pH range for the XBridge Premier Protein SEC column is 2.5 to 8.0, and for the XBridge Premier GTx column, the pH range is 2.5 to 8.5.

Q: *Does increasing the number of angles increase accuracy? What other benefits does having 18 angles in the DAWN, as opposed to the miniDAWN with 3?*

A: With 18 angles, we can better capture the angular dependence of light scattering intensity of larger species and increase the upper limit of size (R_g) to 500 nm compared to the 3-angle miniDAWN's 50 nm limit. Three angles are the minimum requirement to fit any linear trend; additional angles give you more confidence in detecting even a small trend. Even relatively small analytes like proteins can benefit greatly from the additional detectors for better data consistency, accuracy, and precision. It also provides more tolerance to a less pristine HPLC system, making the SEC-MALS method more robust and user-friendly.

Q: *Could you highlight the different temperature settings available in SEC-MALS?*

A: We offer multiple temperature-controlled options for DAWN. The heated-cooled DAWN can perform measurements from -15 °C to +150 °C. An ultra-high temperature DAWN is available for high-temperature GPC and temperatures up to 210 °C. More information can be found here. The Optilab differential refractive index detector has a temperature range of 4 °C to 65 °C, as described here.

Q: *If separation isn't required to distinguish the fullness of particles, how can you determine the capsid content (Vg/Cp)?*

A: Similar to our Protein Conjugate Analysis, we collect concentration data from multiple sources simultaneously in addition to the MALS data. For AAV analysis, this can be any combination of UV280, UV260, and/or differential refractive index (dRI). Since the vehicle and the gene payload have different extinction coefficients and refractive index increments (dn/dc), we determine the composition across the entire peak. From the compositional data, we know how much protein and how much DNA is eluting. We can simultaneously determine the total concentration (Cp) and the full-to-total ratio (Vg/Cp) without separating the empty from the full particles. You can find more information here.

Q: *Most SEC columns have close to a 1 MDa exclusion limit or less. Do you recommend any Waters SEC columns for large particles in the range of MW = 10 - 20 MDa?*

A: We offer a wide range of columns; please feel free to explore the website. As shown in the webinar, the XBridge Premier GTx column, which has a molar mass range of 15 kDa to 10 MDa, can successfully separate AAV monomer (~4-5 MDa) from dimer and oligomeric aggregates. We also offer a 1000 Å pore size column that can be used for molecules and particles up to ~20 MDa. However, above a certain particle size (typically ~100 nm in radius), there may be no suitable columns. In this case, FFF can provide a wider range of separation and an effective alternative separation platform.

Q: *Typically, how long does it take to run one sample?*

A: Sample run time depends on the column volume and flow rate. The run time should allow the column to be completely clear of samples and ready for the next injection. For example, a 7.8 mm x 300 mm column has

approximately 14.4 mL of internal volume. Generally, 30 to 40 min runtime is appropriate for a 7.8 mm x 300 mm column at 0.5 mL/min and a 4.6 mm x 300 mm column at 0.3 mL/min.

Q: *What is the nominal pore size of the new AAV SEC columns?*

A: XBridge Premier GTx column utilizes 450 Å pore-size particles. Detailed specifications can be found here.

Q: *Is SEC-MALS accurate to quantify LMW species or not because those species are not very abundant in the sample? More specifically, referring to the molecular weight.*

A: Our MALS instrument can measure molar mass as low as 200 Da. The exact suitability for LMW species depends on the abundance and the retention on the column. We have multiple examples of LMW molar mass determination with MALS, including these application notes: "Polyurethane—absolute molar mass by SEC-MALS" and "Low-molecular-weight Polystyrene."

Q: *What is the typical column longevity for the XBridge?*

A: Columns as a consumable experience wear and tear under mechanical stress and particulate contamination from the sample and mobile phase. We can monitor the column health by measuring the light scattering noise with the health indicator on DAWN and in ASTRA. Columns should be reconditioned or changed when they become noisy. Depending on your usage, the mileage might vary. With this said XBridge columns are designed to be robust and long-lasting.

Q: *Could you please provide me with some protocol for membrane protein analysis in the presence of detergents?*

A: We have several examples of membrane protein analysis on our website, including these application notes: "Lipid/membrane protein complexes analyzed by SEC-MALS" and "Conjugate Analysis: Lipid Transport Proteins." Successful analysis of these protein-detergent complexes requires ASTRA's Protein Conjugate Analysis. You can find more information about conjugate analysis, including step-by-step instructions for how to use the functions in ASTRA, in this technical note.

Q: *Is there a mass limit for SEC-MALS to accurately detect large aggregates?*

A: The 18-angle DAWN instrument can measure up to 1 GDa (1 x 10^9 Da) or 1 µm in R_g. However, the practical limitation in SEC-MALS may be lower than this since the SEC column can block or filter out large aggregates. FFF-MALS can utilize the full spec of the MALS detector since FFF doesn't remove large aggregates.

Q: *What MALS instrument was used for the 1st part of the presentation: DAWN or miniDAWN?*

A: The 18-angle DAWN was used throughout the presentation.

Q: *What is the minimum flow rate for DAWN and Optilab?*

A: Both DAWN and Optilab can be operated in batch mode, where the flow is stopped during the measurement. However, when connected to LC systems for chromatography or separation mode, we recommend a minimum flow rate of 0.3 mL/min. This is to ensure a uniform concentration across the diameter of the flow cell.

Q: *What would you recommend while seeing BSA or samples with the correct molecular weight for the monomer but higher than usual molecular weight for dimers?*

A: There are several possibilities for this observation. For example, it is possible that the dimers are co-eluting with other higher-order species. Alternatively, the concentration of the dimer may be very low, introducing

concentration error, which impacts the measured molar mass. For specific recommendations for your data, please contact support@wyatt.com.

Q: *Would it be a good idea to re-calculate UV extinction coefficients because of the increased baseline separation between single AAV and aggregates?*

A: Detailed instructions for measuring capsid and genome extinction coefficients are provided in the *AAV SOP Guidance Manual*. In general, the control samples used to determine the extinction coefficients should be relatively free of aggregates, so the difference in resolution should not impact the calculation of the extinction coefficients.

Q: *With the introduction of this BEH column, how will the method perform overall compared to FFF-MALS?*

A: The XBridge Premier GTx columns offer superior resolution for monomers and oligomers. However, its 2.5 µm particle size and 450 Å pore size can remove large aggregates present in the samples. And it is unsuitable for large modalities such as lipid nanoparticles (LNP), lentiviruses, liposomes, and others. FFF-MALS is a more suitable technique for these very large modalities or aggregation studies.

Q: *How easy is it to keep the light-scattering cell 'clean' when using AAV samples? Is the analysis of crude samples possible?*

A: We have not had any issues keeping the flow cell clean with AAV samples in our lab. SEC-MALS analysis of AAVs is typically limited to DSP (downstream processing) samples. The USP (upstream processing) samples could contain too many other materials that co-elute with AAV. For general maintenance and ensuring clean HPLC and MALS detectors, we provide a thorough tech note and video guide on the Wyatt Support Center that guides users through the maintenance of cleaning of the flow cell.

Q: *How well does this system estimate the % full/empty of mixed AAV samples?*

A: With our SOP guidance manual, the viral vector analysis procedure provides precision in Vg/Cp ± 3% and a minimal quantifiable change of ± 5%. More information can be found here.

Q: *Is there another way to measure the dn/dc value without using six different concentrations?*

A: Besides using a series of concentrations to calculate the dn/dc value, which could require the use of a large sample volume, We also can use the peak area of the RI signal to estimate dn/dc; this is called the on-line method. However, this method requires assuming 100% mass recovery from the column and known injection mass. The tech notes will walk you through the steps of setting up the experiments.

Q: *Is there any suggested cleaning protocol, including suggested buffer and supplies? Specifically for the system and column?*

A: We provide multiple technical notes on the Support Center with recommended best practices for the startup and shutdown of the HPLC System. In addition, the SEC-MALS Noise Assessment Guide provides detailed information for assessing and determining the source of noise in the system. Additional information for in situ cleaning of the MALS detector is provided in this technical note. Contact your HPLC vendor for specific information and compatible solvents to clean the LC system.

Q: *Do you recommend a guard column; how often should it be changed when analyzing well-purified and formulated AAVs?*

A: Although they are not strictly required, guard columns provide protection for the analytical column, and we recommend their use, such as the GTx guard column for AAV. We recommend using an in-line filter post-pump and before-sampler to prevent debris or particulate contaminations from entering the column or the DAWN instrument. Consider replacing the guard column or the in-line filter if you observe increased light scattering noise.

Q: *Currently, most publications and application notes are using 2x PBS buffer, sometimes with ethanol or surfactants as additives. Have you done some extensive mobile phase screening or optimization? Or is the mobile phase less important for AAVs?*

A: Our SOP guidance manual for AAV provides a platform method for AAVs that eliminates the need for extensive mobile phase screening.

Q: *Do you have success separating AAV particles of varying levels of "fullness"?*

A: Since the different fullness of the particle doesn't change the hydrodynamic size of the AAV, it is impossible to separate under Size Exclusion Chromatography (SEC). However, with our SEC-MALS method, separation of empty and full is not required. In some cases, Ion-Exchange Chromatography (IEX) can separate empty AAVs from full AAVs. IEX-MALS can be used to determine the goodness of separation and the purity of these peaks, as well as the titer, as shown in this publication.

Q: *Please explain how SEC-MALS is for absolute molecular weight and not relative molecular weight.*

A: Absolute molecular weight is solved from the physics behind light scattering. A more detailed physics explanation can be found here. In short, the light scattering intensity that we measure depends on the analyte's concentration, the scattering angle, and the molar mass of the analyte. Since we can measure the concentration, the light scattering intensity, and the angle of the scattering, we can determine the molar mass of the analyte at every point across the chromatogram from the first principles. No reference standards or assumptions on the molecular shape are necessary.

Q: *Is the DAWN able to hook up to UHPLC or UPLC systems?*

A: Typically, UPLC systems utilize columns with very small particle sizes of less than 2 µm and with a small column volume. For these < 2 µm columns, we recommend using microDAWN. For UHPLC that utilizes columns with particles between 2.5 µm and 5 µm and for HPLC systems with column particle sizes ≥ 5 µm, we recommend the DAWN.