



Live Webinar Q&A Sheet:

Quantify Viral Vector Attributes with Light Scattering

The recorded webinar may be viewed from the [SEC-MALS](#) webinars 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 web site.

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

SEC-MALS and light scattering with other chromatography

Q: Which column did you use for the SEC-MALS analysis?

A: Most of the analysis was done with the Wyatt AAV column, which is available in standard and narrow bore versions.

Q: Does your software support using UV at 260 and 280 nm?

A: Yes, ASTRA 8 can use the signals from two UV wavelengths in the Viral Vector Analysis method as well as either one of these plus the data from a differential refractive index detector (like Optilab). There are two options for acquiring UV data at two wavelengths: with Wyatt's HPLC CONNECT module using supported Agilent HPLC UV detectors, or read in via the MALS instrument if the UV detector has two analog outputs.

Q: What is the lower concentration limit (capsids/mL) than can be measured with SEC-MALS?

A: The lowest measurable concentration for a typical AAV with SEC-MALS is in the order of 5×10^{10} particles/mL and for DLS, $\sim 6 \times 10^{10}$ particles/mL.

Q: Can MALS be used with gradient chromatography?

A: In general, yes - MALS detectors may be placed downstream of virtually every type of column chromatography including size exclusion, ion exchange, hydrophobic interaction and reverse phase. However, there are certain caveats relevant to an HPLC separation using gradient elution:

- analyses that require the use of a differential refractive index detector such as the Optilab may be limited or not feasible at all, first because of variation of dn/dc and second because the changes in solvent refractive index may exceed the Optilab's overall range;

the specific refractive index increment, dn/dc , which is necessary for calculating molar mass, depends on solvent refractive index and may vary across the chromatogram. If this is the case



then it will be necessary to enter different dn/dc values for different elution volumes in order to obtain accurate molar mass values. In general, variations in dn/dc are fairly low for salt and pH gradients.

For IEX-MALS of AAVs, UV signals at 260 and 280 nm are used as concentration sources.

Q: Can MALS be used with ion-exchange chromatograph (IEX) to confirm that empty and full peaks are pure?

A: Definitely. We can send you information on the specifics of this application if you contact us directly at info@wyatt.com.

FFF-MALS analysis

Q: How does FFF-MALS analysis compare to AUC?

A: Both techniques employ free-solution separation and “first principle” characterization. However, the resolving capability of AUC (SV) is based on one speed that is chosen prior to beginning the run, while in FFF resolution may be varied across the run by adjusting the ratio of cross flow to channel flow. This enables a much greater size range of separation by FFF in a single run.

The techniques also differ in the physics behind the separation: AUC utilizes an induced gravitational field while FFF uses an induced solvent-flow field. Hence AUC separates molecules on the basis of their sedimentation and diffusion coefficient, whereas FFF separates molecules on the basis on their diffusion coefficient only.

Perhaps the biggest advantage of FFF-MALS over AUC is in characterization following the separation. MALS has the ability to characterize the molar mass (M_w) and the rms radius R_g as well as (with the addition of a WyattQELS embedded dynamic light scattering module) the hydrodynamic radius R_h ; it can utilize both UV and differential RI detectors for concentration. This allows for more of a three-dimensional characterization of macromolecules and nanoparticles, including gene therapies: FFF-MALS may determine % of empty and full capsids, fragmentation and aggregation, and particle concentration.

It’s true that AUC can separate empty and full AAV, and even partially full, but the characterization capabilities are a bit more rudimentary: it must use the measured sedimentation coefficient and a modelled diffusion in order to calculate the molar mass.

FFF-MALS and AUC also differ in their ability to collect fractions. FFF-MALS is an open system that can be coupled to an automated fraction collector, but AUC is a closed system that does not allow for any fraction collection. Another difference is the required sample volume: ~400 μ L in the standard sedimentation velocity cell for AUC versus ~ 5 μ L injected for a typical FFF-MALS run.



In terms of time spent on a measurement, an AUC run takes ~ 3 to 5 hours to complete and another 1 to 2 hours to clean and load the cell and analyze the data. FFF-MALS run times are comparable to SEC-MALS—typically 30 to 60 minutes per run—and they use much of the same hardware and software, so manual cleaning and loading are not required, data analysis is much simpler than AUC, and the learning curve is very flat.

FFF-MALS can be implemented from R&D through QC: all of the technology is very flexible, yet the analysis software is fully 21 CFR part 11 compliant and the instruments can all be validated through an IQ/OQ procedure. The same certainly cannot be said for AUC.

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 newly available DCM (dilution control module) can effectively concentrate the fractions without compromising resolution and other adverse effects.

Q: How does the run time of an SEC-MALS experiment compare to that of an FFF-MALS?

A: SEC-MALS analyses of AAVs and other biologics generally run 20 – 30 minutes. For FFF-MALS, it largely depends on the channel volume and the complexity of the separation method. Typical methods that we employ take less than 30 minutes but may run up to 60 minutes.

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. More may be loaded in order to characterize low-abundance species such as aggregates.

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

A: They should be very similar, as the mobile phase is the same and membrane interactions in FFF can be minimized by selecting the right membrane material. The recovery of some analytes such as lipid nanoparticles will benefit from the lower shear in FFF, especially with the dispersion-inlet channel.

Dynamic light scattering

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

A: No, we can use the same constants for all of the AAV serotypes in DLS.



Q: How is the number concentration measured using DLS? How accurate is the result?

A: DLS measurement of particle concentrations requires knowledge of the particle's refractive index. It assumes that the particles are spheres, calculates the volume of the sphere using measured R_h and combines that information with the scattered intensity to determine concentration in particles/mL. If the refractive index and shape assumptions are accurate then the values for a pure and very homogeneous sample are typically in good agreement with other techniques that are used to determine particle concentration.

The really neat thing about the concentration determination in DYNAMICS is that it can calculate the concentration for each resolvable peak in the normalization graph. However, if there is polydispersity such as dimers or trimers that cannot be resolved from monomers in a given peak, the accuracy of the concentration decreases.

Application-specific

Q: I see how the technique can characterize empty and full AAVs but what about partial AAVs? Also, how does residual DNA affect empty:full measurement?

A: Both SEC-MALS and FFF-MALS separate molecules on the basis of their hydrodynamic radius so an empty AAV, a full AAV and a partially filled AAV, which all have the same hydrodynamic radius, cannot be separated. But ASTRA's Viral Vector Analysis method can measure the MW of each component in the resulting peak including the AAV protein capsid and the nucleic acid payload. The MW of the nucleic acid component can then be compared to the MW that is expected if the AAV is completely full, and the resulting ratio of empty vs. full AAV can be calculated. While this technique can perform the calculation without physical separation of the empty, full and partials, it cannot specifically calculate the amount of partially filled AAV. A sample consisting of 50% full AAVs and 50% empty cannot be discriminated from 100% half-full vectors. However, the SEC-MALS method can reliably detect and quantify small changes (3%) in the ratio of full and partial AAVs while the differences may be within the experimental error of the other techniques.

Residual DNA is typically resolved from the main AAV peak and does not affect the analysis of the AAVs themselves. Eluting peaks consisting of pure DNA are readily identified on the basis of UV/RI or UV_{260}/UV_{280} ratio; their molar mass can be measured, and related to the number of base pairs.

Q: How do you quantify capsid and DNA amounts (i.e., how to select ϵ_{280} , or dn/dc) for Cp/Vg calculation? What are the ext coeff. and dn/dc values used for the analysis?

A: We have some starting values for the calculation in ASTRA 8 that are fairly accurate. For optimal results it is helpful to have an empty AAV sample and use ASTRA to determine the extinction coefficients for your particular serotype.



Additional information regarding dn/dc and extinction coefficients is provided via the *AAV Training and Method Transfer Service* and in the *AAV Standard Operating Procedure Guidance Manual*. Please contact support@wyatt.com for additional information on these products and services.

Q: Can any of these methods reliably differentiate between monomers and dimers/trimers/smaller aggregates of AAV?

A: Yes, both SEC-MALS and FFF-MALS are ideal for this type of characterization since they separate those species and characterize each one individually. FFF will generally offer better resolution of AAV aggregates and will also have higher recovery for larger aggregates which could be removed by the SEC column. Batch DLS (in cuvettes or microwell plates) cannot differentiate between monomers and small aggregates.

Q: Can we use the same SEC-MALS method for lentivirus?

A: SEC columns typically work for viruses up to 50 or perhaps 100 nm in diameter. Due to the size of lentivirus (~ 100 to 150 nm) it is advisable to employ an alternative separation technique like FFF, which can of course also be coupled to MALS and other online detectors.

Wyatt offerings

Q: Does Wyatt offer any AAV-specific training to help with method development?

A: Yes: there is an AAV-specific *Training and Method Transfer Service*, which includes on-site setup, training and implementation of the AAV SEC-MALS characterization method, and also a very comprehensive 65+ page standard operating procedure (SOP) guidance document that describes all aspects of AAV SEC-MALS method development and characterization, with guidance on how to design and perform validation procedures. Usually customers take advantage of both of these.

Q: Is your software 21 CFR part 11 compliant?

A: Yes, both ASTRA (MALS) and DYNAMICS (DLS) offer 21CFR(11) compliant versions.

Q: Is the Viral Vector Analysis method available in ASTRA 6, or will I have to use the regular protein conjugate method?

A: This method is only available in ASTRA 8. You can extract some of the results using the protein conjugate method, but it will be quite limited in terms of providing the complete CQAs and will not be able to use the dual-UV analysis, only UV-RI. Please contact us if you have questions about the upgrade.



Miscellaneous

Q: *Are there any CROs that offer SEC-MALS, FFF-MALS, and DLS according to Wyatt protocols?*

A: We do not have that specific information, but we do have a list of CRO's that utilize Wyatt instruments and software. Please see <https://www.wyatt.com/ContractLabs>.