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Live Webinar Q&A Sheet: Quality attributes and extended characterization of lentiviral and gammaretroviral vectors using light scattering

The recorded webinar may be viewed from the [FFF-MALS webinars page](#). These questions were submitted by live viewers. Additional information on the [Eclipse™ FFF instrument](#), [DynaPro® Plate Reader](#), [dynamic light scattering \(DLS\)](#) and [the FFF-MALS technique](#) 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.

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FFF-MALS instrumentation

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Light scattering basics

Q: How do you measure particle concentration in FFF-MALS and DLS? What relation are you using to calculate the particle concentrations from R or R_h ?

A: Static light scattering relates the total scattered intensity at angle 0° to the particle concentration, particle size and refractive indices. These are the equations that we use for determining particle concentration:

$$I_{particle}(0^\circ) \propto \left(\frac{n_{particle}}{n_{solvent}} - 1 \right)^2 V_{particle}^2 \quad \text{and} \quad N_{particles} = \frac{I_{total}(0^\circ)}{I_{particle}(0^\circ)}$$

Volume is determined from the particle size, either R_h (DLS, using a [DynaPro® NanoStar](#) or [DynaPro Plate Reader](#)) or geometric radius (MALS). The scattered intensity is extrapolated to 0° either directly (MALS, using a [DAWN® multi-angle light scattering instrument](#)) or with the specific detector angle, shape assumption and measured R_h (DLS).

Q: The graphs appear to show that empty viral particles are larger than full viral particle, is this true?

A: This apparent behavior is the consequence of the different types of particle sizes that affect elution and measurement. FFF separates by hydrodynamic radius, so at any given slice in the fractogram, empty and full particles have the same hydrodynamic radius. If the graph showed R_h versus elution time then the two particles would overlay.



But the size displayed is measured by MALS and is related to the distribution of mass within the particle, in other words to the structure. The mass distribution of empty particles is, by nature, more localized at higher distances from the center of mass than occurs for full particles with the same hydrodynamic radius, so an overlay of fractograms of R by MALS is fully expected to show larger radius for empty particles at a given elution time than for full particles.

Q: Which standards do you use to qualify the DLS and MALS systems?

A: We like using BSA and polystyrene latex particles, but any sample that you know well will suffice.

Q: How can you tell if the dimer of AAV is an aggregate, since its molar mass is huge?

A: MALS measures both the size and molar mass of each species, so a dimer will be indicated when the molar mass is doubled.

FFF basics

Q: Do you dilute your virus prior to SEC- or FFF-MALS?

A: No, samples were used as received, without any special preparation

Q: In FFF, does the polarity of the molecule have any impact or is it only the size of the fractionated molecules? What is the membrane made of?

A: In theory, FFF separates solely on the basis of size. However, molecule properties such as polarity that may lead to particle-membrane interactions can affect both retention time and recovery. FFF-MALS overcomes the issues of retention time since MALS provides an independent measurement of size regardless of specific elution properties.

Q: Which buffers can be used with FFF? which mobile phase do you recommend for viral particles? PBS, PBS + glycol, water...? Can we use our formulation buffer?

A: FFF can work with a wide variety of buffers, including formulation buffers. We recommend using the mobile phase that is the best suitable for your sample; FFF can even accommodate formulation buffers though depending on the formulation, there may be a concern that the buffer components impact downstream detectors such as refractive index, UV or light scattering.

Q: Can we compare the mass recovery of our sample between different FFF methods?

A: Yes, a variety of techniques can be used for this. You can measure particle number density for each run, dRI concentration if the signal is strong enough, and finally, you can compare UV peaks areas keeping in mind that sample size should be similar between each run.



Dilution control module

Q: *What does DCM stand for?*

A: Dilution control module

Q: *Is DCM is a pre-concentration unit? You get more sample concentration and higher S/N ratio with DCM correct?*

A: No. DCM does not pre-concentrate the sample. Samples separated by FFF are inherently diluted upon exiting the FFF channel, and DCM reduces the degree of dilution. Ultimately the sample elutes at higher concentration and provides higher S/N ratio.

Q: *Can you elaborate a bit on how the DCM module works, and why it is important for increasing S/N ratios?*

A: The channel's height is anywhere between 250 and 900 μm while the eluting particles are limited to the lower 10 μm of the channel. Upon exiting the channel, the solvent in the top $\sim 95\%$ - 99% of the channel combines with the eluting particles, leading to high dilution. DCM removes the top part of the mobile phase and reduces dilution, therefore improving the signal from the sample and providing higher titers in fractions that are collected.

Q: *Is the benefit of DCM size-dependent? Would it be the same for an IgG or a lentivirus?*

A: DCM removes solvent from the top part of the channel, which does not contain the sample. The benefit of DCM is not size-dependent since disparate species such as IgG or lentivirus both stay quite close to the bottom of the channel during elution.

FFF-MALS applications

Q: *Which other viral vectors can the FFF-MALS method be used for?*

A: FFF-MALS is best suited for any large viral vectors and VLPs including adenoviruses, lenti- and gamma retroviruses, VSVs, AAVs, and large AAV aggregates

Q: *Would FFF-MALS be helpful for drug nanoformulations (drugs encapsulated in polymers) sizes ranging from 300 - 500 nm?*

A: Yes, this is an appropriate size range for FFF. For more information, I recommend visiting www.wyatt.com/#analyze-5.

Q: *Why were those specific wavelengths used in the FFF example?*

A: We usually use 280 and 260 nm for viral vectors to differentiate protein absorption (max at 280 nm) from nucleic acid absorption (max at 260 nm).



Q: How accurate are LV and/or AAV concentration and empty/full measures using FFF-MALS?

A: While LV concentration measurement depends on knowledge of the sample's refractive index while AAV concentration depends on knowledge of the UV extinction coefficient of the protein, accuracy is usually 5% or less. The empty/full ratio of LV cannot currently be measured accurately, but the empty/full ratio of AAV can be determined to within ~ 5% or less with accurate extinction coefficients.

Lentivirus quantification

Q: SEC of LVVs: Which column did you use that cuts off everything above 87 nm?

A: We used OHPak columns. The problem in cutting off at 87 nm is probably caused by sample degradation due to high shear forces

Q: Can we use UV absorption to measure the concentration of lentiviruses?

A: When particle radii exceed 25 – 30 nm, scattering of the UV light begins to be significant. The O.D. value reported by the detector reflects total extinction, which is the sum of absorption and scattering, and therefore the apparent absorption of a lentivirus sample by UV will be larger than the actual absorption. Therefore the concentration measurement will be incorrect.

Q: What was the molar mass data for the empty vs full LV samples?

A: Calculation of molar mass from light scattering requires measuring mass concentration by, e.g. UV or refractive index. Because mass concentration measurement is challenging for the LV samples, we are still working on its measurement. Be on the lookout for the upcoming publications.

Quantifying empty/full ratio

Q: Can you use ratio of UV absorbance peak areas A260 nm and A280 nm to confidently assess viral full/empty ratio?

A: For small viral particles such as AAVs, if the viral particles have been separated by e.g. SEC from free proteins and nucleic acids then this is a reasonable approach to determining the empty/full ratio. It is not reliable for absolute concentrations of empty and full virions, for that it is better to add light scattering to the mix using ASTRA's viral vector method.

For viral particles larger than about 50 nm in diameter, the A260/A280 method cannot be used reliably because scattering from viral particles affects the values of A260 and A280 differently – nominally according to λ^{-4} , but actually it's more complex than that. We are looking into methods that overcome this challenge; one of them, using the ratio of R_g/R_h , was presented in the webinar.



Q: How to distinguish empty, partially empty and full viral vectors in the same sample using MALS and determine their relative abundance?

A: For AAVs and other small viruses, SEC-MALS and ASTRA's viral vector module determine the average genomic content at each eluting fraction. If we assume that the virions can be either empty or full, but not partially full, then the empty/full ratio can be calculated. However, SEC does not resolve empty, partial and full and therefore cannot provide an absolute relative abundance of all three.

MALS can also be coupled to a separation technique such as ion-exchange chromatography that does resolve empty and full AAVs, at least partially, but as long as the different species overlap in the chromatogram it is still impossible to provide a robust ratio of empty, partial and full. So far the only method that has been shown to provide good separation and quantification of all three is sedimentation-velocity analytical ultracentrifugation – a technique that is tedious and difficult to perform, but clearly necessary, at least in early R&D.

For larger viruses, we are not aware of a good method for doing so.

Dynamic light scattering

Q: What is the difference between measuring size by standard DLS and online DLS?

A: Standard DLS covers a larger size range and does not impart dilution to the sample, but offers low size resolution. Online DLS benefits from upstream separation to provide high resolution size distributions, but the measurable size range is lower and the sample is inherently diluted during separation. In addition, online DLS can be used in conjunction with the MALS to infer particle shape or structure from the R_g/R_h ratio where R_g is determined by MALS and R_h by DLS.

Q: Are DLS size measures of single nano-beads changed when several nano-beads are combined? Any correction factors?

A: DLS measures the hydrodynamic radius, which is derived from the diffusion coefficient. If several beads clump together then they will diffuse more slowly in a manner that corresponds to the arrangement of the beads in the aggregate. The reported value of R_h corresponds to the radius of a sphere with the measured diffusion coefficient, and may be a reasonable estimate of the correct aggregate radius if the clump is roughly spherical.

Q: What is the viscosity effect on the particle size using DLS?

A: DLS measures diffusion coefficient, and the conversion to particle size includes a solvent viscosity factor. Wyatt's DYNAMICS and ASTRA softwares that are used for DLS analysis offer an extensive library of common solvents that provides viscosity as a function of temperature.



Stability and formulation studies

- Q: Which light scattering technologies are most suitable for evaluating virus stability under freeze-thaw and other stressors?*
- A: You can start with high-throughput DLS using a [DynaPro® PlateReader](#) to evaluate a wide variety of samples, for example after multiple freeze-thaw cycles. For more detailed information regarding what happens to a sample under those conditions, FFF-MALS is recommended.
- Q: Since lentivirus is highly polydisperse, what is the best analysis technique within DLS for a formulation screen?*
- A: Even though lentivirus is highly polydisperse, we can still monitor size changes in a formulation screen. Additionally, stability of size and particle concentration over time also help to assess different formulations.

Technology comparisons

- Q: How do FFF-MALS and DLS compare with particle tracking analysis?*
- A: PTA has the advantage that it counts individual particles and therefore provides a true size histogram. However, it requires careful preparation of the sample to within a narrow concentration range, and is limited in terms of the sizes it measures.

Both DLS and FFF-MALS have no need for special sample preparation, cover a wider range of particle sizes while providing accurate particle concentrations even though individual particles are not counted. DLS, while lower in resolution than PTA, is a fast measurement, imparts no dilution to the sample and can be carried out in microwell plates with temperature ramping or stress applied temperatures. and can 'see' the entire particle population. FFF-MALS includes upstream separation for high resolution and the option of adding multiple detection modes including UV, fluorescence, and even mass spectrometry. While FFF-MALS takes more time and is more complex, it provides higher size resolution and much deeper insight into sample distribution and other properties such as shape or composition.

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