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Live Webinar Q&A Sheet:

Optimizing Development and Release Efficiency of Protein, AAV, and LNP Therapies Using Multi-Attribute Quantification (MAQ) 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, FFF, CG-MALS and RT-MALS 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.

Questions & Answers

General Light Scattering Questions

Q: *What is the difference between DLS and MALS?*

A: Dynamic light scattering (DLS) measures time-correlated intensity fluctuations in the scattered light resulting from Brownian motion of the illuminated molecules or particles. By analyzing the time dependence of the fluctuations, one can obtain the translational diffusion coefficient, which is used to calculate the hydrodynamic radius or R_h.

Multi-angle light scattering (MALS) measures the time-average intensity across multiple scattered angles. It provides absolute molecular weight measurements of proteins and other biomolecules in solution, independent of shape. Molecular weight is determined from the total scattered intensity together with weight concentration, usually measured by a UV or differential refractive index (dRI) detector.

Q: *Did you see any inconsistency between SEC- and FFF- MALS when running the same samples because of mobile phase or some other factors?*

A: Both SEC-MALS and FFF-MALS separate molecules on the basis of their hydrodynamic radius and for many samples the results from these two different separation techniques should agree with each other. But for some complex samples especially with large sized molecule or particle fractions, FFF provides more mobile phase options and a gentler separation approach thus more authentic size distribution than SEC.

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

A: The recovery is sample dependent and column dependent. FFF-MALS is the optimal technique for large aggregates that are shear sensitive and could be perturbed by the column resin. The two separation techniques are not exclusive of each other, and many users work in a “switching mode”. 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.

Q: *In using DLS DynaPro plate reader to test the denaturation of a protein in the presence of various concentrations of denaturant, do the results provide enough information to calculate Gibbs free energy at zero concentration of denaturant? (DeltaG(H20))?*

A: Using a chemical denaturation gradient is a useful means to quantify a molecule's stability against conformational changes. A denaturant such as urea or guanidine HCl is titrated into the protein solution in order to determine the denaturant concentration at which the molecule is present in equal amounts as folded and unfolded species, and hence the Gibbs free energy of unfolding. The common method for assessing unfolding in a chemical denaturation measurement is intrinsic fluorescence. However, DLS is a superior quantifier of unfolding since it does not rely on the presence of fluorophores, does not lead to photon-induced damage, and is a positive, reliable indicator of unfolding via changes in actual size. Chemical denaturation screening of a multi-domain protein has been demonstrated by using a DLS Plate Reader in this publication: Yu, Z., Reid, J.C. and Yang, Y.P., 2013. Utilizing dynamic light scattering as a process analytical technology for protein folding and aggregation monitoring in vaccine manufacturing. Journal of pharmaceutical sciences, 102(12), pp.4284-4290.

Software Questions

Q: *Can you explain some software features that Wyatt provides to make SEC-MALS appropriate for a QA/QC environment?*

A: Both ASTRA (MALS) and DYNAMICS (DLS) offer 21 CFR (11) compliant versions which would support its use in QC. We also offer a comprehensive standard operating procedure (SOP) document that guides a user through multi-attribute quantification (MAQ) of AAV samples by the Wyatt SEC-MALS method.

Q: *Is Astra software compatible with Agilent 1260 and 1290 and what firmware is required?*

A: Users can run their instruments within ASTRA using HPLC CONNECT 3.

Application-Specific Questions

Q: *Can I use UV at 260 and 280 nm as the concentration source for my protein-DNA complexes?*

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 largest size of aggregates that can be accurately quantified using SEC-MALS?*

A: It depends on the aggregate shape and the columns used. When the aggregate radius is great than 30 nm, we would recommend confirming the results by FFF, preferably, or at least by batch DLS.

Q: *Would you say it is a "must" to use SEC or FFF-MALS for QC testing of a viral vector or would it also be possible to use a DLS method as a QC release assay?*

A: Depending on the CQAs of your product and 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. But during the development cycle, we'd recommend to also check with SEC/FFF-MALS or other orthogonal approaches to make certain that DLS is sufficient in detecting quality variation and in providing product and process comparability.

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

A: For a stable and relatively homogeneous sample, the DLS inter-assay repeatability is typically 1-2%, and 5% for reproducibility. The acceptable polydispersity index depends on many factors and is primarily determined by safety and efficacy concerns rather than by the 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 the development cycle, 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: *Are there limitations in LNP size analysis when using online DLS for AF4 Rg/Rh evaluation? For example, are there issues with particles < 100nm size?*

A: The MALS R_g ranges in batch and online mode are the same, approximately from 10 to 500 nm. The batch DLS size range is 0.3 to 1000 nm. However, the online DLS size range is much narrower, mainly because the upper size limit is much lower than that in batch mode. The upper size limit depends heavily on the flow rate and the concentration of the data slice. The Dilution Control Module in our Eclipse FFF system lowers the detector flow rate and increases the upper Rh limit. As a result, I will highly recommend using dilution control module for bioNPs like LNPs. I also want to point out that R_g/R_h ratio is actually a low-resolution approach for payload assessment, MWs from different moieties of the NPs provide much more details and with higher resolution when they are measurable.

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

A: Yes, absolutely, the DLS and MALS methods discussed in the webinar are also be used to characterize lentiviral vectors but with two precautions we would like to emphasize. First, SEC is not appropriate for lentivirus due to low mass recovery and poor resolution, so we recommend FFF for size-based separation. Second, the multi-detector methods used to determine LNP payload cannot quantify the lentiviral genome content. But qualitative or semi-quantitative assessment on payload is still possible for some lentiviral vectors. We have an application note on lentivirus characterization by FFF-MALS for high resolution size distribution, particle concentration, aggregation, and product/process comparability.

Q: *Sometimes large particle vaccines can be difficult to analyze via SEC and a 2000 angstrom pore column might be useful. Would FFF work in this situation?*

A: Yes, for those large particle vaccines, we suggest that you try FFF in addition to these large pore columns.

Q: *Can you calculate/estimate empty LNP concentrations in the same way that was done for AAV?*

A: The particle concentration of a pure empty LNP sample can be determined using either the Number Density calculation with MALS data or the MW and weight concentration method, similar to the one used for AAVs. However, calculating the empty LNP concentration in a sample containing both empty and full LNPs is more complex due to the heterogeneous nature of loaded LNP samples. Unlike AAVs, loaded LNPs exhibit varying MW, size, and payload across different eluting data slices. While our approach, which combines MALS, UV, and dRI signals, can determine the average payload, it cannot provide the payload distribution for each slice.

Nevertheless, it enables the determination of size-based payload and online lipid and payload concentrations by integrating the entire peak.

Q: For AAV empty/full analysis, 50% vs 100% full show 0.6 MDa and 1.2 MDa molecular weight of DNA respectively. Would the MW of DNA be constant no matter the capsid is full or empty?

A: Yes, the MW of transgene DNA in an AAV sample should be constant when partial AAVs are neglected. Since our SEC-MALS method measured the average DNA MW from both empty and full AAVs in the sample, the apparent DNA MW can be used to calculate the empty/full ratio.

Q: Is there a specific zeta potential value that would indicate better encapsulation of LNPs with DNA or RNA?

A: I think more measurements from more labs are needed in order to provide a solid answer to this question.