



Live Webinar Q&A Sheet:

Scientific Town Hall Meeting: Biophysical tools for vaccine characterization and virus research

The recorded webinar may be viewed from the [Biotherapeutics](#) webinars page. These questions were submitted by live viewers before and during the webinar. Additional information on SEC-MALS, FFF-MALS, CG-MALS, DLS 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.

Table of contents:

General.....	2
MALS and DLS basics	3
Chromatography	6
General vaccines and viruses	9
Specific vaccines: VLPs	11
Specific vaccines: Adenovirus.....	12
Specific vaccines: glycoconjugates	12
Specific vaccines: AAV	13
Proteins and therapeutic antibodies	14
Measuring particle concentration	15
Process monitoring.....	17
Validation and QC.....	19



General

Q: *What are the different techniques required for characterization of antiviral products during development?*

A: Antivirals come in different modalities, including small molecules, peptides, proteins and monoclonal antibodies. DLS can be helpful in certain aspects of small molecule discovery and formulation, for example those described in [AN5002: High-Throughput Optimization of Drug Nanosuspensions](#) and [Non-Specific Small Molecule Aggregation](#).

For larger therapeutic molecules, in particular monoclonal antibodies, light scattering plays an important role in development:

1. **HT-DLS** evaluates candidates for quality (see [Protein Quality Control in SPR and BLI High-Throughput Screening Studies](#)) and developability, and formulation buffers for optimal stability and minimal aggregation, as described in [WP5003: Automated dynamic and static light scattering in microwell plates](#) and [WP5004: The Diffusion Interaction Parameter \(\$k_D\$ \) as an Indicator of Colloidal and Thermal Stability](#).
2. **SEC-MALS** is used to characterize monomers, aggregates and fragments, as well as glycosylation or PEGylation, as described in [WP1615: SEC-MALS for absolute biophysical characterization](#).
3. **FFF-MALS** is used to characterize large aggregates which may be filtered or sheared by the SEC column. In many cases **FFF-MALS** can establish binding of neutralizing antibodies to target viruses.
4. **CG-MALS** is essential in quantifying self-association, and may also be able to test [binding affinity and absolute stoichiometry](#) of complexes between neutralizing antibodies and target viruses.

Q: *What are best practices for evaluating protein aggregation?*

A: Light scattering techniques are extremely sensitive to small amounts of aggregate since the light scattering intensity is proportional to the molar mass and the concentration of the sample. Accordingly, batch **DLS** is an ideal tool to determine the presence of large aggregates, in particular when screening a large number of samples. For smaller aggregates such as oligomers, **SEC-MALS** will have a higher resolution than batch DLS, however, the stationary phase of the column may filter out large aggregates. In these cases, **FFF-MALS** can be applied.

In addition to determining the aggregate content of solutions, the propensity of proteins to aggregate can also be evaluated. Some common techniques include **HT-DLS** and **CG-MALS** (see previous question).



MALS and DLS basics

Q: What's the reproducibility of the measurement in molar mass, size, and protein-protein affinity? What's the accuracy of a molar mass measurement? How much difference can be distinguished?

A: Molar mass and rms radius measurements performed by SEC-MALS or FFF-MALS have an expected accuracy of 5%. Note that this is a system error and includes uncertainties from the chromatography system (e.g. flow rates, chromatography noise), light scattering instrument and concentration detectors. The repeatability of molar mass and radius of a given sample is often within 1%. In the ASTRA® software, the fit of the angular dependence of scattering is indicated by an uncertainty, which indicates the accuracy of the measurement. The difference in molar mass or size that can be resolved depends on the quality of the fractionation itself and can be as low as 5%.

For batch DLS, we typically expect an accuracy of 5% and repeatability of 2% for well-behaved samples (this will be lower for heavily aggregated samples). For [CG-MALS](#), affinity measurements, like K_d , are expected to be within a factor of 2-3.

Q: Can you give some examples of difference in molecular weight determined by SEC alone as compared to SEC-MALS? How is the correct molecular weight calculated by SEC MALS?

A: There are several reasons why analytical SEC without MALS might give incorrect MW values:

1. If the column calibration curve is established with typical proteins standards, which are globular, but the sample does not have a globular conformation, e.g. glycoproteins, PEGylated proteins, non-globular oligomers (even if the monomers are globular, oligomers need not be), inherently disordered proteins, and polypeptides or nucleic acids that do not fold and so acquire a random coil conformation.
2. If the sample undergoes non-steric (non-ideal) interactions with the column due to electrostatic charge, hydrophobic residues, etc.
3. If the sample elutes in, or close to, the void volume.

Unlike standard SEC analysis by column calibration, SEC-MALS does not depend on retention time, but rather determines MW from first principles. It only uses the SEC column for separating the different species in solution. As the various molecules pass through the MALS and concentration detector (UV, RI or both), each is analyzed by comparing the light scattering intensity $I_{\text{scattered}}$ with concentration c . The ratio of these is related to the molar mass M by the Rayleigh-Gans equation, $I_{\text{scattered}}(0) = KMc(dn/dc)^2$ where K is an optical constant and dn/dc the specific refractive index increment (closely related to the difference in the refractive indices of the sample and solvent).



Q: What are the upper limits on size and molar mass by MALS?

A: For MALS measurements with the DAWN multi-angle light scattering detector, the maximum measurable size is 500 nm in radius. This corresponds to a molar mass $\sim 1 \times 10^9$ g/mol. Additional specification information can be found at <https://www.wyatt.com/Products/MALS>.

Q: How can buffer composition influence results?

A: In this answer we will address how buffer influences the measurement technique per se. Please see the section on chromatography to obtain answers on how buffer composition may affect apparent size, molar mass, or aggregation or stability during chromatography.

For multi-angle light scattering, the only buffer parameter required to calculate a correct molar mass and rms radius is the refractive index (RI). This parameter is provided for common solvents and buffers in the ASTRA software. The buffer composition will also influence the refractive index increment dn/dc (this parameter is related to the difference in RI between the solute and the solvent) which is necessary to calculate molar mass. dn/dc is not necessary to determine size (R_g or R_h).

To determine the hydrodynamic radius by DLS, two solvent parameters are required: RI and viscosity. Viscosity is strongly dependent on buffer additives and temperature. ASTRA and DYNAMICS software provides viscosities and temperature correction models for many common buffers.

Q: How to calculate hydrodynamic size by SEC-MALS?

A: In order to determine the hydrodynamic size of eluting macromolecules, the WyattQELS™ embedded dynamic light scattering module must be installed in the MALS detector. The SEC-MALS software, ASTRA, can acquire data from the WyattQELS in tandem with MALS data and calculate R_h , the hydrodynamic radius, from 0.5 nm up to 30 nm using the measured autocorrelation function.

In addition, the MALS detector can determine the rms radius R_g from 10 – 500 nm, even without the WyattQELS.

Q: What is the maximum resolution with DLS?

Q: How do you differentiate between closely sized viral particles, e.g., 8 nm viral particle and 10 or 12 nm viral particle?

A: This depends on whether fractionation is part of the experiment. In a batch experiment (no fractionation) multiple populations can be resolved if they are 3-5x different in size. This is ideal for detecting aggregates, but not sufficient to resolve monomer from dimer and higher oligomers, or an 8 nm from a 10 or 12 nm viral particle. The presence of such particles will result



in an increase of the average hydrodynamic radius and polydispersity of a sample measured in batch. If fractionation by either SEC or FFF is employed, the resolution will be determined by the quality of the chromatography, and a separation of particles of 8 and 10 nm viral particles can be readily achieved.

Q: R_g should normally correlate with molecular size. What can cause the lack of correlation between R_g and MW using MALS detection?

A: The relationship between R_g and MW is related to the molecular **conformation**. For example, for globular molecules such as well-folded proteins or viruses, $M \sim R_g^3$, whereas for random coils such as DNA and RNA, $M \sim R_g^2$, and for rod-like molecules $M \sim R_g$. If, for example, your sample includes proteins, viruses and free RNA, you will see a discrepancy in the correlation between R_g and MW due to the different conformations.

Q: How do we interpret differences between TEM measurements of particles and DLS measurements? How do high-throughput batch DLS measurements of particle concentration compare to other methods, such as TEM and NTA?

A: Dynamic light scattering and microscopy are both used to provide a measure of particle size. DLS directly measures the diffusion coefficient of particles in solution, and this is converted to a hydrodynamic radius via the Stokes-Einstein equation. For a spherical particle, the hydrodynamic radius corresponds to the geometric radius of a sphere and should correlate to the TEM-measured radius. DLS measurements may be influenced by a hydration layer about the particle. Moreover, for polydisperse samples DLS reports the z-average hydrodynamic radius whereas TEM reports a number-average radius. Aggregates and large particles will have a larger influence on the z-average radius reported by DLS as compared to the number-average radius reported by TEM. Additional information can be found in <http://www.wyatt.com/Theory/DLS>.

For a monodisperse population, a quick DLS analysis compares very well with more involved orthogonal methods, such as TEM, NTA and MALS, and we report within ~30% agreement. For polydisperse solutions, image-based techniques such as microscopy and NTA can provide more detailed insight into the size distribution. Similarly, SEC-MALS or FFF-MALS may be used to separate the differently sized particles and determine the distribution of sizes and number of particles of each size.

Q: Why and when is a refractive index detector needed during MALS analysis?

A: In order to determine molar mass, both a MALS and a concentration detector are required. A UV absorbance detector might be adequate for determining concentration, but there are several instances when an **Optilab® refractive index detector** might be preferred or required:



1. The analyte does not have a chromophore. All molecules will have a dRI response in most solvents.
2. The extinction coefficient is unknown. Since most unmodified proteins have the same dRI response (i.e., dn/dc value), it is not necessary to know which protein is eluting in each peak or to estimate extinction coefficients from the sequence, which can be unreliable.
3. The analyte is a conjugated macromolecule such as a glycoprotein or protein-DNA complex (the latter includes RNA-bearing viruses, etc). In this case, two concentration detectors, sensitive to different properties of the constituent molecules, are required for correct analysis by the *Protein Conjugate Method*. Even if only one concentration detector is used, typically the MW result using dRI is much closer to the correct value than by using UV.

Chromatography

Q: Will the SEC-MALS methods work for sub-micron viral particles in a gradient chromatography such as ion-exchange chromatography (IEX)?

A: MALS, in general, can pair nicely with IEX, but certain caveats must be considered.

First, it is quite straightforward to determine R_g (radius of gyration), R_h (hydrodynamic radius) and particle concentration/number density distributions with IEX-MALS, since the small changes in solvent refractive index in the course of a salt gradient have very little impact on these measurements. It is also quite straightforward to determine the oligomeric state of a protein, if the protein is not heavily conjugated and UV absorption is used for concentration.

Analysis of capsid content in AAVs and other small viruses or virus-like particles can be carried out quite readily with ASTRA's AAV method using two UV wavelengths such as 260 nm and 280 nm. For larger viruses, added UV extinction due to scattering must be accounted for and this is not necessarily straightforward, but with some additional method development it should be possible.

Additional difficulty arises when RI measurements are needed, for example in determining the nucleic acid content of a lipid nanoparticle. The dRI signal will change significantly over the course of an ion-exchange gradient, often by more than the height of the particle RI signal. ASTRA offers a *Baseline Subtraction Method* to overcome some of this difficulty: in addition to measuring the sample chromatogram, you would also measure a blank chromatogram, load both into ASTRA and subtract the blank dRI signal from that of the sample in order to isolate the signal from the sample.

While most HPLC refractive index detectors would saturate as a result of an IEX gradient, the Optilab has a very large range and supports gradients of ~ 350 mM NaCl. For larger gradients, the Optilab HC (high concentration) model is available; it supports gradients up to 2 M NaCl.



However, the RI signal may have relatively high baseline noise due to inefficient mixing of the two buffers used to create the gradient. As a result, higher sample concentration may be required for IEX-MALS than for SEC-MALS when RI signals are utilized.

A significant source of difficulty in analyzing nucleic acid content of LNPs is excess UV extinction due to scattering, which so far has not been adequately addressed. If you are interested in this kind of analysis, please contact Wyatt and we would be happy to collaborate on solving this challenge.

Q: How can you ensure that viruses are stable in the mobile phase for SEC-MALS?

A: Batch DLS measurements may be used for a fast pre-screen to inform whether particles are aggregating in the mobile phase, and may be directly performed in disposable cuvettes in a [NanoStar[®]](#) or microwell plates in a [DynaPro[®] Plate Reader](#). Measuring the size distribution at $t=0$ and then after an hour will suffice to confirm stability in the course of a SEC run. As most virus formulations are stabilized by electrostatic repulsion, measuring the zeta potential using the [Mobius[™]](#) may also inform about the stability in a particular mobile phase.

Q: How can I ensure there are no interactions between the column and the viral capsids?

A: Column interactions may manifest themselves in asymmetric peak shapes, low mass recovery and unexpected retention times. Online DLS measurements with a WyattQELS can determine whether a sample is interacting with a column, by comparing the elution time with that expected for a particle of the same hydrodynamic radius. Note that the MALS detector is still able to determine molar mass correctly, irrespective of elution volume.

Varying the ionic strength, pH, and polarity of the running buffer are all strategies to reduce column interactions. Field-flow fractionation is an excellent alternative to SEC for samples that are challenging to elute by SEC. The lack of a stationary phase and the lower surface area of the FFF membrane significantly reduce the potential for non-ideal elution of the sample.

Q: How do I decide whether to use SEC vs FFF?

A: The primary consideration for SEC or FFF is the size range of the analyte. Common vaccine delivery systems are viruses (live-attenuated or inactivated), virus-like particles, liposomes and lipid nanoparticles. With the exception of AAV, which is below 30 nm in diameter, the size of these particles exceeds the separation range of SEC columns and therefore FFF is the preferred method of separation. FFF may also separate successfully smaller samples that elute poorly from SEC because of column interactions. Additionally, FFF-MALS may be used as an orthogonal technique to SEC-MALS.



Q: What are the limitations of SEC and DLS versus FFF?

A: We have already discussed that FFF is clearly advantageous over SEC for samples above 30 nm diameter. To compare SEC and DLS to FFF, we need to consider not only the size range of the analyte but also the desired resolution, and the ability to separate a very broad range of sizes in a single experiment.

As an example, consider a vaccine with a colloidal component well above 30 nm size plus the requirement to resolve a smaller component like host-cell proteins or free oligonucleotide. DLS is an excellent method for quickly screening the quality and average size of a sample. However, resolution is low and, even if resolution is achieved, the relative concentration of the various components cannot be quantitated very well. SEC does have high resolution, but the separation depends on the stationary phase and the large surface area of the column may interact with the sample. There are limitations of the upper separable size because of the exclusion limit; above 30 nm the separation becomes increasingly challenging. For large polymers, such as high-MW polysaccharides, the column packing creates shear stress and potential degradation. The packed bed can even act as a filter for larger samples.

FFF does not suffer from these limitations. The webinar slides show an example of a detailed analysis of a lipoplex sample where the amount of mRNA and the size distribution is determined with one injection using FFF.



General vaccines and viruses

Q: How can DLS, SEC-MALS, and FFF-MALS characterize protein-based SARS-CoV-2 vaccines? Which critical quality attributes of mRNA-based vaccines can be determined by the Wyatt tools?

A: The major attributes each of these techniques can measure for different vaccine types are summarized in the table below.

Vaccine type	DLS	SEC-MALS	FFF-MALS
Protein based	Screen size, aggregation, formulation, and stability	Determine oligomeric state, degree of glycosylation, aggregation, and structural information	Used as orthogonal method to SEC, especially for aggregation characterization.
Adenovirus based	Screen size distribution, particle concentration, formulation, and stability; also useful for at-line process monitoring	Not recommended	Size, size distribution, high resolution quantification of aggregation; particle concentration; estimation of encapsulated DNA
LNP-mRNA based	Screen LNP size distribution, particle concentration, formulation, and stability; also useful for at-line process monitoring	Measure MW, size, and conformation for mRNA; not recommended for LNP	For LNP, high resolution differentiation of different preparations; particle concentration; estimation of encapsulated mRNA

Q: How easy is it to quantify/differentiate genomic material encapsulated in a particle using MALS techniques?

A: It depends on several factors including particle size and composition. FFF-MALS and SEC-MALS provide a few different ways to differentiate and quantify the genetic payload encapsulated in a particle: the apparent MW, R_g and R_h ratio, and potentially more in-depth analysis using the approach similar to our protein conjugate analysis in ASTRA. Some method development may be required; better accuracy will be obtained if known empty and full particles are available.



Q: Is it possible to use these techniques to quantify the amounts of virus and host cell protein in a virus sample?

A: Generally, it is straightforward to use SEC-MALS or FFF-MALS to quantify the amount of virus and host cell protein in a virus sample, if both species elute cleanly.

Q: How do you correct for light scattering when using Absorbance as your concentration detector when working with large particles?

A: This is certainly challenging; reliable analysis depends on the type of the large particles, and some of the development work is still in progress. Please contact support@wyatt.com to discuss your specific applications.

Q: Can FFF characterize very heterogeneous vaccines and viruses with lipid shells?

A: FFF is an ideal technique to characterize heterogeneous vaccines over a wide size range with high resolution. Specifically, the fractionation power may be tuned through the FFF method itself, and software tools exist to aid in method development. As an example of a large, heterogeneous virus with a lipid shell, Orthopox virus has been successfully separated by FFF-MALS. The size from 60 to 400 nm diameter was calculated using MALS and is consistent with the values expected for this virus.

Q: Could we discriminate specifically the COVID-19 infectious from the non-infectious particles using your technology? Would it be possible to specifically detect antigenic vs non antigenic virus particles by light-scattering based techniques?

A: While light scattering quantifies biophysical properties including size and overall nucleic acid content, it is not a bioassay or a genetic sequencing method, and hence cannot determine if a virus is antigenic or infectious.

Q: Do you have examples of adjuvanted vaccine characterization with light scattering?

A: Dynamic light scattering (DLS), electrophoretic light scattering (ELS), and multi-angle light scattering (MALS) have all been used to characterize vaccine adjuvants. The following examples were presented in the Town Hall Meeting.

1. Simultaneous DLS and ELS have been used to characterize alum particles and other metal-based adjuvants. Size and zeta potential are measured simultaneously in the Wyatt Mobius to ensure that the samples are stable under the measurement conditions. In addition, the Mobius enables rapid measurements under gentle conditions to prevent redox reactions on the surface of the particles and incorrect measurement of the zeta potential. You can find more information in this webinar: [Charge it! Electrophoretic Mobility as a Tool for Characterizing Nanoparticle Stability](#).



2. DLS can be used to investigate environmentally responsive adjuvants. For example, a polymer adjuvant may exist as a monomer at 25 °C but assemble into a larger structure at physiological conditions, and the size change and critical micelle temperature may be measured by DLS. Similarly, adjuvant molecules may assemble or change structure in response to pH or the presence of certain ions, all of which may be probed by high-throughput DLS.
3. SEC-MALS is used to characterize molar mass, size, and conformation of polymeric raw materials that are used in the production of adjuvants. In addition, SEC-MALS can provide the molar mass and critical micelle number for self-assembling adjuvants, such as lipoproteins and amphiphilic peptides.

Specific vaccines: VLPs

Q: How is MALS used in virus-like particle (VLP) analysis?

A: Depending on the specific VLP, it might best be analyzed by SEC-MALS or FFF-MALS. In any case, MALS can help determine that the VLP capsid has assembled into the correct size and molar mass, as well as identify any free capsomeres or aggregates. Separation by SEC or FFF with analysis by MALS can even resolve capsids with differences in radius of just 15%. This capability is invaluable in process development and stability testing.

In addition, the *Protein conjugate analysis* method can quantify the VLP's cargo, e.g. a DNA or RNA strand.

Q: Regarding enveloped virus-like particles: Is it possible to describe the lipid contribution in eVLPs using conjugate analysis?

A: Yes. *Protein conjugate analysis* can be readily adopted to quantify many two-component conjugates such as eVLPs, which are conjugates of lipid and protein, similar to membrane protein-lipid conjugates. Unlike membrane protein conjugates, due to its larger size, the UV signal from an eVLP may contain noticeable scattering contribution which needs to be corrected.

Q: What tools should I be using to screen for virus capsid assembly or misassembly?

A: FFF-MALS is ideal for monitoring dissociation or assembly of virus capsids or virus-like particles. There is a good example in this poster: [VLPs Packed with DNA Payload](#). For smaller capsids such as AAV, SEC-MALS may also be used.



Specific vaccines: Adenovirus

Q: How can AF4-MALS characterize clustered and aggregated adenovirus?

A: Virus particles can form clusters of spheres, going from dimers up to pentamers and higher. Because separation is based on size rather than on molecular weight, the fact that the size increase becomes incrementally smaller for higher oligomers, and depends on the specific configuration, means that separation becomes increasingly challenging. In most cases the monomer can be baseline-separated from the dimer and the higher aggregates, whereas full separation of higher oligomers may not be achieved and the size distribution becomes continuous. A quantitative analysis of the relative amounts of monomer and aggregate is usually possible.

Q: Adenovirus has been used as a vaccine carrier. How can Wyatt's FFF-MALS instruments characterize adenoviruses?

A: Successful analysis of adenovirus with FFF-MALS has been published in multiple peer-reviewed journal articles. The main application is to determine the amount of aggregates, particle concentration of the virus and its aggregates, and to verify the correct size of the main capsid population. In downstream processing, FFF-MALS can help to identify problems in the purification process.

Specific vaccines: glycoconjugates

Q: How might one determine the level of glycosylation of a purified protein using SEC-MALS? How is light scattering/MALS applied in polysaccharide & conjugate vaccines?

Q: If the stoichiometric analysis of conjugate vaccines is unknown, how can I use optimum parameters for biophysical analysis? What dn/dc value should I use for the complexes/mixtures of capsid and cargo or conjugate vaccine?

Q: How can we analyze vaccine conjugates that use UV active linkers? One example is protein-Maleimide-polysaccharide.

A: Ideally you would use ASTRA software's Protein conjugate method which only requires knowledge of the UV extinction coefficients and dn/dc values of the components; for protein-polysaccharide conjugate vaccines, all of these are well-known and universal except for the protein's UV extinction coefficient: the UV extinction coefficient of the polysaccharide is zero, the protein $dn/dc = 0.185$ mL/g and dn/dc of the polysaccharide is generally $0.14 - 0.15$ mL/g.

This method utilizes data from MALS, dRI and UV to determine the molar masses of the protein and polysaccharide in the conjugate at each elution volume. It provides the protein fraction in the conjugate as well as the total molar mass.



The method is subject to limitations at high molar masses, in the range of tens of millions and above, due to excess UV extinction resulting from scattering. If the *Protein conjugate method* cannot be applied correctly, then the standard MALS analysis is applied and the best estimate for dn/dc is the weight-average of dn/dc of the two components.

Like dn/dc , the UV extinction coefficient of a conjugate can be approximated as the weight average of the extinction coefficients of each component. When the MW of the linker (Maleimide) is negligible as in the protein-maleimide-polysaccharide conjugate and the linker extinction coefficient at 280 nm is low compared to protein, the effect from the linker can be neglected.

Specific vaccines: AAV

Q: *What are the advantages and disadvantages of DLS, AF4-MALS, SEC-MALS, CG-MALS, real-time MALS, and PALS for analyzing AAV vectors?*

A: The table below summarizes the advantages and limitations of each of the techniques for AAV analysis.

Technique	Advantages	Limitations
DLS	Fast screening tool to determine size and estimate size distribution, particle concentration, formulation, and stability.	Mostly used for screening, less accuracy, precision, and resolution compared to SEC and FFF.
SEC-MALS	Robust and rapid quantification of 3 AAV critical quality attributes: total AAV concentration, empty to full ratio, and degree of aggregation.	Cannot provide information on partially filled AAV. Column may remove some large aggregates (radius larger than 25 nm).
FFF-MALS	Better recovery and resolution of large AAV aggregates compared to SEC.	Compared to SEC, need to inject more sample in order to get sufficiently high peak signals for low-abundance aggregates.
Real-time MALS (ultraDAWN)	To monitor AAV attributes including aggregation and physical titer during production, for process development and control. Most applicable to down-stream purification process.	No fractionation in the analysis, only measures average values



CG-MALS	To analyze association between capsid proteins and antibodies/solubilized receptors.	Relatively low throughput and requires more sample than some other commonly used interaction analyses.
PALS (Mobius)	To measure bulk charge and zeta potential of AAV particles.	Cannot resolve charge distribution on the AAV surface or in a sample with different species.

Q: What is the best way to characterize/quantify partially packed AAV particles in a SEC-MALS experiment? How can we distinguish aggregates from monomers, empty/full/partials?

A: In analysis of the monomers, the presence of partials does not affect the total AAV particle concentration (physical titer) measured by the SEC-MALS method; it determines the particle concentration regardless of whether the AAV is empty, full, partial, or mixture of these. However, the SEC-MALS method cannot tease out the percentage of partially filled AAVs from a mixture of empty and full. It considers a population of partials as a fraction of empty AAVs and a fraction of full AAVs.

Aggregates are generally well-separated from monomers by SEC, due to their different physical sizes.

Proteins and therapeutic antibodies

Q: Can light scattering be used to measure binding between neutralizing antibodies and multimers like trimeric coat proteins? What is the difference between CG-MALS and SEC-MALS?

A: SEC-MALS involves first separating the molecules by size and measuring the individual species as they elute. It can be used to measure the stoichiometry of tightly-associated complexes that survive dilution on an SEC column via their molar mass. You can find more information at www.wyatt.com/SEC-MALS.

CG-MALS is a batch measurement, meaning that there is no separation of the different components before measuring the weight-average molar mass by MALS. CG-MALS uses the [Calypso® composition-gradient system](#) to prepare different concentrations and compositions of biomolecules and deliver them to a MALS detector. The molar mass as a function of composition is used to quantify the affinity and stoichiometry of the reversible interactions taking place in solution. You can find more information at www.wyatt.com/CG-MALS.



Both CG-MALS and SEC-MALS use light scattering to measure the molar mass and size of molecules, particles, and their complexes. This includes complexes formed by interactions between antibodies or antibody fragments and their target antigens as well as the interactions between other biomolecules, such as protein-DNA complexes. Several references are provided below:

- Hastie, K. M. *et al.* Convergent Structures Illuminate Features for Germline Antibody Binding and Pan-Lassa Virus Neutralization. *Cell* **178**, 1004-1015.e14 (2019).
- Pallesen, J. *et al.* Structures of Ebola Virus GP and sGP in Complex with Therapeutic Antibodies. *Nat Microbiol* **1**, 16128 (2016).
- [WP3001: SEC-MALS and CG-MALS: Complementary Techniques to Characterize Protein-DNA Complexes](#). White paper.

Q: How can I study complex interaction patterns like cooperativity-driven oligomerizations?

A: CG-MALS is an excellent tool to characterize binding stoichiometry and affinity of supramolecular complexes. In some cases it can also help study cooperativity. Please contact support@wyatt.com to discuss specific applications with an application expert.

Q: How do I interpret non-specific interactions measured by light scattering: k_D and A_2 ?

A: Both of these quantify weak and nonspecific interactions that are indicative of colloidal stability, and arise from various sources, such as electrostatic interactions, induced dipoles, and excluded volume effects. These interactions may be quantified by static light scattering (SLS or MALS) using the second virial coefficient (A_2 or B_{22}) and by dynamic light scattering (DLS) using the diffusion interaction parameter (k_D). A comprehensive explanation of k_D and A_2 , and how to measure them, is available to Wyatt customers in [TN9102: Assessing Nonspecific Interactions with Light Scattering: \$A_2\$ by SLS and \$k_D\$ by DLS](#).

Measuring particle concentration

Q: How can light scattering be used to measure particle concentration?

A: Light scattering provides three methods for quantifying particle concentration. Depending on the type of sample and analysis needs, one or more may be applicable.

1. Rapid measurements in batch (without separation) may be performed with dynamic and static light scattering (DLS and SLS) in multi-well plate or cuvette. This method provides a rapid (<30 seconds) assessment of the size and size distribution and quantitation of the number of particles. The hydrodynamic radius measured by DLS combined with the SLS intensity enables direct quantification of the number of particles in solution. Although this technique can provide information about multiple populations whose sizes differ by at least



3x, it cannot resolve solutions into their monomer, dimer, and other oligomeric species for detailed characterization. More information can be found in the application note [AN5007: AAV quantitation and stability analysis by batch DLS](#).

2. Multi-angle light scattering (MALS) data provide a direct measurement of particle concentration for a variety of particle sizes and shapes. The light scattering intensity at 0° scattering angle is proportional to the square of the volume of the particle. By measuring the light scattering intensity as a function of angle, the size of variously shaped particles can be measured accurately and their concentration determined. When combined with a separation technique, like size-exclusion chromatography (SEC) or field-flow fractionation (FFF), this method may be used to quantify the number of monomer, oligomer, and aggregate species in a solution.
3. A recently introduced method combines SEC with UV, MALS, and dRI detection to determine the concentration and capsid content of small viral particles, such as adeno-associated virus (AAV). By using all three detectors, the total concentration of protein and nucleic acid is measured directly and provides accurate quantitation of total particle concentration, particle molar mass, and the fraction of particles that are filled with DNA or RNA. More information can be found in the application note [AN1617: AAV critical quality attribute analysis by SEC-MALS](#).

Q: What is the precision of particle concentration analysis used by ASTRA software? Which parameters affect the precision, and to what extent?

A: Calculating the particle number concentration requires knowledge of the particle shape and the refractive index of the material. In the ASTRA software, the light scattering intensity as a function of angle is fitted to an appropriate shape model to determine the geometric radius and the total amount of light scattered by the particles. The measured size and light scattering intensity are combined with the user-provided refractive index to calculate a particle concentration. The reproducibility of this measurement is consistent with other light scattering measurements, and we have recorded reproducibility within 2% for a variety of particles.

There are two main sources of error: the particle shape and the particle refractive index. The light scattering intensity is proportional to the radius to the sixth power (R^6) and the number of particles in solution. As a result, an error in radius of 5% results in ~30% error in the number density calculation. This error may result from uncertainty in the shape of the particle (e.g., the particle is not perfectly spherical) or from the co-elution of multiple sizes in the MALS detector flow cell. The particle concentration is also sensitive to the choice of particle refractive index. For example, a ~2% error in refractive index can lead to a 1.5× difference in calculated concentration.



Q: What is the limit of detection for MALS? How does that limit apply to a virus like SARS-CoV-2?

A: For SEC-MALS measurements under typical conditions using our DAWN multi-angle light scattering detector, the limit of quantitation is 200 ng BSA ([DAWN specifications](#)). This corresponds to a BSA concentration inside the MALS flow cell below 1 µg/mL.

Since the light scattering signal is proportional to the concentration and size of the particle, the limit for larger virus particles is lower than this concentration. Scientists at the CDC published the limit of quantitation for influenza virions with average radius of 52 nm in as 2.3×10^6 total viral particles (Bousse, T. et al. *Journal of Virological Methods* **193**, 589–596 (2013)). Assuming an average radius ~ 40 nm for SARS-CoV-2, we can calculate the limit of quantitation as 2×10^7 total viral particles.

Process monitoring

Q: Can viral aggregation during process development be monitored using these technologies? How would light scattering help in vaccine production?

A: Light scattering is the primary means for evaluating aggregation, and there are several ways to apply it during process development:

- 1. Off-line:** Aliquots may be collected and brought to the analytical lab for analysis by FFF-MALS. This provides the most detailed characterization in terms of size, concentration and aggregation, but is the slowest: in addition to the time needed to collect and transport the aliquots, each FFF-MALS run typically takes 30 – 60 minutes.
- 2. At-line:** Aliquots may be collected and pipetted into microwell plates or disposable cuvettes for DLS analysis by the DynaPro Plate Reader or DynaPro NanoStar, respectively; the Plate Reader even offers a programmable interface so the entire process can be automated by liquid-handling robotics. DLS can distinguish aggregates from the primary size mode if the aggregate size (radius) is at least 3 – 5x more than the monomer. If dimers or trimers form, they will not be resolved from the monomer but will lead to higher values of polydispersity and average size. DLS can also determine particle concentration, i.e. viral physical titer. The time per DLS measurement is typically just 30 – 60 seconds.
- 3. In-line/on-line:** Aggregation monitoring and determination of particle concentration can be fully automated and continuous using [real-time MALS](#) and the [ultraDAWN™. OBSERVER™ software](#) reads the MALS signals and calculates particle size and concentration 30 times per minute. However, only the average particle size is measured, so the appearance of a small fraction of dimers will not be as apparent as it would be with FFF-MALS. At the end of the process, the total titer in the pooled fraction is also calculated.



Q: Can process monitoring by light scattering provide critical quality attributes of vaccines and eliminate the need for QC lot release testing? How to monitor, real-time and in line, the size and the titer of viruses as part as purification process using MALS coupled to FPLC? How can it be automated? Can it be scaled up from the lab to pilot and full production?

A: RT-MALS can measure average particle size and concentration in real time, during the process. If average size or viral physical titer are CQAs of the product, then the answer is yes, at least some aspects of QC lot release may be reduced.

In any case, real-time size measurement can be extremely valuable during process development and production, first for optimization, and then for process control as a process analytical technology (PAT). With an ultraDAWN either in-line or on-line, OBSERVER software calculates the particle size 30 times per minute, providing rapid feedback on process changes.

For control purposes, OBSERVER can be programmed to generate a trigger signal related to particle size in order to begin and end fraction collection. Under the control of a OPC UA host software such as DeltaV or SIPAT, it can transmit those measurements to the host for storage and automated decisions. Since the control signal is based on an attribute that is unique to viruses (proteins and nucleic acids usually have much smaller sizes), it is far more reliable than non-specific signals such as UV absorption.

Typically, the ultraDAWN would be placed in-line during initial lab-scale purification, and on-line (using a pump to draw a small fraction of the process flow through the detector) during processes with larger flow rates such as pilot production, ultrafiltration and full-scale production.

Q: What are the upper and lower limits of virus concentration that can be measured in process monitoring by light scattering?

A: The answer depends on size and, to some degree, on other sample properties. Our estimated ranges are outlined in the table below. Actual values for the larger particles (R = 100 and 150 nm) will vary with flow rate – the lower limit will decrease if the flow rate through the ultraDAWN is above 1 mL/min, and increase if the flow rate is lower.

R (nm)	Minimum (particles/mL)	Maximum (particles/mL)
30	6×10^6	5×10^{13}
50	2×10^5	2×10^{12}
100	1×10^4	3×10^{10}
150	1×10^4	3×10^9



Q: *Any examples of real time MALS applications in process development for biotherapeutics?*

A: The use of RT-MALS in development of a protein purification process was published in 2018: Bhumit A. Patel et al, *Multi-angle light scattering as a process analytical technology measuring real-time molecular weight for downstream process control*, *mAbs* 2018, vol. 10, no. 7, 945–950 <https://doi.org/10.1080/19420862.2018.1505178>. In this paper, hydrophobic interaction chromatography was used as a polishing step to remove aggregates. RT-MALS provided the indication for ‘breakthrough’, i.e. the point when the column effectively was saturated and could no longer retain aggregates.

RT-MALS is used for biotherapeutic and pharmaceutical process development in other contexts, but we are not free to share the information. In lieu of real data, we have simulated the kind of data one might see in these processes using standard macromolecules and nanoparticles, and presented such case studies in [AN8005: RT-MALS end-point determination of a polysaccharide depolymerization process](#) and [AN8006: In-line monitoring of liposome size by RT-MALS](#).

Validation and QC

Q: *How robust and reproducible are light scattering measurements? Can they be used in QC? Is the technology acceptable by regulatory bodies?*

A: Light scattering data have been submitted and accepted by regulatory bodies for a variety of applications. An example in was provided in the Town Hall Meeting for characterization of PLGA used as a drug-delivery polymer for peptide drugs (Hadar, J. et al. *Journal of Controlled Release* **304**, 75–89 (2019).). In addition, Wyatt provides installation and operational qualification (IQ/OQ) for our detectors and software as well as GMP-compliant software for use in a QC environment.

The methods themselves are robust and reliable, and the accuracy is specified within 5% and reproducibility within 2%. Authors at the NIH have published long-term stability data for a monoclonal antibody, including molar mass data measured by SEC-MALS over a three-year period (Soman, G. et al. *mAbs* **4**, 84–100 (2012).). The measured molar mass of their BSA standard was accurate with reproducibility of 1.3% over 3 years (average and standard deviation of (65 600 ± 888) g/mol). The mAb being studied in this paper was found to be stable for 5 years, and the measured molar mass over 2 years was reproducible within 1.1%. Similarly, the hydrodynamic radius measured by DLS over the course of two years exhibited reproducibility within 1.5% over this time period.

Q: *How can we qualify the DLS method for release testing?*

A: We would be happy to discuss details with you regarding your qualification process. Please contact us at support@wyatt.com.



- Q: What additional method validation parameters are required to establish that the MALS can be a routine method in a QC laboratory?*
- A: Wyatt provides installation and operational qualification (IQ/OQ) for our detectors and software as well as GMP-compliant software for use in a QC environment. Researchers and administrators are able to set up methods that technicians can perform. The data are analyzed with automatic or SOP-defined baselines, peaks, and other preferences. Wyatt also offers certified standards that may be run as part of your performance qualification procedures. We would be happy to discuss this with you in more detail. Please contact us at support@wyatt.com for details.
- Q: Which light scattering methods are moving to being QC methods?*
- A: Both multi-angle light scattering (MALS) and dynamic light scattering (DLS) have been implemented in QC environments. ASTRA and DYNAMICS software products are available in 21 CFT Part 11 compliant options. For more information about moving your light scattering methods into QC, please contact support@wyatt.com.
- Q: How is the FFF-MALS solution placed in the workflow of vaccine (and other nanomedicines) development?*
- A: FFF-MALS has become a standard method in pharmaceutical research environments, and is now becoming increasingly important as a method for routine analysis of QC of nanodrug delivery systems. The push for vaccine development will the demand for FFF-MALS in QC applications under GLP environments, and Wyatt is working with industry partners to develop solutions for FFF-MALS for use for QC and release assays.
- Q: What is the status of AF4 data acceptance with regulatory agencies?*
- A: The need for FFF-MALS-DLS in characterization for regulatory filings, and eventually quality control of nanomedicines, is fully recognized by regulatory agencies. Government entities and standardization committees are developing are protocols and standards for standardization of FFF methods. As AF4-MALS is becoming an essential characterization tool for nanoDDS and gene vectors, the process of standardization and acceptance by regulatory agencies will accelerate and Wyatt is participating in this effort.