

Characterizing Vaccines with the Light Scattering Toolkit

Biophysical analysis aids in discovery, development and production

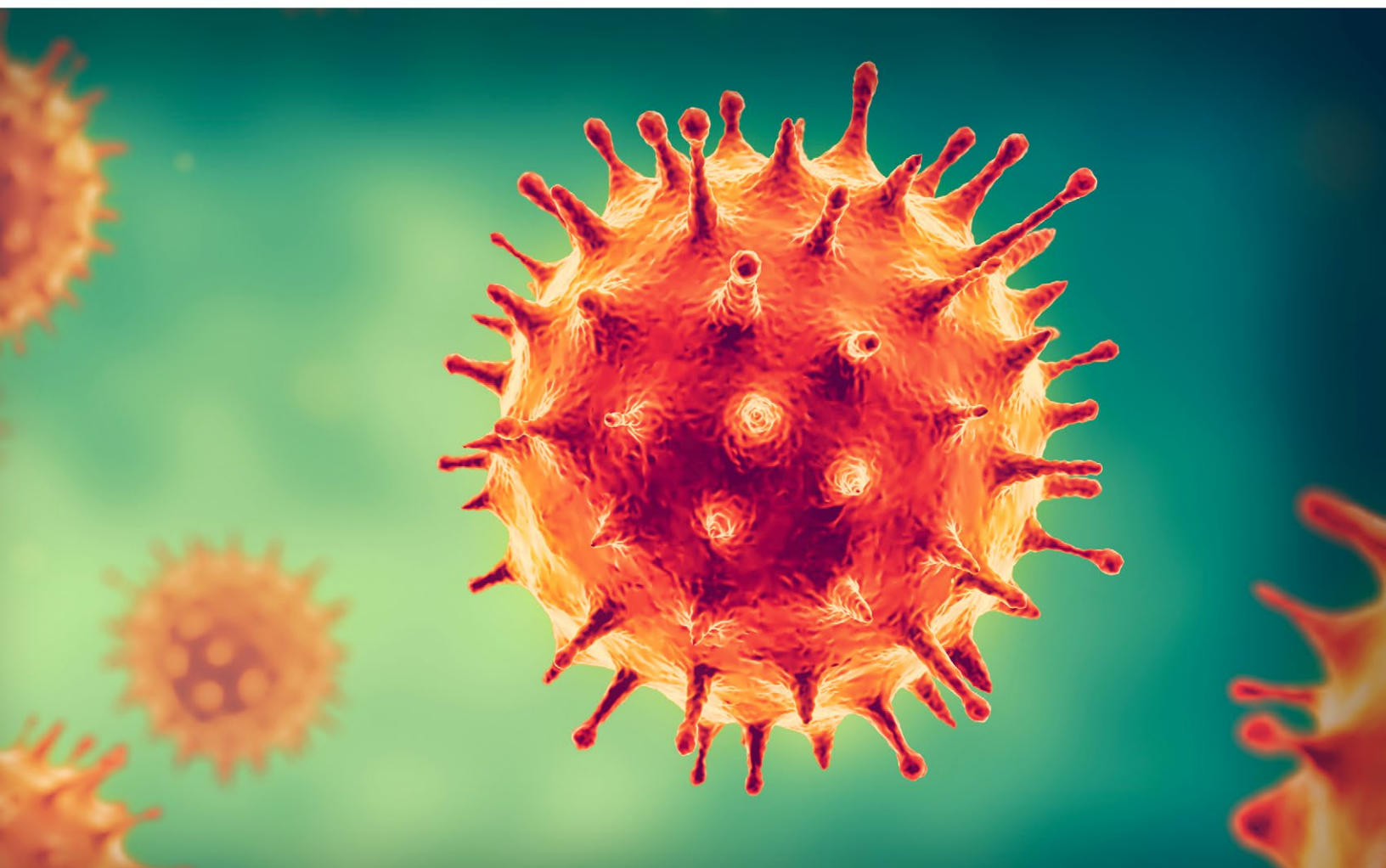


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Discovery, manufacturing and testing

Vaccines are an indispensable weapon in the fight against human and animal disease, and countless lives have been protected by them since the first one was invented over 200 years ago. Biophysical characterization is an essential component in the development and commercialization of biotherapeutics of all types. This publication will explain how light scattering, across its various flavors, is used to characterize vaccines. By measuring attributes such as size, shape, molar mass, particle concentration and parameters derived from these fundamental characteristics, light scattering aids in vaccine discovery, development and manufacture. Several classes of vaccines, spanning a large range of physicochemical properties, will be covered.

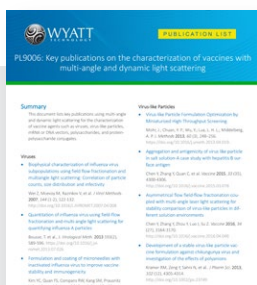
The first vaccines were either **inactivated virus** or **attenuated virus**. While vaccines of these types are still in use, they can pose issues with safety, and generally require a very long time to develop, manufacture and test. Alternative approaches have since been employed. Listed from the most mature to the newest, in terms of development and adoption, modern vaccines include:

- **subunit vaccines**, which are usually protein antigens designed to challenge the immune system;
- **polysaccharide conjugates**, typically for bacterial pathogens;
- **virus-like particles (VLP)**, which are self-assembled, usually recombinant, viral capsids that mimic whole viruses (these may also include membrane elements); and
- **nucleic acids** coding for antigens, which usually require a viral or non-viral carrier nanoparticle that can deliver them to cells where the antigen is expressed.

Almost all vaccines are in the size range that are amenable to interrogation by static, dynamic and electrophoretic light scattering. The biophysical properties accessed by these techniques are radius, molar mass, zeta potential and composition of conjugates. Size and zeta potential are strong determinants of delivery success for certain vaccines, especially for those requiring nanocarriers. Radius, molar mass and derivative parameters help to characterize aggregation of proteins or viruses, molecular conformation, biomolecular interactions, glycosylation and payload, for example a VLP with nucleic acid cargo. Particle concentration and payload are relevant to physical and genomic titer, and so are crucial for proper dosing.

We start here with a description of the basic principles of light scattering and instrumentation, followed by case studies highlighting characterization of the various classes of vaccines by light scattering, and the relevance of these results to their discovery, development and manufacture.

View key publications on vaccine characterization



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Camille Lawrence received a BS in Biochemistry at the University of California, Santa Barbara. Her doctoral work in Biophysics, also at UCSB, focused on understanding the contributors to protein and biopolymer folding kinetics. She also has bioinformatics industry experience, where she worked on the first draft of the human genome project. At Wyatt, she has served in roles in software, support and sales and marketing. She is also the dean of Light Scattering University.



Chapter 1

The light scattering toolkit



There are three types of analytical light scattering relevant to bionanoparticles: multi-angle light scattering (MALS), which measures size (spherical radius R or root-mean-square radius, R_g) and—in conjunction with a concentration detector—absolute molar mass M ; dynamic light scattering (DLS), which measures hydrodynamic radius, R_h ; and electrophoretic light scattering (ELS), which measures charge and zeta potential. These may be applied to unfractionated samples or, for more detailed information, to samples that have been fractionated by size.

Multi-angle light scattering: Molar mass, radius and beyond

MALS is a form of static light scattering (SLS). By measuring the fraction of incident light scattered by a sample in a solvent—the Rayleigh ratio—over a range of angles, one obtains the molar mass based on the intensity and radius based on the angular dependence of the scattering. The simplest representation of the relationship is:

$$\frac{R_\theta}{K^*} = McP(\theta) \quad (1)$$

where R_θ is the Rayleigh ratio at angle θ , M is molar mass, c is concentration in mg/mL, and $P(\theta)$ is a function which is fit to determine R_g . K^* contains physical constants, including wavelength, solvent refractive index, and the specific refractive index increment of the sample in solution, dn/dc .

This is the most generalizable analysis. If the shape of the particle is known along with the sample's refractive index, then the particle concentration, i.e., particles/mL, can be measured. In this mode, the shape models are fit to provide geometric parameters, like radius for a sphere or length for a rod.

DAWN, microDAWN, miniDAWN

With 18 detectors, from 22° to 147°, the [DAWN®](#) instrument measures R or R_g over a large range, from 10 nm to 1000 nm. Paired with an online concentration detector like the [Optilab®](#) differential refractive index detector, it also determines molar mass from 200 Da to 1 GDa.

For samples under ~50 nm radius, including most proteins, oligomers and complexes, 3 angles are sufficient to fit the data to measure radius. Wyatt Technology offers two 3-angle MALS instruments: the [miniDAWN®](#), for standard HPLC and the [microDAWN™](#), for UHPLC. See details about the differences between HPLC and UHPLC in the next section.

Dynamic light scattering: Hydrodynamics revealed

Dynamic light scattering determines hydrodynamic radius, R_h , by measuring fluctuations in the scattered intensity on microsecond time scales (Figure 1, top). These fluctuations are caused by Brownian motion of the scattering particles and the rate of fluctuation is related to the particles' diffusion properties.

The raw data produced by these fluctuations are processed to produce a plot, called an autocorrelation function (ACF), with a characteristic exponential decay along the time axis, τ (Figure 1, bottom). As shown in Equation 2, the decay rate is determined by the hydrodynamic radius of the particle (R_h is in green, other experimental parameters are captured in the constant k) with smaller particles diffusing more rapidly and hence leading to a faster decay.

The DAWN, miniDAWN and microDAWN may be outfitted with an embedded [WyattQELS™](#) module for simultaneous online DLS measurements. MALS

combined with DLS informs on molecular conformation, as the physical bases for R_h and R_g are different and their ratio contains information about how mass is distributed over the molecule.

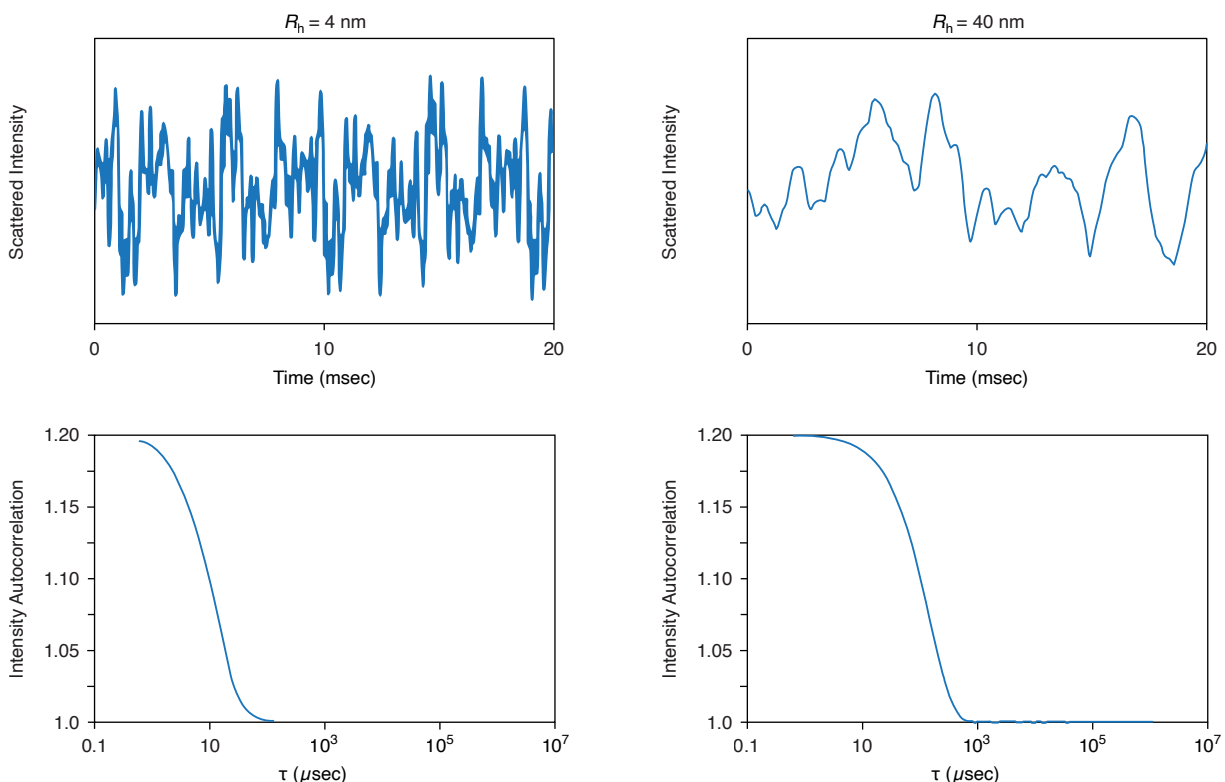


Figure 1. Raw DLS data (top) shows fluctuations in scattered intensity due to Brownian motion, which is converted into an autocorrelation function (ACF; bottom). The decay rate of the ACF is faster for smaller particles.

$$g^2(\tau) = 1 + \beta e^{-\frac{k}{R_h \tau}} \quad (2)$$

DynaPro® Plate Reader, DynaPro NanoStar®, Mobius™

Online measurements usually involve separation of species in solution. DLS measurements can also be carried out in “batch”, that is, without fractionation, yet still determine a size distribution. Wyatt Technology offers three batch DLS

instruments: the [DynaPro Plate Reader \(DPR\)](#), [DynaPro NanoStar](#) and [Mobius](#).

In addition to measuring R_h , the DPR and NanoStar also perform single-angle SLS measurements to obtain the solution's weight-average molar mass, with an upper limit of 1 MDa, and the particle concentration of each population present.

Electrophoretic light scattering: Charges in motion

In the context of modern vaccines, charge and zeta potential are critical properties that impact cellular transfection with nucleic acid cargo, as well as stability of formulations and interactions with plasma components. Electrophoretic light scattering (ELS) determines charge and zeta potential by measuring the Doppler shift in the light scattered from a sample that is moving in response to an applied electric field.

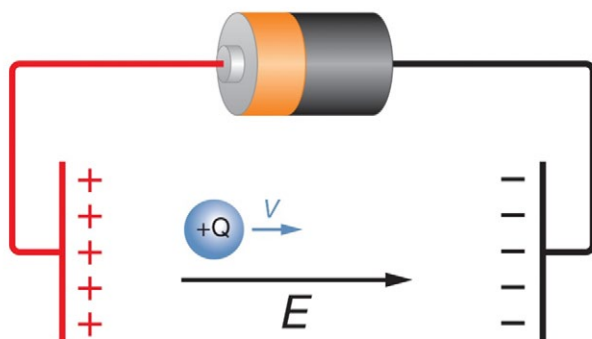


Figure 2. Electrophoretic Light Scattering (ELS) applies an electric field to a charged particle while shining a laser on it. The scattered light is Doppler shifted, and this frequency shift is measured to obtain mobility (i.e., speed) and charge.

[Mobius](#)

Simultaneously with DLS, Mobius performs ELS measurements to directly determine size and electrophoretic mobility, from which zeta potential and charge are calculated.

Fractionation: Divide and conquer

Two auxiliary techniques that enhance and complement light scattering are size-exclusion chromatography (SEC) and field-flow fractionation (FFF), both of which separate macromolecules and nanoparticles according to size. Following separation, each fraction is characterized by inline detectors – MALS, DLS and others. The combination of separation and characterization, denoted SEC-MALS or FFF-MALS, provides high-resolution distributions of size and molar mass, as well as more detailed information on structure and conjugate content (i.e. cargo, in the case of gene vectors).

Size-exclusion chromatography

SEC, a type of high-performance liquid chromatography (HPLC), is a common method for fractionation of macromolecules that utilizes a packed column as stationary phase. Recently, UHPLC has improved upon traditional HPLC with specialized columns and pumps. The advantages of UHPLC are higher resolution, faster runtime and lower minimum required sample. The microDAWN instrument is specially designed to couple with UHPLC pumps and columns and to preserve the excellent resolution afforded by this technique, together called UHP-SEC-MALS.

Field-flow fractionation

FFF uses an open channel rather than a packed stationary phase and offers certain advantages over SEC: 1) Its separation range, 1 to 1000 nm radius, is much larger than that of SEC and more suitable for typical viruses and other vaccines, which are roughly 100 nm in diameter; 2) With no stationary phase, FFF imparts little to no shear and will not disrupt fragile bionanoparticles or polymers. Like SEC-MALS, an FFF-MALS system incorporates HPLC components—pump, autosampler and UV detector—but replaces the column with an Eclipse™ FFF instrument and channel.

Light scattering, refractive index, fluorescence and other inline detectors may be added downstream for comprehensive characterization.

Composition-gradient MALS: Rules of attraction

By pairing a MALS instrument with a [Calypso®](#) composition-gradient delivery system, binding is characterized in detail via a technique called [CG-MALS](#). In CG-MALS, the weight-average molar mass of a solution is measured as a function of concentration and composition in order to determine the absolute stoichiometry and binding affinity of complex formation. It is a label-free method for analyzing interactions in solution and is suitable for self-association, hetero-association and complex multivalent interactions.

Real-time MALS: Molar mass live!

Production processes for vaccines, similar to other biologics, are complex and should be monitored closely to maintain product quality. While process analytical technology (PAT) of bioreactors and downstream purification processes typically measure process parameters, it is far more beneficial to monitor, in real time, actual product attributes. [ultraDAWN™](#) is a specialized MALS detector engineered for process development and process control. With [OBSERVER™](#) software, the ultraDAWN reports molar mass, particle size and particle concentration via real-time MALS ([RT-MALS](#)). The system may be used in-line for low flow rates, or on-line for high flow rates or stationary reactor vessels and may be programmed to trigger fraction collection or process endpoints according to criteria related to these quantities.

Chapter 2

Vaccines, great and small



The following sections are case studies of the representative classes of vaccines: subunit, polysaccharide conjugate and nanoparticles including viruses, virus-like particles and lipid nanoparticles. These categories were chosen based on applicable light scattering analyses; for example, subunit vaccines are generally smaller than the other two classes and work well with SEC-MALS and protein conjugate analysis, while polysaccharide conjugates are very polydisperse and often too large to separate by SEC, which favors FFF as a separation technique. Nanoparticle vaccines, in addition to analysis by FFF-MALS, are typically characterized by batch DLS or ELS.

Subunit vaccines

Unlike the original form of a vaccine, which is a whole pathogen, subunit vaccines are a fragment of the pathogen, usually surface proteins. The antigen is either paired with an adjuvant or embedded in the surface of an innocuous virus. Often these are oligomeric, and often they are glycoproteins. Light scattering analyses characterize these attributes: binding, oligomeric state and glycan content.

Binding

The adaptive immune response is mediated by recognition of an antigen by an antibody or T-cell receptor. Characterization of binding of immunoglobulins to putative immune targets like viral surface glycoproteins is an important early stage in vaccine research. These interactions depend not only on the strength and specificity of binding, but also on the oligomeric state of the pathogenic targets, many of which are not monomeric. Since MALS measures absolute molar mass, it is well suited to characterize unambiguously the strength and stoichiometry of binding interactions. Complexes can be analyzed either with fractionation, via size-exclusion chromatography (SEC-MALS), or without, using

composition-gradient MALS (CG-MALS). Different information about the interacting species is accessed by the two approaches.

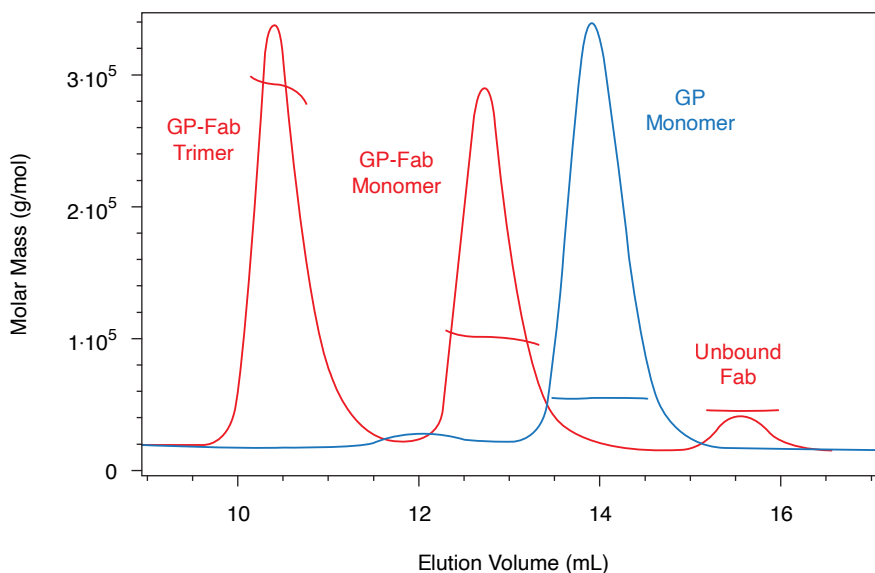


Figure 3. Both monomer and trimer forms of Lassa virus surface glycoprotein, GPCysR4, bind tightly to the Fab fragment of a neutralizing antibody. Adapted with permission from Hastie, et al.¹.

In SEC-MALS, the presence of molecular complexes and determination of their oligomeric state is based on multiples of a given monomer's molar mass. Figure 3 presents two SEC-MALS chromatograms of a Lassa virus glycoprotein (GP) and of the species formed upon incubation of GP with an IgG Fab domain that binds to GP¹. The molar masses determined by MALS at each point in the peak are overlaid on the chromatogram.

In comparing the GP monomer and unbound Fab, it is notable that these proteins, with quite similar molar masses, elute at quite different volumes. This is a consequence of different conformations: the glycoprotein contains a significant fraction of glycans, which occupy a much larger hydrodynamic volume compared to an equal mass of well-folded protein and hence elute earlier in the size-exclusion

column. The analysis of conjugated proteins such as this viral surface moiety is described in the next section.

The GP-Fab monomer complex is unambiguously identified as such by its molar mass, which is just the sum of the masses of Fab and GP. While it could, in principle, consist of a GP dimer (which would have very similar molar mass), this possibility is dispelled via the conjugate analysis which shows that the glycan content is equivalent to that of GP + Fab rather than of GP + GP. Further, the GP-Fab trimer is identified the same way.

Identifying the complexes requires the affinity to be high enough that they do not dissociate on the column. The decreasing molar mass on the trailing shoulders of GP-Fab monomer and dimer peaks is a result of the complex partially dissociating due to dilution while passing through the column. Because the solution is not in equilibrium, an accurate equilibrium dissociation constant K_D cannot usually be measured by SEC-MALS.

Coupling a MALS detector to a Calypso composition-gradient delivery system (CG-MALS) creates a unique means for measuring both stoichiometry and K_D of protein interactions. CG-MALS works for complicated combinations of interactions as well as simple heterodimer formation. For example, if the sample in the red trace of Figure 3 were to be analyzed by CG-MALS, all three populations would be identified, along with the respective binding strengths and stoichiometries for the two complexes.

Unlike FRET or other dye-based binding assays, no labeling is required in CG-MALS. This technique also has important advantages over ELISA, SPR and other surface-bound assays, which may introduce artifacts when the orientation of immobilization or surface properties alter binding characteristics.

Protein conjugate analysis quantifies glycan content of antigenic glycoproteins

Glycosylation is a key property of most viral antigens. In the biochemical arms race between pathogens and the host immune system, glycosylation of viral antigens may serve to cloak the invading pathogen². In rational design of subunit vaccines, a first step may take into account overall glycan content.

Standard SEC-MALS analysis uses either UV absorption or refractive index detection to measure concentration. In a glycoprotein, the glycans are invisible to UV and the dn/dc values of glycans and proteins differ. Hence this analysis will not provide an accurate molar mass result.

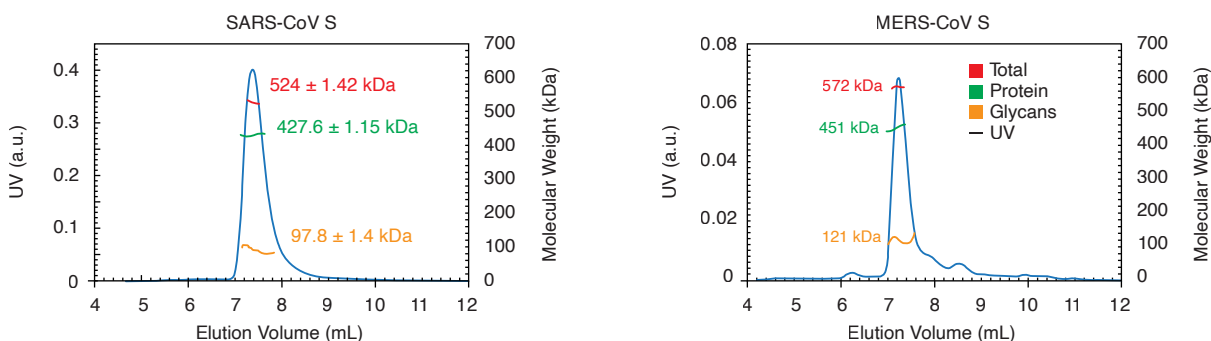


Figure 4. Glycosylation is one strategy employed by pathogens to evade the immune system. Using the MALS-UV-RI Protein Conjugate Analysis method to distinguish between the total proteinaceous molar mass and that of glycans, total glycan content in both SARS-CoV and MERS-CoV spike protein is found to be 25% by mass. Adapted from Walls, et al.³ with permission.

The **ASTRA**[®] software used with Wyatt's MALS instruments includes a method for analyzing glycoproteins and other conjugates, known as the *Protein Conjugate Analysis* (though it is also applicable to other types of conjugates). This method quantifies the molar masses of both glycan and protein components (as well as the total molar mass) at each eluting fraction in a SEC-MALS experiment that acquires signals from both RI and UV concentration detectors.

Figure 4 shows the results of a study exploring structural determinants of antibody binding and neutralization. As a starting point to characterizing the extent and nature of glycosylation in the spike (S) target antigen, the total glycan content of both SARS-CoV and MERS-CoV was found to be ~25% of the total mass³.

These data were collected by the UHPLC-compatible microDAWN and microOptilab. With the higher resolution afforded by UHPLC columns and pumps, as little as 1 μ L of sample can be analyzed in a fraction of the run time as traditional HPLC.

Protein conjugate analysis is also effective in quantifying nucleic acid cargo in carrier nanoparticles, as elucidated in the VLP example in [Viruses, VLPs and Nanocarriers](#).

View how ACROBiosystems
uses SEC-MALS for
2019-nCoV therapeutics



Polysaccharide conjugate vaccines

Polysaccharide conjugate vaccines, which are most often developed against cellular pathogens like bacteria, typically contain a weaker polysaccharide antigen conjugated to a stronger antigen, such as a protein, to boost immune response. The polysaccharide component can be very polydisperse and heterogeneous, with varying conformations and ranging in molar mass from kDa to many MDa. For these vaccines, SEC-MALS and FFF-MALS quantify several quality attributes.

Stability is of critical importance for efficacy. Degradation of the product over time can be monitored by SEC-MALS with high resolution of the distribution of sizes present⁴.

SEC-MALS was used in a recent study of the effect of vaccine size on immunogenic response, and to elucidate the relative importance of polysaccharide loading, stoichiometry and size. Light scattering determined the molar masses and oligomeric state of several MenC-TT conjugates, with the MenC portion ranging from 24 to 441 kDa. It was found that the conjugates with longer polysaccharide chains were more effective at generating an immune response in mice⁵.

Viruses, VLPs and lipid nanocarriers

These vaccine classes are combined in this section because they share multiple characteristics relevant to light scattering. They are all much larger than proteins and they usually comprise some combination of protein, nucleic acid and lipid. In terms of function, these classes include traditional live or attenuated viral vaccines, sub-unit vaccines where the antigen is expressed on the surface of a virus or virus-like particle and gene vectors.



Prof. Jon Pokorski, Associate Professor at University of California, San Diego, was an avid user of Wyatt instrumentation during his postdoctoral stint at Scripps Research Institute in La Jolla, CA. His primary use was the characterization of viral nanoparticles used as delivery vehicles for cancer therapies. He states, *“Wyatt instruments provide us the experimental tools to push research forward very rapidly. Particle sizing is extremely reproducible and accurate, while the team at Wyatt is always willing to help to resolve any experimental obstacles.”*

Sizing

Size is one of the strongest predictors of pharmacokinetic behavior, successful delivery and potency of nanocarriers, especially if entry into a cell or nucleus is necessary or if the particle needs to appear like an infectious virus to the immune system. For example, for lipid nanoparticles (LNPs) used in mRNA vaccines, those smaller than 75 nm in radius are most successful at reaching target tissue and getting taken up by antigen-presenting cells (APCs)⁶. Size is also a common attribute in assessing quality and reproducibility for regulatory purposes.

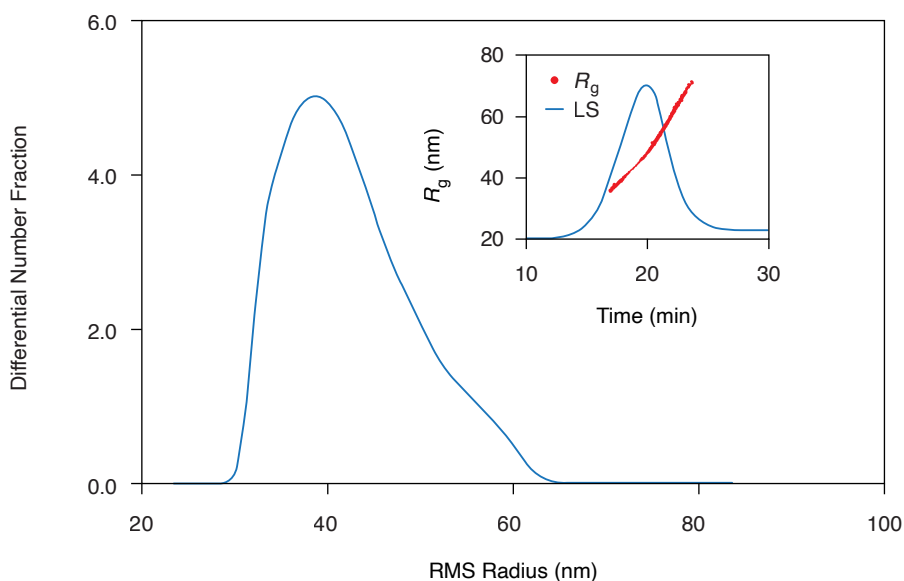


Figure 6. With the aid of FFF to fractionate the sample, the size distribution of a lipid nanoparticle preparation is determined with high resolution. It is relatively monodisperse, with 99% between 35 to 70 nm R_g . Inset: Original fractogram, with R_g overlaid. Source: Wyatt Technology Corporation.

Depending on how nanoparticles are synthesized, they may be monodisperse or highly polydisperse. Both SEC-MALS and FFF-MALS, optionally including online DLS, provide high-resolution size distribution information. Hydrodynamic radius, R_h and/or spherical geometric radius R may be measured simultaneously (see [WP2608](#) for LNPs and [WP9001](#) for nanoparticles). Particle concentration

is determined directly and accurately using only a MALS detector, as described in the next section.

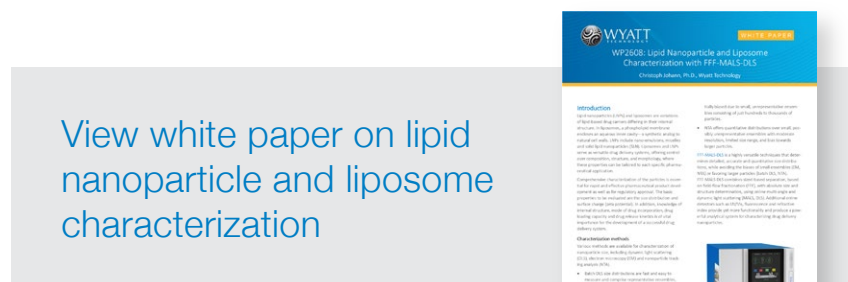


Figure 6 presents the size distribution of an LNP sample, analyzed by FFF-MALS. Over 90% of the particles are between 35 to 70 nm R_g , with no aggregates detected.

Particle count of virions

In producing safe, effective vaccines, it is important to know the total physical titer of virions and the level of aggregation at each point in the process. FFF-MALS accomplishes this without specialized reagents or tedious sample preparation. Once a method has been established, a run takes 30-60 minutes with little preparation time and can be automated with a standard HPLC autosampler.

SEC-MALS may also be used for this type of analysis, but SEC is limited to much smaller radii than FFF. In either case, an accurate particle concentration is obtained, requiring only a MALS detector with the refractive index of the particle as input.

MALS does not rely on the presence of a target nucleic acid sequence for amplification. In contrast to PCR methods, which are used to estimate genomic titer, FFF-MALS detects all particles including defective virions or those lacking genetic material. Moreover, as discussed below, the addition of UV and RI detectors enables quantification of the nucleic acid payload and therefore genomic content.

Table 1 shows a comparison of total count and percent aggregate of an influenza virus sample, measured by four techniques, two of which are microscopy based, and two that use MALS⁷. The count is determined in an FFF-MALS or SEC-MALS run by integrating concentration, determined in particles/mL, across the peak. The total count from FFF-MALS agrees well with TEM, yielding 5-fold better precision. The SEC-MALS result slightly underestimates the total count, probably because larger particles are held up in the column or poorly resolved. FFF-MALS also detects aggregate content that SEC-MALS misses, and % aggregate is in excellent agreement with TEM.

| Method | log ₁₀ Total Virus Count (mL) | | % Aggregate | |
|----------|--|------|-------------|------|
| | Average | %CV | Average | %CV |
| FFF-MALS | 10.4 | 1.8 | 25 | 24.5 |
| SEC-MALS | 9.9 | 0.8 | 19 | 4.4 |
| AFM | NA | NA | 23 | 8.9 |
| TEM | 10.2 | 10.0 | 25 | 8.2 |

Table 1. Based on data presented in Reference 7.

Detecting and quantifying nucleic acid cargo

During expression, purification, packaging and assembly, carrier nanoparticles like VLPs or LNPs can be monitored for properties including size, incomplete assembly, aggregation and nucleic acid content using analytical techniques in the light scattering toolkit.

DNA cargo of VLP via conjugate analysis

Introduced in the previous section, protein conjugate analysis reports the molar masses as well as relative amounts of two moieties. While often used to quantify protein and glycan in the context of glycoproteins, it also determines protein and nucleic acid in the context of gene vectors and other protein-nucleic acid complexes. Coupled with a fractionation technique like FFF, a high-resolution size distribution will indicate the presence of dimers, oligomers or aggregates.

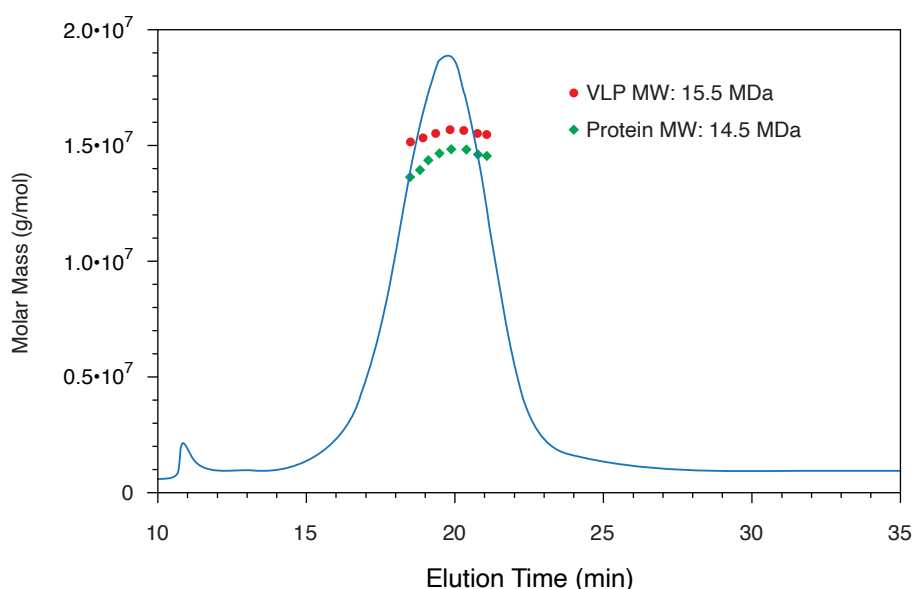


Figure 7. FFF-UV-MALS-RI confirms the expected molar mass of protein capsid and genetic content for a VLP sample. Data courtesy A. Citkowicz, Bayer Healthcare Pharmaceuticals.

In the example of FFF-UV-MALS-RI shown in Figure 7, a final VLP product with DNA cargo is verified to have a total mass of 15.5 MDa: 14.5 MDa protein capsid, with 1 MDa of DNA (not shown) corresponding to the full genome of 1.6 kbp⁸. The R_g radius is also verified at 22 nm (not shown).

mRNA cargo of LNP via conjugate analysis

The protein conjugate method was so named because it was originally applied to glycoproteins, but it can be used for any conjugated particle, as long as one or both of the UV extinction coefficient and the specific refractive index increment dn/dc of the two moieties have sufficiently different values.

The same principle and method employed in the previous VLP example are used to quantify RNA cargo in lipid nanoparticles (LNPs). However, for particles above about 30 nm in radius, UV scattering will confound concentration measurement. A proprietary method developed by Wyatt Technology corrects for UV scattering, and the molar masses of both RNA and lipid are determined for each fraction.

The fraction by weight, F_w , is calculated as $M_{w, \text{ RNA}} : M_{w, \text{ total}}$.

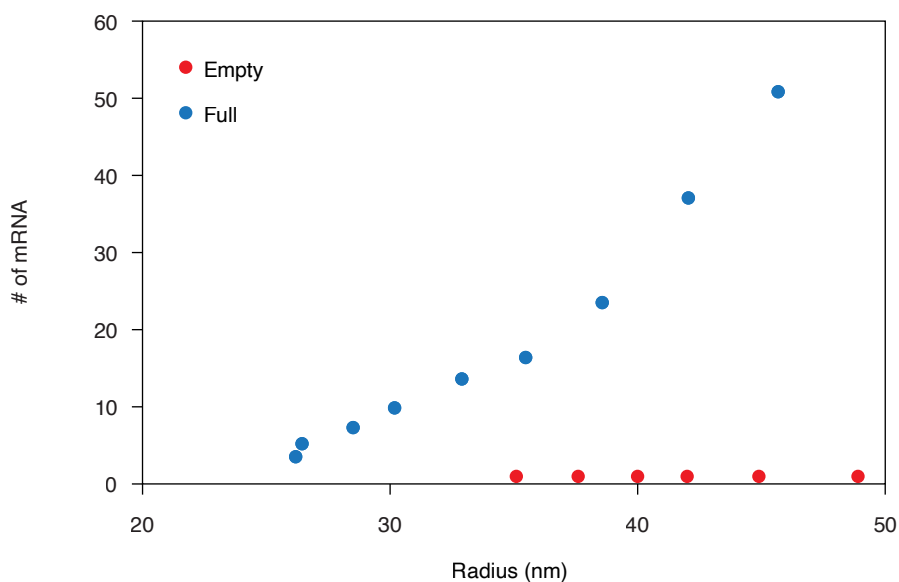


Figure 8. Conjugate analysis, with correction for UV scattering, shows two different preparations of LNPs, one with and one without mRNA cargo. It is confirmed that there is no mRNA detected in the Empty sample. In the Full sample, more mRNA molecules are taken up by the larger LNPs. Source: Wyatt Technology Corporation.

Figure 8 shows a comparison of LNP preparations with and without mRNA encapsulated. Using the specialized LNP analysis, the number of mRNA molecules loaded is quantified. In the empty control, it is confirmed that no mRNA is detected. In the encapsulated sample, the total mRNAs in each LNP increase as the LNP size increases.

Stability, aggregation and formulations

For vaccine candidates with diverse chemistries and sizes, from proteins to VLPs, aggregation is a major concern. At best it may reduce efficiency and yields of industrial processes, and at worst, it may cause toxicity. It is also indicative of unstable native tertiary and quaternary protein structure.

To reduce the possibility of aggregation and to ensure overall stability during the early formulation process, panels of hundreds of combinations of buffer conditions and excipients are screened. For late-stage formulation stability and shelf-life prediction, samples are typically stressed by agitation, freeze-thaw cycling or thermal ramping, followed by quantification of aggregates.

DLS offers many advantages in aggregation screening:

- 1) Results are obtained in seconds.
- 2) Few parameters are required for the analysis, no matter the chemical composition of the analyte. Only the solvent refractive index and viscosity are needed and the DLS software provides these values for the most common solvents and buffers.
- 3) All three of Wyatt's batch DLS instruments support automated temperature ramping.

- 4) Low volumes are required: as low as 4 μL in a 1536-well plate, and as little as 1.25 μL for the NanoStar in a quartz cuvette or 4 μL in a high-optical-quality disposable cuvette. The DynaPro Plate Reader is compatible with standard well plates of 96, 384 or 1536 wells.
- 5) The DPR in particular supports high-throughput analyses, with each measurement requiring as little as 10 seconds.

Beyond DLS screening, SEC-MALS and FFF-MALS add fractionation to the analysis, providing much more detail about size distribution and relative concentrations of oligomers and aggregates.

In a study optimizing formulation conditions of recombinant murine polyomavirus (MuPyV), the virus was analyzed by FFF-MALS, TEM and batch DLS in a DynaPro Plate Reader. Analyses were performed before and after exposing the sample to an elevated temperature of 48 °C for 1 hour.

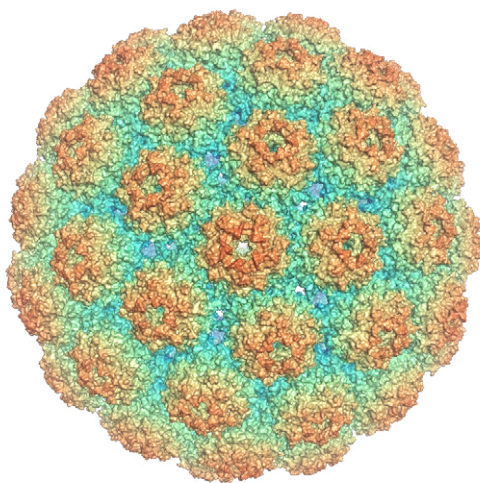


Image credit: Opabinia Regalis, Wikimedia Commons.

In all three analyses, the naïve sample indicated the expected radius of ~25 nm. Following heat stress, the sample was centrifuged and the supernatant subjected

to analysis by FFF-MALS, which failed to detect an appreciable amount of material, either as monomer or aggregate. Post-heat-stress material without further treatment (i.e., no centrifugation) was also tested by TEM and DLS; TEM detected some amorphous aggregates and DLS indicated large, micron-sized particles as well as high overall scattered intensity.

Fractionation generally yields higher resolution of size and/or molar mass distributions than a batch measurement such as DLS. But in this case thermal stress precipitated the sample, so FFF-MALS did not detect any VLPs in the supernatant of the centrifuged sample. A batch DLS experiment, unlike FFF, does not require centrifuging the sample for the sake of protecting a channel or inline detector from clogging or fouling. Batch DLS detected large particles that were present in an uncentrifuged sample, with an approximate average R_h of 2300 nm⁹. The precipitate was detected using only 2 µg of sample and 100 sec per measurement.

In the same study, a temperature ramp was performed in the DPR to determine the best formulation conditions for thermal stability. Twenty-eight formulations were sampled. They included varying concentrations of sucrose or trehalose and the polyols mannitol or sorbitol, both with and without 0.5% polysorbate 20.

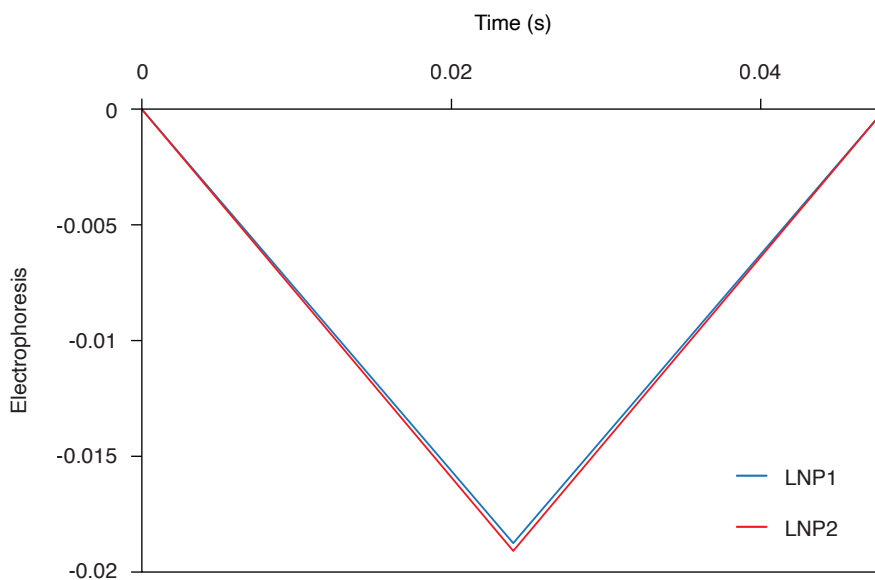
In a single, automated experiment, the condition that yielded the maximum T_{agg} was found to be 40% sorbitol or sucrose, plus 0.5% polysorbate 20. The total sample amount used in the experiment was less than 150 µg.

Zeta potential of LNPs

Zeta potential is a predictor of stability against aggregation in solution. Along with overall charge, it is one of the most important predictors of successful delivery of carrier nanoparticles to target tissues. For lipid nanoparticles in particular, which

are often used to deliver mRNA, there seems to be an optimal charge: some positive charge is required to reduce repulsion from the negatively charged nucleic acid cargo, as well as to facilitate localization to the negatively charged cell membrane, but too positive may be toxic⁶.

While zeta potential is critically important in predicting stability of carrier nanoparticles, adding DLS to the analysis gives a more complete picture. In Figure 9, two formulations of LNPs were analyzed with a Mobius DLS/ELS detector, which simultaneously measures zeta potential and charge (using ELS) and hydrodynamic radius (using DLS).



| Item | Temp | Mobility ($\mu\text{m cm/s V}$) | Zeta Potential (mV) |
|------|------|--------------------------------------|------------------------|
| LNP1 | 25.0 | -0.75 | -10.73 |
| LNP2 | 25.0 | -0.76 | -10.92 |

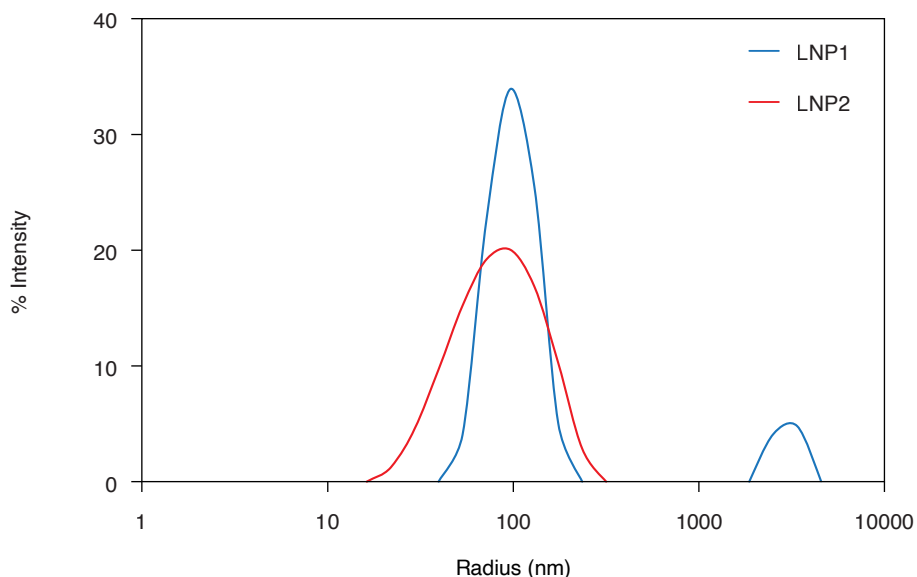


Figure 9. The graph on the previous page and the graph here above show the Mobius zeta potential and R_h simultaneous measurements. This allows distinction between two LNP samples that appear identical in terms of zeta potential; the DLS reveals trace aggregate in LNP1. Source: Wyatt Technology Corporation.

The raw mobility graphs, from which zeta potential is derived, are shown on top. The two graphs are virtually identical, with a zeta potential of ~ -11 mV. However, the DLS results on the bottom show the presence of aggregates in the LNP1 (blue) sample and their absence in LNP2 (red). Zeta potential alone is important but insufficient for a comprehensive stability study.

Real-time MALS in process analytical technology

In industrial processes, the aim of process analytical technology (PAT), as defined by the FDA, is to create a dynamic manufacturing process that compensates for variability to produce a consistent product¹⁰. Among the criteria is collecting data at appropriate intervals. In this regard, live monitoring of critical quality attributes, like size or molar mass, makes for a streamlined, reproducible process.

The ultraDAWN, though similar to the DAWN in terms of measurement range, is designed for real-time MALS (RT-MALS) measurements. The accompanying

OBSERVER software reports molar mass, size and particle concentration as the process proceeds. The user provides an acceptable target range, for either molar mass, radius or particle concentration and OBSERVER will generate a trigger for automated collection within that range or to indicate a process endpoint.

RT-MALS in virus purification

Figure 10 illustrates an example of virus purification, wherein the viruses are expected to be between 85 to 105 nm while smaller particles correspond to other cellular components in the lysate such as inclusion bodies and nucleic acids. Sample exiting the ion-exchange column is slipstreamed to the ultraDAWN, and OBSERVER software records live data, with a lag time of 10 seconds from the chamber exit. The radius is plotted in blue with the light scattering intensity (corresponding to the product of particle concentration and the sixth power of the radius) in red. The triggers to start and stop collecting are indicated by the green and red vertical lines, respectively.

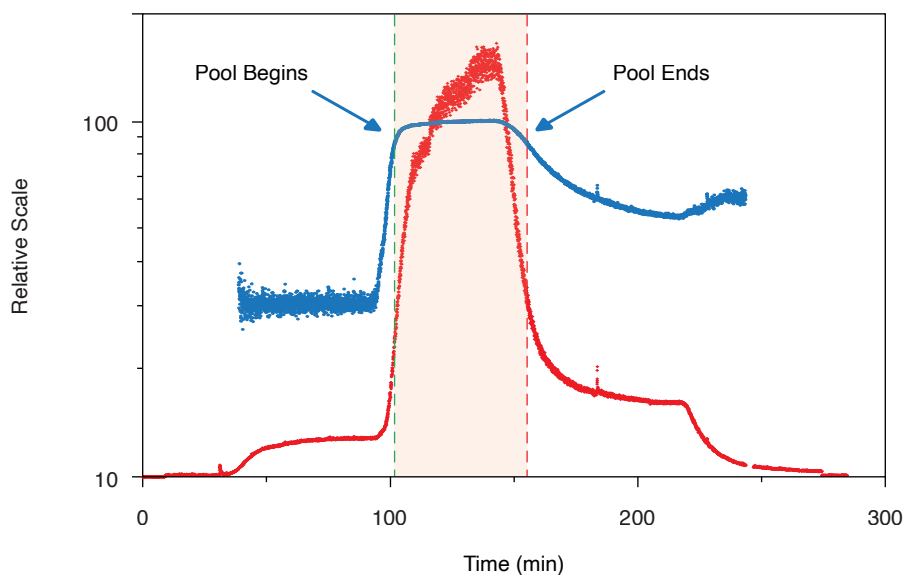


Figure 10. Trace of radius (blue) and light scattering intensity (red) in OBSERVER software, measured by ultraDAWN, used to control collection of viral fractions during ion-exchange purification. Source: Wyatt Technology Corporation.

Collection of virus fractions begins at roughly 100 minutes, when R reaches 85 nm and stops at 150 min. During pooling, viral concentration is integrated to estimate the total number of virions in the pool.

View application note on
in-line monitoring of
liposome size by RT-MALS



Adjuvants

The light scattering toolkit can probe diverse chemistries and conformations, as it directly measures a very fundamental property: molar mass. In addition to the proteins and biomacromolecules described so far, it is also useful to characterize various types of adjuvants.

The most commonly used adjuvants are various forms of aluminum salts, collectively referred to as alum. The size and zeta potential of alum are relevant to its performance as an adjuvant, and the Mobius supports measurements at low enough voltage to avoid damaging samples that may undergo redox reactions at higher voltages. Figure 11 shows zeta potential and hydrodynamic radius results for Alhydrogel (aluminum oxyhydroxide). Only 2 V was sufficient to move the particles without inducing the redox reactions that occurred at higher voltages.

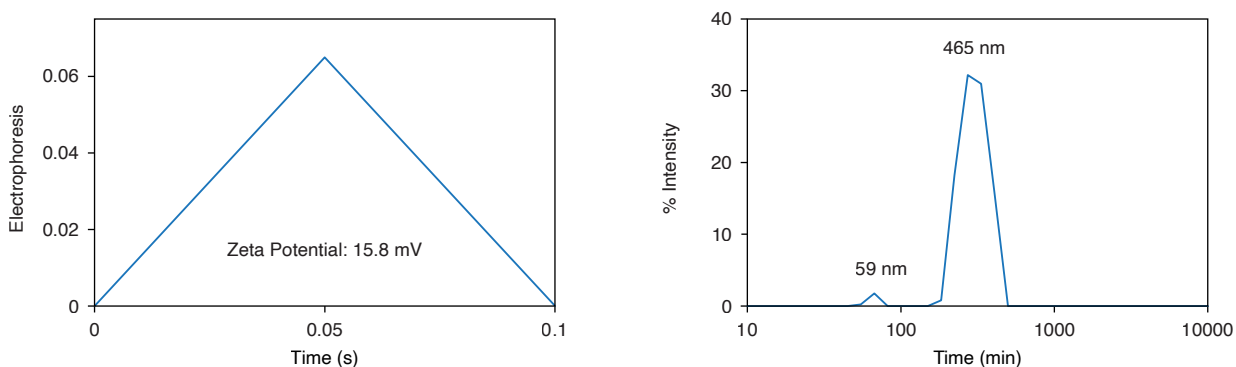


Figure 11. Alum adjuvant Alhydrogel, analyzed in the Mobius, shows a zeta potential of 16 mV, and the expected radius of 700 nm, with a trace of smaller particles. These data were collected at 2.0 V. Source: Wyatt Technology Corporation.

In a structural vaccinology approach to more exploratory adjuvants, thermoresponsive amphiphilic nanoparticle-forming polymers were employed to generate a supramolecular micelle-like assembly, with the hydrophobic portion of the polymer as the core and subunit antigen protruding into the aqueous surrounding¹¹. A TLR agonist was decorated on the hydrophilic portion of the polymer, serving as an adjuvant. This assembly conferred neutralization of the virus in mice that the antigen alone failed to do.

The thermoresponsive function of the assembly was confirmed by DLS, with a sharp transition from monomers to the supramolecular assembly at 33 °C. The benefit of this transition upon reaching physiological temperature is that during production and storage, prior to assembly, the formulation is more soluble, filterable and colloidally dispersed.

Other examples of the utility of light scattering for characterizing adjuvants include SEC-MALS of polymeric raw materials for nanoparticle adjuvants¹², as well as measuring micelle association number for lipoproteins and other amphiphilic adjuvants¹³.

Limit of detection

The limit of detection in light scattering depends mostly on the molar mass. For the vaccines discussed here, it makes sense to distinguish between two major classes: proteins and larger particles, including VLPs, LNPs and whole viruses.

The minimum injected mass for a 100 kDa protein in SEC-MALS is about 0.6 µg. For DLS, the minimum concentration is about 10 µg/mL. For the larger viral-type particles, using influenza as a representative example, which has a radius of 52 nm, the lower limit of detection using FFF-MALS is $\sim 2 \times 10^6$ particles¹⁴.

Summary

The physicochemical properties of various classes of vaccines and adjuvants lend themselves well to light scattering techniques, including SEC-MALS, FFF-MALS, CG-MALS, DLS and ELS. These methods facilitate rapid development, assured quality and industrial scale production for safe, efficacious vaccines.

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