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WHITE PAPER

WP9007: Characterizing vaccines with light scattering

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Introduction

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.

This paper will explain how the various types of light scattering can be used to characterize vaccines. By measuring several attributes including size, shape, molar mass, and other parameters derived from these fundamental characteristics, light scattering aids in vaccine discovery, development, and manufacture. In this paper, we cover several classes of vaccines that span a large range of physicochemical properties.

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, they include:

- **subunit vaccines**, which are usually protein antigens designed to be targeted by the immune system;
- **polysaccharide conjugates,** typically for bacterial pathogens;
- **virus-like particles,** 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 some carrier nanoparticle.

Almost all vaccines are in the size range that can be interrogated by static, dynamic, and electrophoretic light scattering. The fundamental measurements accessed by these techniques are radius, molar mass, and zeta potential. Size and zeta potential are strong determinants of delivery success for certain vaccines, especially for those requiring nanocarriers. Radius, molar mass, and other derivative parameters help to characterize aggregation of proteins or viruses, molecular conformation, biomolecular interactions, and composition of conjugates, for example a VLP with nucleic acid cargo.

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

The Light Scattering Toolkit

There are three types of analytical light scattering relevant to bionanoparticles: multi-angle light scattering (MALS), which (in conjunction with a concentration detector) measures absolute molar mass *M* and size

(root-mean-square radius, R_g); dynamic light scattering (DLS), which measures hydrodynamic radius, R_h; and electrophoretic light scattering (ELS), which measures charge and zeta potential.

Light scattering instrumentation

All online MALS instruments measure the scattered intensity at multiple angles, which is extrapolated to 0° to calculate the molar mass. For samples with radius > 10 nm, the dependence of scattered intensity vs. angle can be fit to measure R_g.

With 18 detectors, from 22° to 147°, the DAWN™ instrument measures 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, like proteins, 3 angles are sufficient to fit the data to measure R_g. There are 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.

Any of the MALS instruments may be outfitted with an embedded WyattQELS™ module for simultaneous online DLS measurements. MALS combined with DLS can inform 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.

Online measurements usually involve separation of species in solution. DLS measurements can also be carried out in "batch", that is, without fractionation. Wyatt offers three batch DLS instruments: the DynaPro™ Plate Reader (DPR), NanoStar™, and ZetaStar™. In addition to measuring R_h, the DPR, NanoStar and ZetaStar can also perform single-angle static light scattering (SLS) measurements to obtain weight-average molar mass, with an upper limit of 1- 10 MDa.

Simultaneously with DLS, ZetaStar performs ELS measurements which directly determine electrophoretic mobility, from which zeta potential and charge can be calculated. ZetaStar replaces Wyatt's venerable Mobius™ DLS/ELS product.

Light scattering with separation

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 downstream 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).

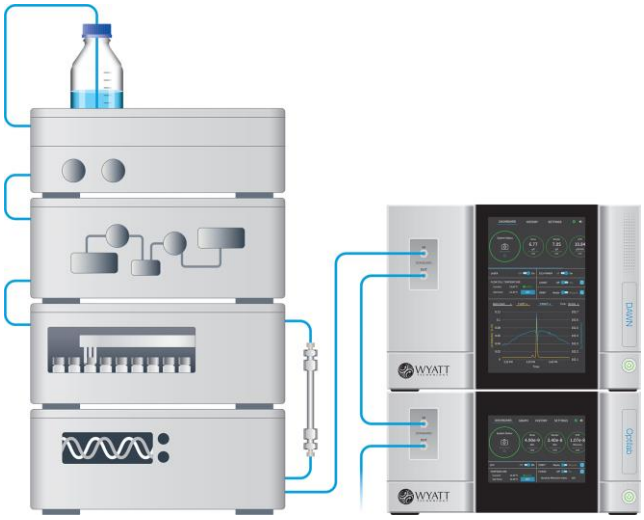

Figure 1. Typical SEC-MALS setup, with chromatography pump, autosampler, DAWN MALS detector, and two concentration detectors: UV HPLC module and Optilab RI.

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. 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.

FFF uses an open channel rather than a packed stationary phase, and offers certain advantages over SEC: 1) its separation range, 1 – 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 controller and channel. Light scattering, refractive index, fluorescence and other inline detectors may be added downstream for comprehensive characterization.

Measuring biomolecular interactions

By pairing the DAWN MALS instrument with a Calypso™ composition-gradient delivery system, binding can be characterized in detail by means of 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 multi-valent interactions.

Real-time MALS for process analytics

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 measure, 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, and may be programmed to trigger fraction collection or process endpoints according to criteria related to these quantities.

Case studies

The light scattering toolkit quantifies many attributes of biological macromolecules and other nanoparticles that are important during discovery, product and process development, manufacture and quality assessment of vaccines. These include:

- Protein-protein and protein-nucleic acid binding
- Glycan content of glycoproteins
- Nucleic acid cargo content of carrier nanoparticles
- Size and structure
- Particle concentration
- Aggregation and stability
- Zeta potential of carrier nanoparticles

The remainder of this white paper discusses each measurement in further detail, including examples and comparisons with other techniques. Finally, applications of MALS as process analytical technology are described.

Antibody-antigen binding

The adaptive immune response is mediated by recognition of an antigen by an antibody or T-cell receptor. Characterizing 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 on the oligomeric state of the pathogenic target, many of which are not monomeric. Since MALS measures absolute molar mass, it is well-suited to unambiguously characterize 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 between these two approaches.

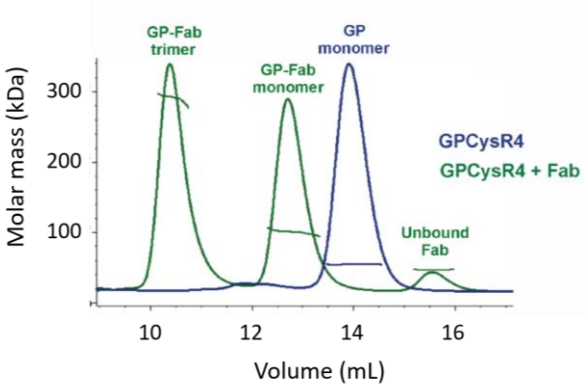

Figure 2. Both monomer and trimer forms of Lassa virus surface glycoprotein, GPCysR4, bind tightly to the Fab fragment of a neutralizing antibody. Data courtesy Kathryn Hastie, Scripps Research Institute.

In SEC-MALS, the presence of molecular complexes and their oligomeric state is measured, based on multiples of a given monomer's molar mass. Figure 2 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 GP[1]. The molar

masses determined by MALS analysis 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. 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 complex dissociation due to dilution while passing through the SEC 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 green trace of Figure 2 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 pathogen[2]. As such, 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.

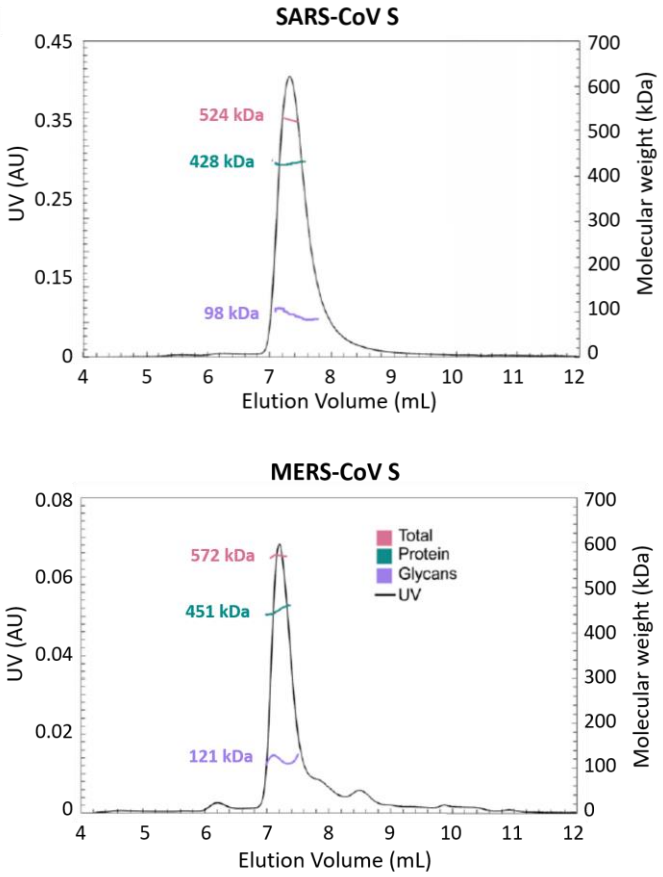

Figure 3. Glycosylation is one strategy employed by pathogens to evade the immune system. Using the MALS-UV-RI *Protein Conjugate Analysis* method to distinguish between the total proteinaceous molar mass and that of glycans, total glycan content in both SARS-CoV and MERS-CoV spike protein is found to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

For glycoproteins and other conjugates, the molar masses of both glycan and protein components (as well as the total molar mass) can be easily quantified using ASTRA's

Protein Conjugate Analysis in a SEC-MALS experiment that uses both RI *and* UV concentration detectors.

Figure 3 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 for both SARS-CoV and MERS-CoV, the total glycan content was found to be ~25% of the total mass[3].

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 µL 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 section.

Detecting and quantifying nucleic acid cargo in carrier nanoparticles

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 different techniques in the light scattering toolkit.

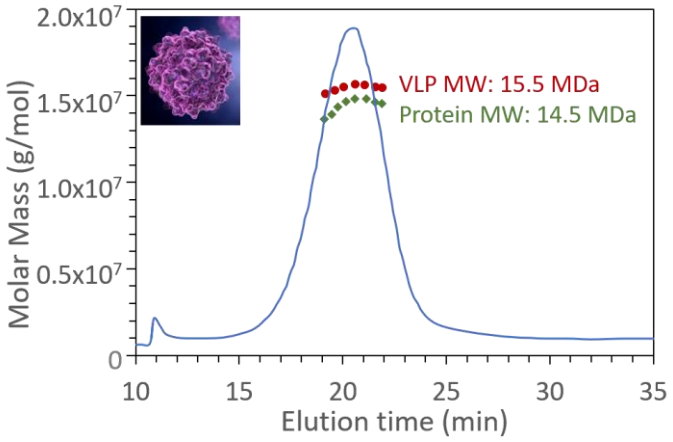

Figure 4. FFF-UV-MALS-RI confirms the expected molar mass of protein capsid and genetic content for a VLP sample. The R_g also agrees with the expected value.

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 can also measure protein and nucleic acid in the context of gene vectors and other protein-nucleic acid complexes. Coupled with a fractionation technique like FFF, high resolution size distribution will indicate the presence of dimers, oligomers, or aggregates.

In this example of FFF-UV-MALS-RI shown in Figure 4, a final VLP product with DNA cargo is verified to be 15.5 MDa total: 14.5 MDa protein capsid, with 1 MDa of DNA (not shown) corresponding to the full genome of 1.6 kbp[4]. The R_g 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 conjugate particle, as long as either or both the UV extinction coefficient and the dn/dc of the two moieties are different.

The same principle and method as in the previous VLP example can be used to quantify RNA cargo in lipid nanoparticles (LNPs). Both UV and RI concentration detectors are required for this analysis, and for particles above about 30 nm in radius, UV scattering will confound concentration measurement.

A proprietary method corrects for the UV scattering in the extinction coefficient, and the molar mass of both RNA and lipid are determined for each fraction. From that, the RNA mass fraction $F_w = M_{w,\,RNA} : M_{w,\,total}$ can be calculated.

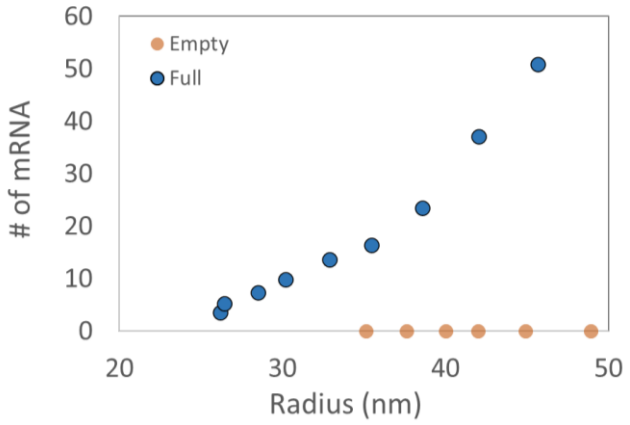

Figure 5. Conjugate analysis, with correction for UV scattering, shows two different preparations of LNPs, one with and one without mRNA cargo. It is confirmed that there is no mRNA detected in the empty sample. In the full, more mRNA molecules are taken up by the larger LNPs.

Figure 5 shows a comparison of LNP preparations with and without mRNA encapsulated. Using the specialized LNP analysis, the number of molecules of mRNA loaded can be 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.

Sizing for LNPs, VLPs, and other carrier nanoparticles

Size is one of the strongest predictors of pharmacokinetic behavior and potency of nanocarriers, especially if entry into a cell or nucleus is necessary. For example, for lipid nanoparticles (LNPs) used in mRNA vaccines, those smaller than 75 nm radius are most successful at reaching target tissue and getting taken up by antigen presenting cells (APCs)[5]. Size is also a common attribute in assessing quality and reproducibility for regulatory purposes.

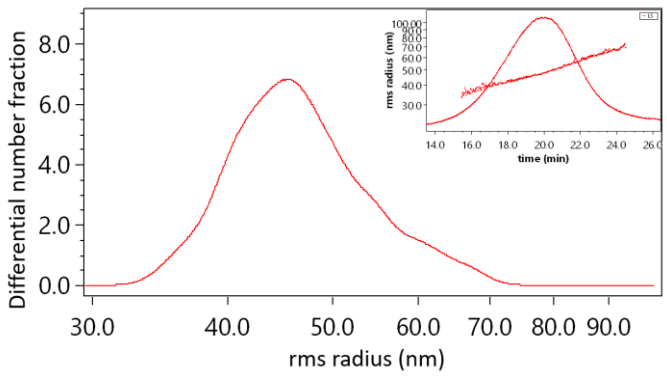

Figure 6. With the aid of FFF to fractionate the sample, the size distribution of a lipid nanoparticle preparation is determined with high resolution. It is relatively monodisperse, with 99% between 35 - 70 nm. Inset: original fractogram, with rms radius overlaid.

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, as hydrodynamic radius R_h and/or rms radius R_g may be measured simultaneously (see WP2608 for LNPs, and WP9001 for nanoparticles). Particle concentration can also be measured 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 35-70 nm R_g, with no aggregates detected.

Particle concentration 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.

In contrast to PCR methods, which are used to estimate infectious titer, FFF-MALS detects all particles including defective virions or those lacking genetic material. FFF-MALS does not rely on the presence of a target nucleic acid sequence for amplification.

Table 1 shows a comparison of total count (by integrating concentration, in particles/mL) and percent aggregate of an influenza virus sample, measured by four techniques, two of which are microscopy based, and two using MALS[6]. The total count from FFF-MALS agrees well with TEM, and with 5-fold better precision. The SEC-MALS result 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 is in good agreement with TEM.

Table 1. In an influenza sample, FFF-MALS outperforms SEC-MALS in terms of detecting aggregate and in total virus count, as confirmed by microscopy (TEM). Both FFF-MALS and SEC-MALS measure much larger ensembles than TEM with correspondingly smaller %CV in total count.

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

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 structure.

To reduce the possibility of aggregation and to ensure overall stability, panels of hundreds of combinations of buffer conditions and excipients are screened during the early formulation process.

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 Wyatt batch DLS instruments support automated temperature ramping
4) Low volumes are required, as low as 4 µL in the 1536-well plate, and as little as 2 µL for the NanoStar or ZetaStar quartz 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.

In assessing stability, and to predict shelf life, samples are typically stressed by agitation, freeze-thaw cycling, or thermal ramping. Adding fractionation to the analysis provides 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 both before and after exposing the sample to an elevated temperature of 48 °C for 1 hour.

In all three analyses, the naïve sample indicated the expected radius of ~ 25 nm. Post 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. In a batch DLS experiment, unlike FFF, it is not necessary to centrifuge the sample for the sake of protecting a channel from clogging or fouling. Batch DLS detected large particles that were present in an uncentrifuged sample, with an approximate average R_h of 2300 nm[7]. 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 is critical for LNPs and nanocarriers

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 toxic[5].

While zeta potential is critically important in predicting stability of carrier nanoparticles, adding DLS to the analysis gives a more complete picture. In Figure 7, two formulations of LNPs were analyzed with Wyatt's Mobius™ DLS/ELS instrument, which simultaneously measures zeta

potential and charge (using ELS) and hydrodynamic radius (using DLS).

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 (red) sample and their absence in LNP2 (blue). While zeta potential is a good predictor of aggregation, crucial additional details are revealed with the additional DLS detector.

Item	Temp	Mobility (µm cm/s V)	Zeta Potential (mV)
LNP1	25.0	-0.75	-10.73
LNP2	25.0	-0.76	-10.92

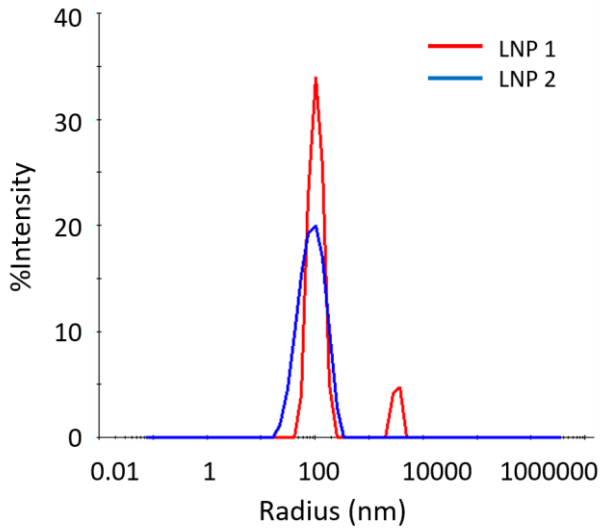

Figure 7. The ZetaStar measures zeta potential and R_h simultaneously. This allows distinction between two LNP samples that appear identical in terms of zeta potential; the DLS reveals trace aggregate in LNP1.

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 product[8]. 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 in real time. 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.

In Figure 8, in an example of virus purification, the viruses are expected to be within 85- 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 22 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 grey. The triggers to start and stop collecting are indicated by the green and red vertical lines, respectively.

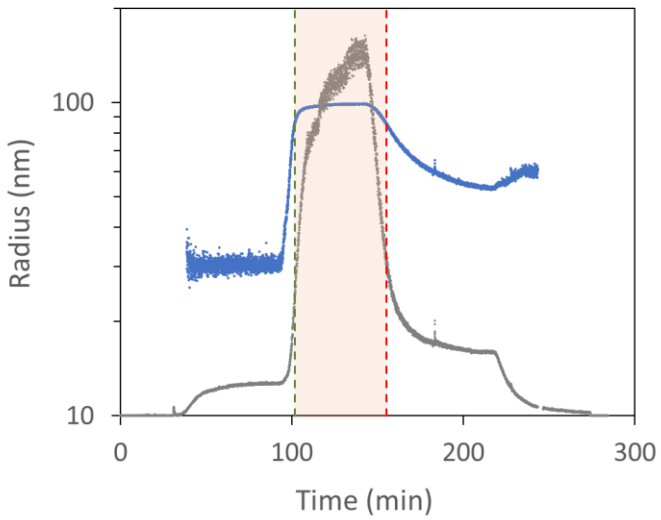

Figure 8. Trace of radius (blue) and light scattering intensity (grey) in OBSERVER software, measured by ultraDAWN, used to control collection of viral fractions during ion-exchange purification.

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 average particle concentration and total number of virions in the pool.

Conclusions

Vaccines encompass a broad range of molecular classes, with diverse physicochemical properties. As they are all in the size regime interrogated by light scattering, MALS, DLS, and ELS can inform on essential properties and quality attributes including size, aggregation, stability, interactions, composition, and conformation. Not only is this applied in research, but many of these attributes are required for production, quality control and verification of lot-to-lot reproducibility in regulatory environments. Tools in Wyatt's light scattering toolkit therefore facilitate safe, efficacious, and rapid vaccine development and production.

For more information on the technology and applications of …

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- *FFF-MALS, please visit* www.wyatt.com/FFF-MALS.
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* Updated January 2024 to reflect Wyatt's newest products.

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