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Solutions for Lipid Nanoparticle Characterization in Product and Process Development

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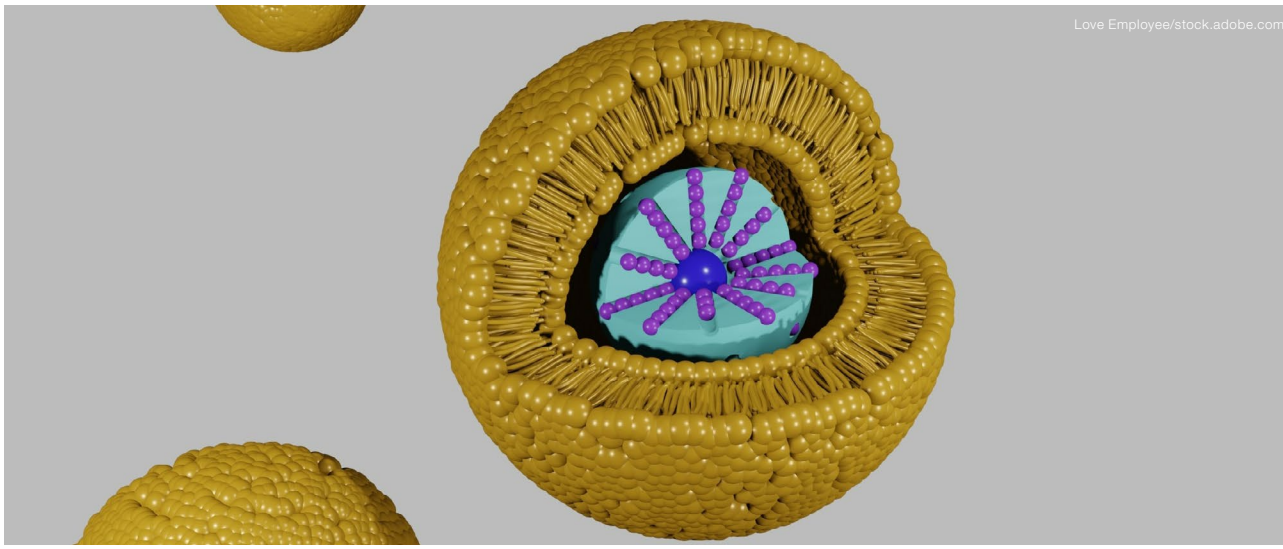

Using Multidetector Asymmetric-Flow Field-Flow Fractionation to Efficiently Analyze Nanopharmaceuticals

Roundtable with Michelle Chen, Fanny Caputo, Jeffrey Clogston, and Xiujuan Jia

Today's nanopharmaceuticals require a highly flexible separation technique.

The characterization of nanopharmaceuticals is a crucial analytical process for quantifying nanomedicine formulations, including important quality attributes such as stability, particle size, and more. Multidetector asymmetric-flow field-flow fractionation (MD-AF4) provides a robust, full-characterization method for a range of applications, while also adhering to regulatory guidelines. *BioPharm International* spoke with Michelle Chen, PhD, Vice President of Analytical Sciences at Wyatt Technology; Fanny Caputo, PhD, Research Scientist with the Department of Biotechnology and Nanomedicine at SINTEF Industry; Jeffrey Clogston, Principal Scientist of Physicochemical Characterization at National Cancer Institute Nanotechnology Characterization Laboratory (NCI–NCL); and Xiujuan Jia, Business Engineer at Merck & Co. to take a deeper dive into MD-AF4 and how to utilize this technique to simplify complex nanopharmaceutical formulations.

BIOPHARM: What benefits does MD-AF4 offer for the analysis of nanopharmaceuticals?

CHEN: Nanopharmaceuticals include liposomes bearing small-molecule drugs, self-assembled protein nanoparticles (NPs), gene vectors, and extra-cellular vesicles, with a diameter between 10 and several hundred nanometers. This size range is the sweet spot of what MD-AF4 can separate and characterize. AF4 provides a gentle fractionation of the nanopharmaceuticals based on their hydrodynamic radius; there is no concern that the large size fractions may be removed or altered, as may occur in size-exclusion chromatography (SEC).

As we know, from small molecules to proteins to nanopharmaceuticals, the difficulty level in full characterization increases exponentially with analyte size. The multiple online detectors following AF4, however, ease the characterization challenges tremendously. The cohort of online sensors usually includes multi-angle light scattering (MALS), dynamic light scattering (DLS), ultraviolet absorbance (UV), fluorescence, and differential refractive index (RI) detectors. The rich data from MD-AF4 enable comprehensive and in-depth characterization of these NPs. With further analysis, multiple quality attributes such as the size, polydispersity, shape, particle weight, and payload content of many of the aforementioned nanopharmaceuticals can be quantified in a one-hour run. Because AF4 is a fractionation technique, narrowly sized fractions can be isolated for additional assays and research, including testing the biological effects of different particle sizes.

"With further analysis, multiple quality attributes, such as the size, polydispersity, shape, particle weight, and payload content of many of the aforementioned nanopharmaceuticals can be quantified in a one-hour run."

CAPUTO: MD-AF4 has two main advantages for the analysis of drug products containing nanomaterials that could be of great interest during the R&D step and for quality control (QC) purposes:

The first advantage is the ability to analyze the physical-chemical properties and stability of polydisperse samples, and of samples incubated in complex biological matrices, with high resolution. Generally, drug products containing nanomaterials are moderately to highly polydisperse in nature. The fractionation step prior to detection allows for the separation of different populations of nanoparticles, proteins, or polymeric samples by size. This key feature allows the user to analyze multiple physical-chemical properties of the samples with an increased resolution compared to batch techniques like DLS or NTA.

The second advantage relates to the multi-detector configuration, which provides for

the analysis of multiple physical-chemical parameters in one measurement. This is a very versatile and powerful analytical approach. Multiple detectors are generally used downstream during the AF4 separation step to measure different attributes of drug products containing nanomaterials; this can be done in the same run or in consecutive runs using the same fractionation protocol. Depending on the detectors available:

- Particle size, polydispersity, and particle morphology can be assessed by MALS and DLS
- Drug loading can be estimated by using a combination of concentration detectors such as UV-VIS and RI
- The molecular weight of polymers and proteins and their aggregation propensity can be measured by combining MALS and RI
- Information about the chemical composition of the samples can be obtained by coupling inductively–coupled plasma-mass spectrometry (ICP–MS) and/or Raman online
- Particle concentration measurements can be obtained by MALS and/or nanoparticle tracking analysis (NTA).

Online coupling with other detectors is currently being explored and will become more widely used in the future. For example, coupling AF4 to synchrotron or neutron radiation sources—including small-angle X-ray scattering or small-angle neutron scattering—could be a powerful way to increase the resolution of structural analysis of complex systems, which reduces difficulties linked to sample polydispersity.

CLOGSTON: We have developed methods where we can incubate nanopharmaceuticals with human plasma and use MD-AF4 to separate the nanopharmaceuticals from the free/unbound proteins found in human plasma. With MALS and DLS detection coupled to AF4, protein binding can be assessed. Moreover, peak fractions coming from the AF4 can be collected and analyzed offline by reverse-phase HPLC (RP–HPLC) for API loading.

BIOPHARM: What makes MD-AF4 different from other analysis techniques?

CHEN: Other analysis techniques, such as batch dynamic light scattering, NTA, electron microscopy, and some bioassays such as droplet digital polymerase chain reaction or enzyme-linked immunoassay, can quantify one or two specific properties of NPs. MD-AF4, on the other hand, provides comprehensive characterization and quantitation of multiple quality attributes. Nevertheless, multiple analysis techniques are often used orthogonally and complementarily towards well-characterized nanopharmaceuticals.

CAPUTO: The fractionation step is based on a gentler physical principle than the one used in other widely used fractionation techniques like SEC. This is due to the absence of a stationary phase. Therefore, AF4 fractionation is often the method of choice for the analysis of soft nanopharmaceuticals,

"For example, SEC/gel permeation chromatography, for characterization/separation of liposomes and colloidal metal nanoparticles, is highly dependent on the column, which may become irreversibly saturated with lipids or free metal, respectively."

such as lipid-based nanoparticles, liposomes, extracellular vesicles etc. When compared with batch measurements (in which there is no fractionation prior to the measurement of sample attributes) based on the same physical principle used for online detection, the presence of a fractionation step increases measurement resolution and, in some cases, accuracy. At the same time, control of the AF4 elution method adds a layer of complexity to the measurement protocol. Therefore, a higher degree of technical expertise and a foundation of robust standard operating procedures (SOPs) are key for MD-AF4 analysis.

CLOGSTON: The technique is also very versatile in the sense that the channel and cross-flow rates, spacer thickness and type, membrane molecular weight cut-off (MWCO) and type, and channel dimensions can all be changed and optimized to provide adequate separation for essentially any nanoparticle platform. The addition of multiple downstream detectors such as MALS, RI, UV, DLS, and ICP–MS provides additional characterization data/parameters on a fractionated sample. The fractionated peaks collected post-inline detectors can be further analyzed (for example by RP–HPLC for API content, resistive pulse sensing for particle concentration, phase-analysis light scattering for zeta potential) yielding a complete characterization picture of the nanoparticle formulation.

The MD-AF4 technique has been used in our lab to characterize essentially all types of nanoplatforms (of varying sizes and composition), whereas other techniques are limited to certain nanoplatforms. For example, SEC/gel permeation chromatography, for characterization/separation of liposomes and colloidal metal nanoparticles, is highly dependent on the column, which may become irreversibly saturated with lipids or free metal, respectively.

XIUJUAN: With both channel flow and cross flow and separation on a flat channel membrane, AF4 separation is free of restricted diffusion and shear stress, which is often seen in SEC separation of macromolecules or branched polymers or polymer-protein conjugates, producing high variability, unreliable molecular size measurements, and shortened column life. MD-AF4 can also separate a much wider range of macro/molecules within one run, whereas SEC would require tandem combination of columns with different pore sizes with much longer run time.

Nanoparticles, such as LNPs encapsulated with RNA, usually have molar mass >10 MDa; AF4 would be an ideal tool for the separation of these particles. AF4 coupled with MALS detectors or concentration detectors such as RI and UV allow scientist to measure LNP quality attributes simultaneously without the need of standards, including molar mass, particle size, polydispersity, particle filling, morphology, size-based RNA weight percent distribution, and number of RNA encapsulated per particle (a technique proprietary to Wyatt).

BIOPHARM: Can you highlight some of the key features of the MD-AF4 technique?

CHEN: The MD-AF4 technique offers not only comprehensive and in-depth characterization, but also a potentially robust quantitation method. In our latest Eclipse AF4 system, we implemented a dilution control module to minimize sample dilution and as a result, the detection sensitivity and fraction concentration can be significantly enhanced. This system is robust and easy to use. To improve usability, we added a great deal of automation to our software and made it more intelligent. Wyatt's software is also Code of Federal Regulations (CFR) 21 Part 11 compliant, which makes it suitable for regulatory and QC uses.

CAPUTO: Many reviews have been written to highlight features of MD-AF4 in the pharmaceutical field, and examples in the pharmaceutical field can be almost endless (1–4). Some of the key applicative features in this field are:

- **Measurement of batch-to-batch consistency.** The high resolution of MD-AF4 facilitates accurate measurements of the variation in size and other physical properties within batches of drug products containing nanomaterials. This key feature allows the user to evaluate the reproducibility of the production process and biosimilarity in case of generics containing nanomaterials. Examples are found in various publications that focus on lipid-based nanoparticles and liposomes (2, 4–8).
- **Measurement of nanoparticle-protein interactions.** In physiological media, nanoparticle interactions with plasma proteins endow nanomaterials with new biological properties, which influences their safety and efficacy profile. For example, it is known that the proteins attached to the particle surface (protein corona) determine the biodistribution of nanopharmaceuticals. MD-AF4 allows the user to measure the formation of the protein corona by detecting the increase of the particle size of a few nanometers. This is due to the layer of proteins, as well as any additional drastic phenomena such as aggregation, disaggregation, or destabilization of the particles that may happen as consequence of nanoparticle-proteins interactions. Examples of protein binding assessment performed by MD-AF4 on liposomes and on lipid based nanoparticles are nicely shown in (1, 2, 5, 6) .

- **Measurement of storage stability.** MD-AF4 enables the measurement of aggregation propensity during storage. Widely used sizing techniques such as DLS are not suitable to measure size and polydispersity of highly polydisperse samples (e.g., samples with aggregates), while MD-AF4 can distinguish changes in size and resolve the presence of multiple populations up to 1 µm in diameter. For this reason, MD-AF4 is indicated for studying the physical stability of nanopharmaceutical products during storage.

BIOPHARM: How do you view the role of MD-AF4 in the development and production of lipid nanoparticle formulations of nucleic acids such as small interfering RNA (siRNA) or messenger RNA (mRNA)?

CHEN: MD-AF4 is a powerful tool for RNA therapeutics formulated in LNP. A literature search would show that both SEC and AF4 have been used for LNPs, but AF4 is considered the separation tool of choice for LNP because of its high recovery and versatility. The data from multiple online detectors, following either an SEC or AF4 separation, can be used to measure many of the quality attributes: size distribution, shape, molecular weights from lipids and RNA, mass concentrations from lipids and RNA, and size-based RNA payload distribution.

CAPUTO: MD-AF4 enables the characterization of multiple critical attributes of LNPs, including:

- particle size distribution and polydispersity
- particle concentration
- particle morphology
- batch-to-batch consistency
- stability during storage
- the protein corona formation

Within the pharmaceutical industry, efforts have been made to quantify the percentage of encapsulated mRNA or siRNA, which is a very tricky attribute to measure reliably. The measurement of mRNA payload can either be performed by offline measurement of the particle fractions or by direct online MD-AF4 measurement using a method called conjugate analysis (5). Therefore, MD-AF4 presents a unique setting to get multiple information with one measurement (6,7).

CLOGSTON: In the simplest case, MD-AF4 can be used to evaluate the size distribution (radius of gyration and hydrodynamic size) of LNP formulations. Incubation with human plasma and running MD-AF4 can give information on protein binding to the surface of the LNPs. Moreover, if the AF4 peaks are collected, offline analysis for mRNA/siRNA concentration and particles per mL concentration can be performed to give API distribution as a function of size. Particle concentration can give relative amounts if the sample is polydispersed and can be related to both size and API distribution. Moreover, depending on the AF4 elution profile, LNP, and API, it may be possible to separate free, unencapsulated mRNA from mRNA-loaded LNPs. It is also possible to perhaps examine the shape factor

and see how uniform the LNPs are loaded with API. Such characterization can be used to examine batch-to-batch consistency as well as to assess different manufacturing processes used in the preparation of LNPs. In our lab, we have done all of this for several mRNA-LNP formulations.

BIOPHARM: What are some of the challenges associated with developing robust standard operating procedures for applications using MD-AF4, and how can users address such challenges?

CHEN: AF4 is analogous to HPLC, but is a more versatile technique with user-tunable resolution. The flexibility can be a double-edged sword and it can lead to complexity with more parameters to manipulate. The early AF4 systems were also built with relatively low robustness and automation, precluding widespread adoption by scientists working in the nanopharmaceutical field. Wyatt's latest AF4 systems have seen immense improvements in robustness, automation, and usability, and I am delighted to see that scientists from multiple prestigious organizations are advocating for its adoption and employment. In the meantime, we have collaborated and will continue to collaborate with the industry users to come up with more ready-to-adopt methods.

CLOGSTON: Challenges will be in reproducibility between labs regarding instrument setup and analysis. The AF4 portion will rely on everyone in the lab having similar setups (for example, long channel, 10k Da MWCO RC membrane, etc.). This should be easy to address. The hardest aspect will be the method used to analyze the data. For MALS and DLS, the same input parameters must be used, and the same models must be agreed upon and be consistently used.

SOPs on AF4 methodology should be developed as well as SOPs on which light scattering models to use and for what nanoparticles. ASTM and ISO are working on these issues and are currently developing standard protocol documents to address them. Moreover, test samples or reference materials for MD-AF4 do not currently exist. This is a challenge in the sense that a highly reproducible and thoroughly characterized nanoparticle must be provided. The choice of nanoparticle (for example, liposome versus polymeric micelle), drug-loaded or not, stability issues (shelf-life, solvent compatibility, etc.) must be addressed and agreed upon by the community before even starting to prepare such a standard. This standard would then have to go through rigorous MD-AF4 analysis and validated across several labs. Only when a standard is available can we start to develop SOPs for MD-AF4 analysis.

XIUJUAN: The major hurdle for lab scientist to adopt MD-AF4 would be the broad peak shape that makes the accurate mass analysis difficult, and therefore affect the accuracy of other quality attribute analyses. The other limitation is the type of membranes available. Both RC and polyether sulfone have pKa below 4, which could cause ionic interaction of the analyte with the membrane and,

therefore, affect mass recovery; it may carry positive surface charge, particularly for LNP. A method that could overcome the analyte-membrane interaction would be invaluable. If these factors are addressed accordingly, MD-AF4 would be a very attractive analytical technique for pharma scientists to adopt.

BIOPHARM: What kind of inquiries can researchers using MD-AF4 expect from regulatory authorities?

CAPUTO: A good starting point for meeting regulatory expectations involves following guidelines reported in ISO/TS 21362:2018 and running suitable standards as QC to ensure that the system works correctly. Regulatory authorities usually ask to demonstrate the repeatability, reproducibility, and robustness of the applied methods to characterized specific products. In practice, this means showing the applicability and suitability of the MD-AF4 specific test method to answer your specific research questions. This applies to MD-AF4 as well as any other assays reporting physical-chemical properties of nanopharmaceutics. Validation requirements such as those indicated in the *ICH Q 2 (R1) Validation of Analytical Procedures* could serve as a general guide, but those criteria are developed for other analytical approaches (such as analytical chemistry and chromatography). Specific indication of the validation requirements for methods measuring particle size distribution and other physical properties have been presented by FDA in the SBIA 2020 workshop, Advancing Innovative Science in Generic Drug Development Workshop September 29–30, 2020.

The main criteria to validate the repeatability and reproducibility of methods to measure particle size and other physical properties are the precision, robustness, and accuracy. When performing chemical analysis and assessment of drug loading by MD-AF4, specificity, linearity, and detection limits could also be important to consider.

CLOGSTON: Regulatory bodies will probably want orthogonal techniques, which are FDA-recognized and/or based on ASTM, ISO, or other standards agencies standard documents, to prove the MD-AF4 results.

CHEN: Large particles and aggregates can cause inflammation and other undesirable immune responses. So, for nanoparticle-based therapeutics, regulatory authorities may request data from MD-AF4. The authorities may demand validation data and scrutinize data consistency. Again, our analytical sciences team at Wyatt Technology is suited to collaborate with industry users to help optimize MD-AF4 methods and develop SOPs that can satisfy stringent scrutiny.

BIOPHARM: What will help increase adoption of MD-AF4 across the pharma industry?

CAPUTO: The use of MD-AF4 in the pharma industry has significantly increased in the last 10 years, and there are multiple elements that can support the spread of MD-AF4 within the industry. First, the availability of standard test methods applicable to different

"Collaboration among manufacturers, experienced MD-AF4 labs and researchers, and industrial users to develop methods, workflow, and SOPs will help increase the adoption of MD-AF4 across the pharma industry."

kind of nanopharmaceutical classes is critical. Standards that are recognized by regulatory agencies will streamline the evaluation of the data presented. Currently, only ISO/TS 21362:2018 focuses on the use of MD-AF4 for analyzing nanomaterials, but international standardization bodies are currently working to develop additional standard test methods specific to different nanoparticle classes used as pharmaceutical products and vaccines, including liposomes. Other kinds of lipid-based nanoparticles will also be taken into consideration in the near future.

A second key aspect for the pharma industry is to have available GLP compliant software; such 21 CFR Part 11 compliant software was in fact announced in June 2021 by one of the AF4 instrumental providers. Finally, infrastructures and experts, such as the NCI–NCL, the JRC NanoLab, SINTEF, and European Nanomedicine Characterization Laboratory (EUNCL) that provide characterization services and training on the use of MD-AF4 are very helpful to support the spread of MD-AF4 use in the pharma industry. Collaboration between experts and different stakeholders in the fields is critical to demonstrate the versatility of MD-AF4 and inform the industry about applicability and limitations of the technique.

CLOGSTON: Acceptance by FDA of MD-AF4 will give pharma industry more comfort in exploring MD-AF4. This can be in the form of accepted ASTM and/or ISO standards documents. Pharma collaborations with academia, NCL, and Wyatt, to name a few, will enable the industry to explore and observe the usefulness of MD-AF4 as a characterization technique for their formulations. General knowledge sharing through workshops and conferences showcasing MD-AF4 will also educate pharma.

Ideally, having a nanoformulation accepted by regulators that used MD-AF4 in its chemistry, manufacturing, and control section would also be extremely useful to promote industry adoption.

CHEN: Collaboration among manufacturers, experienced MD-AF4 labs and researchers, and industrial users to develop methods, workflow, and SOPs will help increase the adoption of MD-AF4 across the pharma industry. We would also love to receive feedback on new features and improvements to our MD-AF4 hardware and software, to further improve and pave the way to acceptance of MD-AF4 an essential tool for nanopharmaceutical development and production. A good place to start is a webinar on this topic that is available for on-demand viewing at www.wyatt.com/Webinars.

BIOPHARM What pathways are available for pharma scientists and engineers to explore and adopt MD-AF4?

CAPUTO: As a starting point, it is important to define the most suitable set of instrumental configurations, including available AF4 channels, online detectors, and software specifications according to the measurement purpose and to the requirements of the quality management system. Those requirements may change according to the different stages of development of the product to characterize.

Next, to get the optimal results, the MD-AF4 method and instrumental settings need to be optimized according to the specificity of the formulation analyzed and to the targeted attribute(s). Many documents are available to guide new users for the development of reliable measurement protocols. We suggest to follow the guidelines for method development and optimization provided by the NCI–NCL and by the EUNCL infrastructure (2). Requirements to be followed to develop a robust and accurate method are described in the International Organization for Standardization (ISO) standard ISO/TS 21362:2018 *Nanotechnologies — Analysis of Nano-Objects Using Asymmetrical-Flow and Centrifugal Field-Flow Fractionation*.

CLOGSTON: The best way for pharma and engineers to explore and adopt MD-AF4 is through collaboration. Direct collaboration with Wyatt in terms of running and testing some representative samples would be the first step. Collaboration with NCL in characterizing formulation—either through the white paper process (free) or cooperative research and development agreement mechanism (paid service)—is another option.

"As a starting point, it is important to define the most suitable set of instrumental configurations, including available AF4 channels, online detectors, and software specifications according to the measurement purpose and to the requirements of the quality management system."

Collaborations with academic institutions and labs that share a common research interest in MD-AF4 as well as the capabilities to execute research also an option.

Lastly, attending meetings and workshops given by Wyatt (or scientific conferences where AF4 is utilized) is perhaps the best way to explore AF4 and its applications.

CHEN: I would suggest to pharma scientists and engineers who are interested in exploring and adopting MD-AF4 to contact Wyatt Technology. Our analytical sciences team will work with you to make certain that the technique is the right match for your applications and needs, including sample runs and specific feasibility studies. Working with experienced MD-AF4 labs and experts at NCI-NCL and SINTEF is also a good idea.

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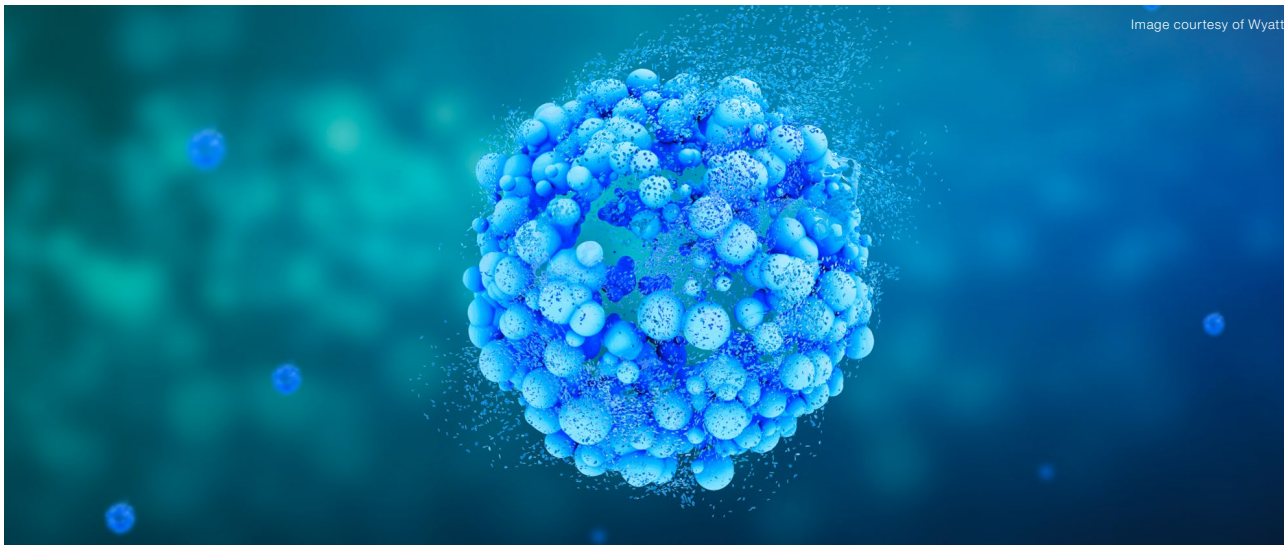

Characterization of Nanopharmaceuticals with Field-Flow Fractionation and Light Scattering (FFF-MALS-DLS)

Dan Some and Christoph Johann

How FFF-MALS-DLS provides solutions to many challenges in pharmaceutical analysis.

Introduction

One of the primary challenges in developing effective formulations for the nanoscale delivery of therapeutics is particle characterization. Standard techniques such as dynamic light scattering (DLS), particle tracking analysis (PTA) and transmission electron microscopy (TEM) typically suffer from tradeoffs between simplicity, detail and sampling efficiency. For example, DLS is simple to use, and can sample a large particle ensemble, but only provides semi-quantitative, low-resolution size distributions; on the other hand, TEM offers exquisite structural detail, at the cost of complexity, laborious sample preparation and very small ensembles that lead to high statistical uncertainty.

Field-flow fractionation (FFF) is a size-based separation technique covering the entire range of macromolecules and nanoparticles from 1-1000 nm in diameter. Coupling FFF to online multi-angle light scattering (MALS) and DLS detectors provides detailed, quantitative

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Field-Flow Fractionation: Virtual Optimization for Versatile Separation Methods

size distributions and structural information, sampled over large ensembles, for good statistical robustness. Spectroscopic and other types of online detectors may be added to obtain compositional information and more. Since FFF systems incorporate standard chromatography modules, the measurements are fully automated, and fractions may be isolated and collected for additional off-line analysis. It is a unique property of FFF that very little sample preparation is needed, because the separation method itself eliminates most impurities, and inherently performs dialysis into the carrier fluid.

These capabilities of FFF-MALS-DLS provide a pathway to meeting the requirements of regulatory agencies for enhanced characterization of liposomal drug formulations and other nanoparticle delivery systems[1–4] and have led to the development of international standards literature for nanoparticle characterization such as ISO TS 21362 and ASTM WK 68060, as well as methods published by the NCI-NCL and EU-NCL.[5,6]

The system described here is implemented in Wyatt Technology's FFF-MALS-DLS platform comprising an **Eclipse™ FFF flow controller** and separation channel, **DAWN® MALS instrument**, **WyattQELS™ DLS module**, **Optilab® differential refractometer** and standard HPLC components, illustrated in FIGURE 1. For particles above 30 nm in radius, a wide-bore flow cell is installed in the MALS detector in order to provide the most accurate online DLS measurements. The entire setup is controlled by the VISION™ software suite.

Nanoparticle Separation with FFF

Field-flow fractionation was invented by Calvin Giddings in 1966.[7] However, it took several decades until commercial FFF instrumentation became convenient, robust and popular in analytical laboratories, primarily in the asymmetrical-flow FFF (FFF) format described herein. The advent of nanoparticle-based drug and gene delivery formats has increased the demand for its unique capabilities.

The flow FFF principle, described in FIGURE 2, was described by Giddings in a review article.[8] Briefly, separation takes place in an open channel consisting of two long, narrow blocks bolted together with a spacer in between. The spacer is a polymer foil with a

"Since FFF systems incorporate standard chromatography modules, the measurements are fully automated, and fractions may be isolated and collected for additional off-line analysis."

FIGURE 1: FFF-MALS-DLS system components and organization. The DLS detector is embedded in the MALS detector.

typical thickness of just 200 to 500 μm. Flow within this thin ribbon-like channel is laminar, with a pronounced parabolic flow profile that drives particle separations.

The bottom block comprises a semi-permeable membrane supported by a frit. The membrane is permeable to solvent but not to the analyte; this essential function is guaranteed by selecting the appropriate membrane pore size, expressed as a molecular-weight cutoff (MWCO) ranging from 1 kDa to 100 kDa. The particle-containing carrier fluid flows parallel to the membrane, but a constriction at the end of the channel forces some of the fluid to pass through the membrane, creating a "cross-flow field" that concentrates the particles towards the membrane. Diffusion acts as a counter-force that drives particles back up into the channel, resulting in a height distribution above the membrane that depends on the particle's translational diffusion coefficient D_t, and hence hydrodynamic radius R_h, as well as the cross-flow velocity.[9]

Separation arises from differential transport velocity in the laminar flow profile according to the particles' height above the membrane.

In FFF, the particle retention time t_R depends on D_t, the channel thickness w, the cross-flow rate F_c (which is controlled by a precise flow controller), and the detector flow rate F_{out}. If the flow rates are constant over time and retention is sufficiently high, the retention time is, in good approximation, given by Eq. 1[9]:

$$t_R = \frac{w^2}{6D} \cdot \ln\left(1 + \frac{F_c}{F_{out}}\right) \quad \text{Eq. 1}$$

Characterization of Nanopharmaceuticals with Field-Flow Fractionation and Light Scattering (FFF-MALS-DLS)

FIGURE 2: Separation mechanism in an FFF channel, consisting of a semi-permeable membrane and a controllable lateral flow restrictor which forces some of the solvent to flow through the membrane. The size-dependent balance between diffusion and cross flow leads to different height distributions for different sizes. Smaller particles are swept out of the channel earlier than larger particles, which remain close to the membrane and experience a lower flow velocity.

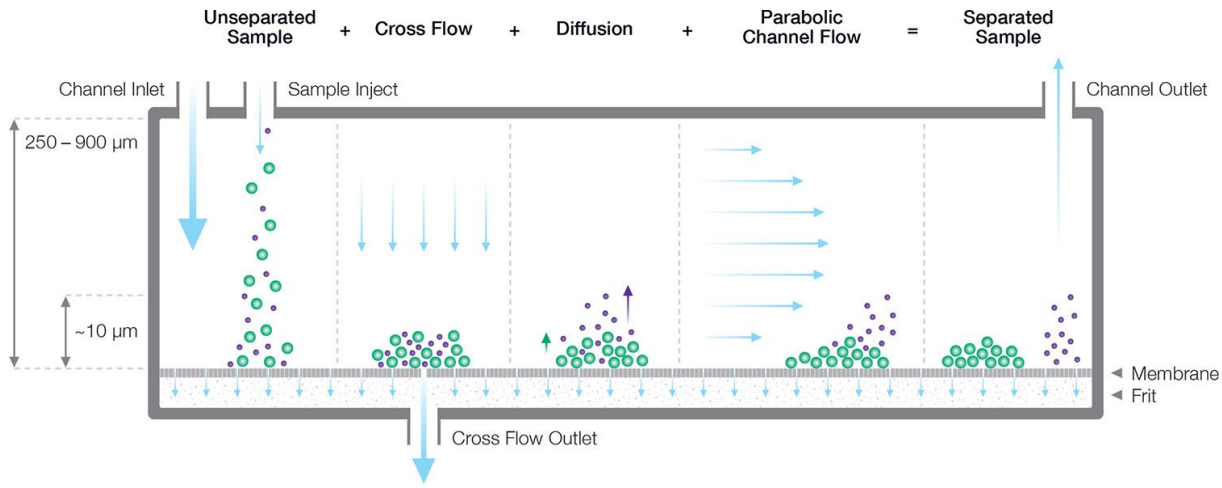
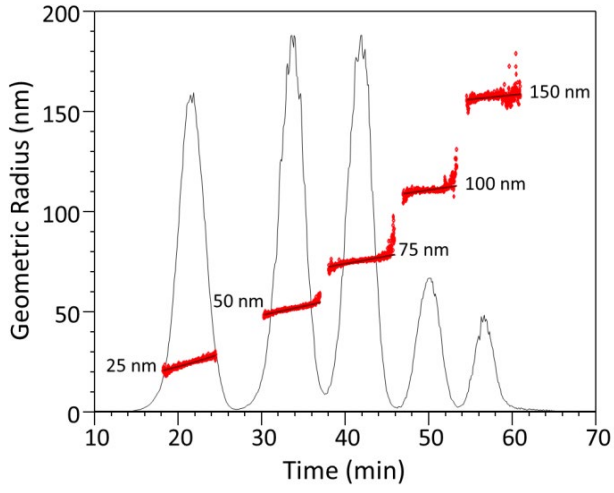


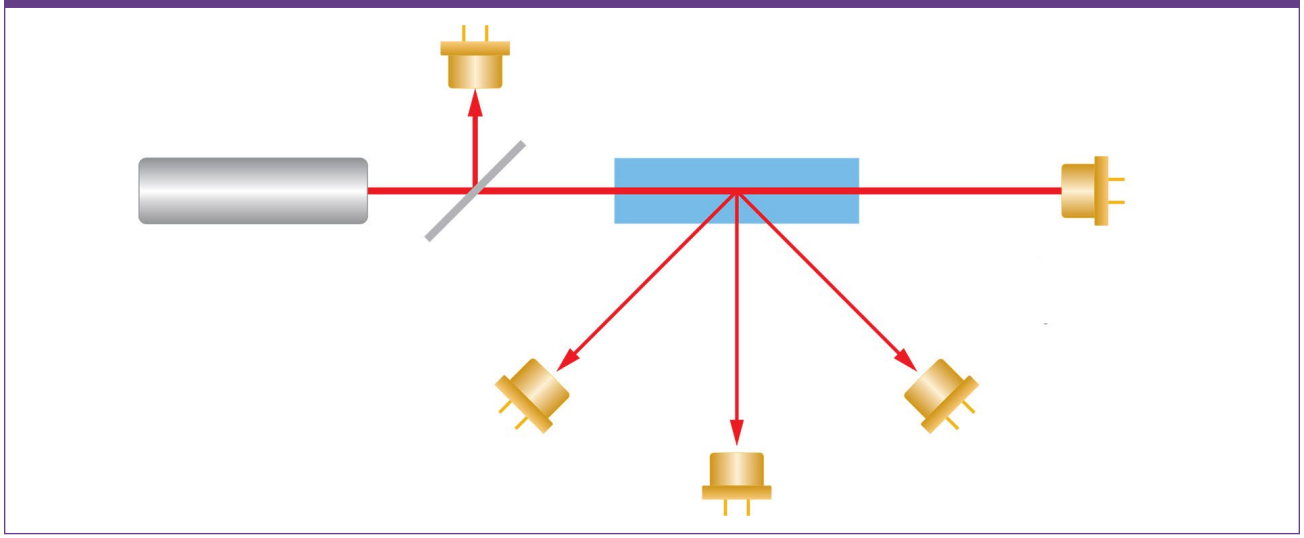
FIGURE 3: FFF separation of a series of latex standards ranging from 25 nm to 150 nm in radius. The size of each eluting fraction, determined online by MALS, is indicated by red symbols.



Retention time, zone broadening and dilution of the sample at the channel outlet for more complex flow profiles can be calculated by standard fluid dynamics.[10] Computer simulation of the separation process enables virtual optimization of the separation method, as well as calculation of diffusion coefficients based on the measured retention time.[11] An example of the excellent separation capabilities of FFF is shown in FIGURE 3.

FFF separation channels include analytical and semi-preparative scales. The latest development in FFF is the incorporation of an electrical field, applied perpendicular to the membrane. Charged particles exhibit a shift in height above the membrane, and a

FIGURE 4: Basic multi-angle light scattering geometry. Detectors are placed at multiple angles relative to the illuminating beam, within the plane perpendicular to the light's polarization vector. Additional detectors monitor the beam intensity and transmission through the flow cell.



corresponding shift in retention time, that varies with applied field strength, from which the electrophoretic mobility μ_E and zeta potential may be calculated. This method can reliably determine μ_E for several components simultaneously, providing an indication of the charge distribution.[12]

Online Nanoparticle Analysis

Separation of nanoparticles is just the first stage in their characterization. The ability to perform a variety of online measurements on the purified and nearly monodisperse fractions flowing through one or more detectors means that each species can be analyzed thoroughly for physical and compositional properties.

Multi-angle light scattering (MALS)

MALS measures the time-averaged intensity of light scattered by the nanoparticle ensemble into multiple scattering angles, defined relative to the direction of propagation of the incident laser beam. A MALS instrument typically incorporates between three and eighteen photodiodes to detect scattered light, though an eighteen-detector configuration is most common for nanoparticle applications. All the photodiodes are placed in a single plane, oriented perpendicularly to the polarization of the laser beam, as shown in FIGURE 4.

The application of MALS analysis to determine molar mass and size (rms radius, a.k.a. radius of gyration) is well known.[13] The relationships between scattered intensity (reduced Rayleigh ratio, R), scattering angle θ and analyte properties such as molar mass M, refractive index increment dn/dc, concentration c and

rms radius R_g, are summarized in Equations 2 and 3, in the limit of dilute solutions. K is an optical constant related to the illumination wavelength λ and refractive index of the carrier fluid n_0.

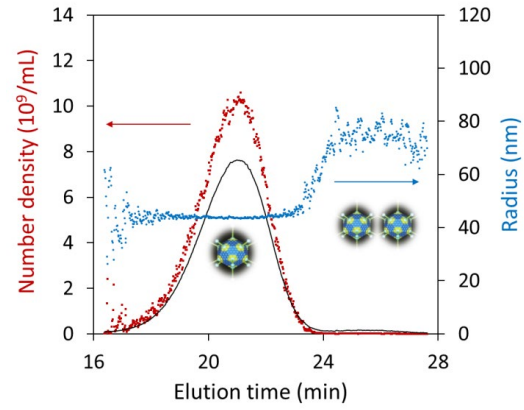
$$R(\theta) = K\left(\frac{dn}{dc}\right)^2 McP(\theta) \quad \text{Eq. 2}$$

$$P(\theta) = 1 - \frac{16\pi^2 n_0^2}{3\lambda_0^2} sin^2\left(\frac{\theta}{2}\right)\langle R_g^2\rangle + \ldots \quad \text{Eq. 3}$$

In the limit of sub-wavelength sizes and assuming $(n_p/n_0\text{-}1) << 1$, a collection of N uniform particles of volume V and refractive index n_p, produces the excess Rayleigh ratio given by Eq. 4[14]:

$$R(\theta) = \frac{8\pi^2 NV^2}{\lambda^4}\left(\frac{n_p}{n_0} - 1\right)^2 P(\theta) \quad \text{Eq. 4}$$

FIGURE 5: Top: size and number density analysis of influenza virus by FFF-MALS, overlaid on LS chromatogram. Bottom: comparison of FFF-MALS size and integrated particle concentration with values determined by imaging (table data based on reference 15).

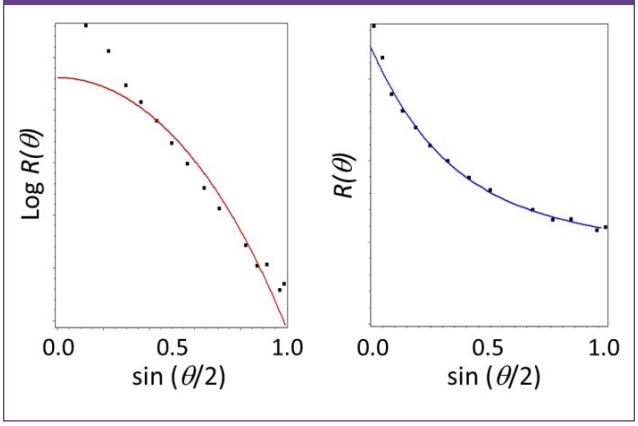


	AFM/ TEM	FFF-MALS	%Δ
Total Particle Count	2.9 x 10^{10}	2.8 x 10^{10}	2
Average Radius	43.0	45.0	5

Analysis of $P(\theta)$ yields the root-mean-square radius R_g, which is related to the particle's overall structure and internal mass distribution. For specific, known particle shapes or conformations, R_g can be related to physical dimensions, e.g. the length of a narrow rod or the radius of a spherical shell, and hence the volume term V can be calculated.[13] If V, n_p and n_0 are known, the number of particles N in the scattering volume—and hence the particle concentration—may also be derived. This analysis has been demonstrated to be quite accurate in comparison with concentration determined by TEM, as seen in FIGURE 5 for influenza viruses.[15]

If the particles are large enough relative to λ then it may also be feasible to determine the general particle shape (spherical, ellipsoidal, etc.) by fitting $P(\theta)$ to known angular functions that include additional terms in the expansion of Equation 3. This capability has been applied to characterization of thin-disk and cylindrical unilamellar liposomes.[16] Explicit forms of the angular dependence of light scattering exist for several basic shapes including spheres, rods, ellipsoids and random coils.[13,14,17] This analysis has been applied to a carbon nanotube in FIGURE 6, demonstrating a distinct failure of the sphere model fit and success of the rod model. In order for the fits to be robust enough

FIGURE 6: Estimating shape from the angular scattering function. The sample is a rod-shaped carbon nanotube. Left: the angular data are fit poorly by a sphere model. Right: the data are fit well by a rod model.



to estimate shape, at least one sample dimension should be an appreciable fraction of the wavelength, e.g. 100 nm if the MALS wavelength is 660 nm.

In addition to particle size and concentration, under dilute conditions typical of FFF the scattered intensity may also be related to the molar mass of a macromolecule flowing through the MALS detector[13] by Eq. 2. The concentration *c* is typically measured by an upstream or downstream detector based on UV/Vis extinction or differential refractive index. Hence the same MALS instrumentation can be used to characterize both nanoparticles and macromolecules in a complex sample, separated by FFF.

Wyatt Technology's **ASTRA® MALS analysis softeware** provides calculations for molar mass as well as size and spherical particle concentration, and these may be determined at each eluting FFF fraction in order to obtain detailed size distributions. MALS is also commonly employed in-line with size-exclusion chromatography to characterize macromolecules such as proteins and polymers, providing absolute molar mass and size that do not depend on calibration of the retention volume with reference standards.

It is noteworthy that MALS measurements can take place on sub-second time scales, making this technology an excellent candidate for process analytics of preparative and full-scale production processes. Using the **ultraDAWN™ real-time MALS detector** for process monitoring and control, the average rms radius may be determined up to 30 times per minute to verify uniform nanoparticle production, identifying process deviations early on in order to minimize waste and discarded lots of precious material. **Real-time MALS** has also been demonstrated for monitoring molar mass and aggregate breakthrough in the purification of therapeutic monoclonal antibodies.[18]

Dynamic light scattering (DLS)

Dynamic light scattering (DLS) is also known as photoncorrelation spectroscopy (PCS) and quasi-elastic light scattering (QELS). In DLS, rather than measuring the time-averaged intensity as a function of angle, size is determined by analyzing the rapid intensity fluctuations occurring on time scales of microseconds and milliseconds. These fluctuations arise through the Brownian motion of the particles: the

wavelets scattered by individual particles combine coherently at the detector, and as the particles diffuse, the relative phases of the wavelets change and the detector experiences rapid intensity fluctuations.

The rate of fluctuations is directly related to the particle's translational diffusion coefficient D_t, which can in turn be converted via the Stokes-Einstein equation (Eq. 5) to a measure of size known as the Stokes radius or the hydrodynamic radius R_h.

$$R_h = \frac{k_B T}{6\pi D_t} \qquad \text{Eq. 5}$$

For a spherical particle, R_h is the radius of the particle's envelope, regardless of internal structure. For a non-spherical particle, R_h is the radius of a sphere with approximately the same overall volume as the particle; the more the particle's shape deviates from spherical, the smaller the actual volume of the particle relative to the hydrodynamic volume $V_h=4\pi R_h^3/3$. For example, when the axial ratio of an ellipsoid is 3:1, the actual volume of the ellipsoid is roughly 30% smaller than the measured V_h , as estimated from material presented in Tanford's classic textbook.[19]

Standard DLS measurements are made on unfractionated samples (batch mode). While an average R_h is simple to determine, size distributions by this technique are subject to low resolution and ambiguity due to the complex, under-determined mathematical analysis involved. On the other hand, FFF fractionation of the sample by size prior to DLS measurement greatly simplifies and enhances size determination, since it can be assumed that each eluting fraction is practically monodisperse. Since FFF can separate particles that are quite close in size, FFF-DLS size distributions are robust and fully quantitative, providing far more detail than batch DLS. The qualitative distinction between size distributions of a liposome sample, measured by batch DLS and FFF-DLS as shown in FIGURE 7, are obvious.

Multi-detection

The combination of MALS and DLS, with the addition of other online detectors, expands the range of analyses possible with a single FFF run. The most common and practical

FIGURE 7: Comparison of liposome sizing by batch DLS and FFF-DLS. Left: batch DLS measurement of two liposome samples, empty and filled; Right: FFF-fractograms with overlaid DLS measurements of the two samples.

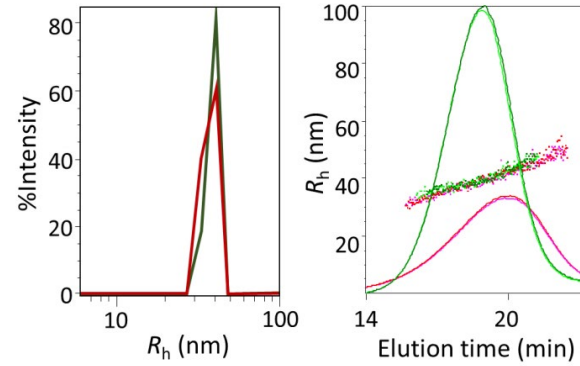


TABLE 1: Some representative shape factor values

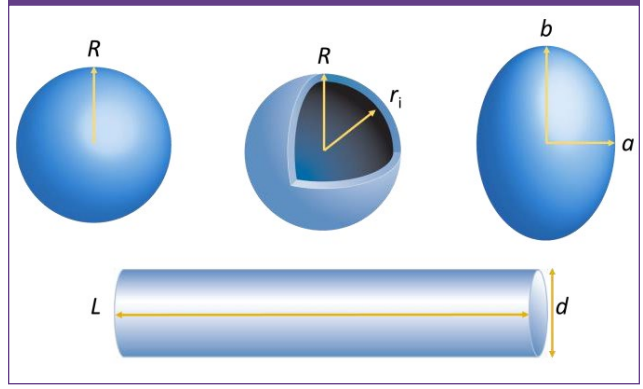
Structure	R_g	R_h	Shape factor
Uniform sphere with radius *R*	$R\sqrt{\frac{3}{5}}$	*R*	0.77
Hollow sphere with radius *R*	*R*	*R*	1
Spherical shell, *p* = ratio of inner radius r_i to outer radius *R*	$R\sqrt{\frac{3}{5}}\sqrt{\frac{1-p^5}{1-p^2}}$ [20]	*R*	$p = 0.5 \rightarrow \rho = 0.82$ $p = 0.9 \rightarrow \rho = 0.95$
Uniform rod, p = length / diameter = *L/d*	$\frac{L}{2}\sqrt{\frac{1}{3}}\sqrt{\frac{1}{2p^2}}$ [21]	$\frac{L/2}{\ln(p)+0.312+\frac{0.565}{p}-\frac{0.1}{p^2}}$ [21]	$p = 2 \rightarrow \rho = 0.85$ $p = 10 \rightarrow \rho = 1.55$
Uniform prolate ellipsoid, *p* = axial ratio *b:a*	$b\sqrt{\frac{1+2/p^2}{5}}$ [22]	$\frac{b\sqrt{1-\frac{1}{p^2}}}{\ln(p+\sqrt{p^2-1})}$ [23]	$p = 2 \rightarrow \rho = 0.83$ $p = 10 \rightarrow \rho = 1.36$

detection methods used with FFF for nanoDDS are described below.

Shape factor

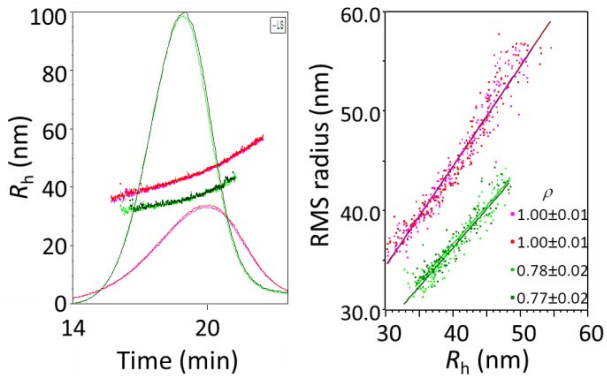
The shape factor ρ is the ratio between R_g, determined by MALS, and R_h, determined by DLS. Its value has been calculated for simple structures such as uniform spheres or ellipsoids, hollow spherical shells, rods, random coils and more. Several examples are provided in TABLE 1 for the shapes depicted in FIGURE 8.

FIGURE 8: Some simple structures for which shape factors have been calculated: uniform sphere, spherical shell, prolate ellipsoid and rod.



In the context of nano-drug delivery, where a hollow delivery particle such as a liposome or VLP is expected to incorporate a drug or gene payload in the core, this structural parameter can differentiate between empty and full nanoparticles. Since the lipid bilayer of a liposome is typically about 4-5 nm thick, an empty liposome with a typical radius of 50 nm can be expected to have a ρ

FIGURE 9: Empty and filled liposomes analyzed by FFF-MALS-DLS. a) fractograms overlaid with R_g values; b) shape factor analysis.



value of roughly 0.95. In fact, a value of 0.93 was found by Vreeland et al. for unilamellar liposomes.[24]

FIGURE 9 illustrates the further analysis of empty liposomes and filled liposomes from FIGURE 7, measured by FFF-MALS-DLS. Since FFF separates according to hydrodynamic radius, it is not surprising that the samples exhibit the same R_h values (FIGURE 7B, as measured by online DLS) at each elution volume, even though the specific size distributions differ. On the other hand, the R_g values for each elution volume in the fractogram clearly differ (FIGURE 9 left), with filled liposomes presenting smaller R_g values relative to empty. The shape factors derived from the ratio of R_g:R_h (FIGURE 9 right) are, as expected, quite different.

If a liposome is known to contain drug—for example by spectroscopic analysis—but still appears as a hollow sphere by shape factor, it can be inferred that the drug is incorporated into the lipid shell rather than the hydrophilic core.

Elongated objects are characterized by shape factors greater than ~1.1, as reflected in the FFF-MALS-DLS analysis of two heterogeneous particles, one spherical and one rod-like, shown in FIGURE 10.

UV and Fluorescence

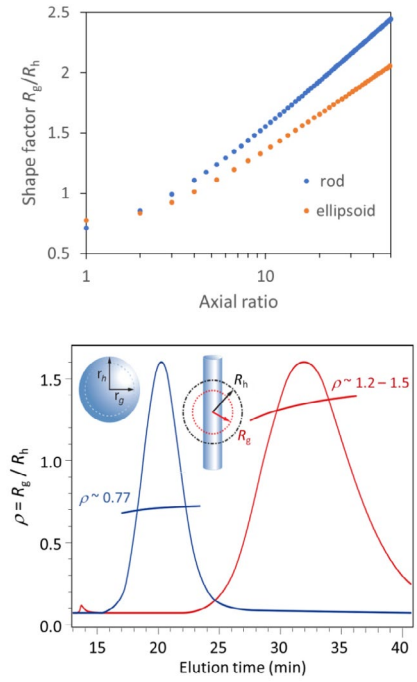
Standard HPLC UV and fluorescence detectors may be added in-line with FFF. They may be used in several ways:

- To determine concentration of a sample containing an appropriate chromophore or fluorophore.
- To determine full or partial sample composition via the absorbance or emission spectrum.
- To determine sample composition by combining the UV or fluorescence data with other signals, e.g. with refractive index data in the “conjugate analysis” below.

Conjugate analysis

The combination of two concentration detectors responsive to different sample properties—for example, a UV detector and an RI detector—can be combined with MALS to determine not only the overall molar mass but the molar mass of each component in the conjugated molecule[25] or bi-component particle. This concept can be applied to a nano-formulated drug to determine the composition

FIGURE 10: Shape factor analysis of uniform spheres and elongated particles. Top: theoretical shape factor values for rods and ellipsoids with given axial ratio (rods: length to diameter; ellipsoid: major to minor axes), based on Table 1. Bottom: FFF-MALS-DLS analysis of two types of particles constituting uniform spheres and rod-like particles, indicating ratios of length:diameter in the range of 5:1 to 10:1.



ratio of a complex drug such as a liposome or polymersome loaded with DNA, RNA, protein or small molecule therapeutics. The greatly different responses of lipids, polymers such as PLGA, nucleic acids or proteins in the UV spectral range ensures sufficient contrast to perform the analysis. This technique has been applied to drug-loaded liposomes[26], virus-like particles containing nucleic acid payloads[27] and polymersomes with proteinaceous cargo.[28]

Encapsulation efficiency

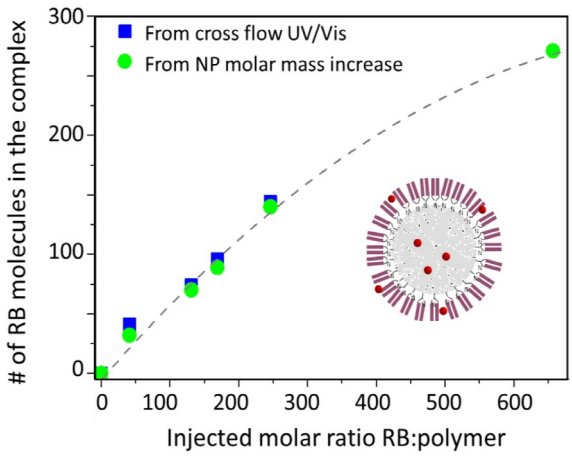
Analyses of encapsulation efficiency and free:bound drug ratio are of singular importance in developing and establishing dosing guidance for a safe, effective drug nanocarrier. Free proteins or nucleic acids can be separated and quantified relative to the content of a nanoparticle carrier quite readily by FFF-MALS[28], but quantifying small molecules is more of a challenge: they will escape the channel by passing through the membrane, but since they are often quite hydrophobic, they will also adhere to the membrane. A method introduced by Boye et al.[29] quantifies the amount of free drug in solution by means of a UV/Vis detector on the cross-flow outlet and compares to the total drug introduced in the formulation; the membrane is pre-conditioned to prevent drug adsorption by saturating it with drug substance. In addition, drug loading in the carrier can be quantified directly using the conjugate analysis. A comparison of these two orthogonal methods in FIGURE 11 indicates close agreement.[29]

Conclusions and Outlook

The challenges posed by complex drugs, such as nano-formulations of small molecules and gene vectors, require advanced approaches to particle characterization. FFF-MALS-DLS provides solutions to many of these challenges, and is increasingly recognized as an important analytical technique for R&D in this field.

While the use of FFF with light scattering for characterization of size distributions is well-established, recent work has offered a glimpse of its potential for more advanced

FIGURE 11: Comparison of two orthogonal methods to determine encapsulation efficiency of a small-molecule drug in a polymersome. Blue symbols: by quantification of free drug passing through the membrane, using a UV/Vis detector; green symbols: by analyzing the molecular weight of the drug-loaded nanoparticles relative to empty polymersomes. Reproduced with permission, Albena Lederer.



analyses that lie at the heart of developing and commercializing pharmaceutical nano-formulations. Further adoption of FFF-MALS-DLS in the pharmaceutical community will go hand-in-hand with advances in the instrumentation and software for increased robustness, ease-of-use and support of extended multi-detector analysis.

To learn more about the theory, technology and applications of FFF-MALS, please visit **wyatt.com/FFF-MALS.**

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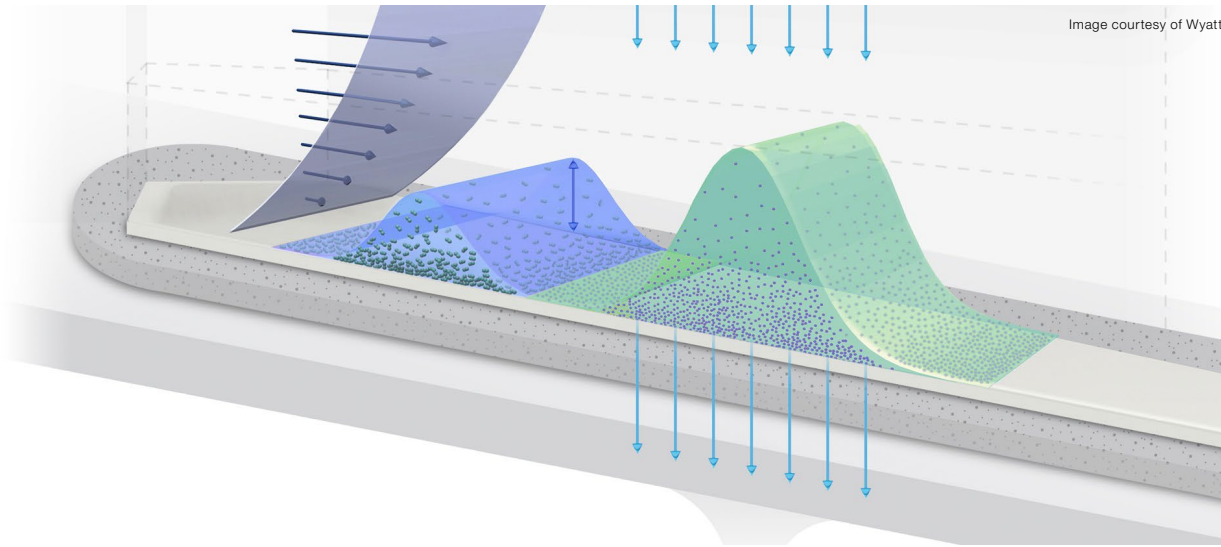


Image courtesy of Wyatt

Meeting Regulatory Needs in the Characterization of Lipid Nanoparticles for RNA Delivery via FFF-MALS

Fanny Caputo and Christian Sieg

A validated FFF-MALS method aligned with technical specification ISO/TS 21362 for the analysis of LNPs encapsulating siRNA and mRNA.

Field-flow fractionation coupled to multi-angle light scattering (FFF-MALS) is a powerful analytical approach for the advanced characterization of nanomaterials (sizes <1 µm). Contrary to size-exclusion chromatography (SEC), no stationary phase is required during the fractionation process, and the optimal separation range can be fine-tuned by varying flow parameters. This flexibility enables reliable measurement of complex pharmaceutical products, such as nanomedicines, monoclonal antibodies (mAbs), and vaccines. For quality control purposes, the FFF-MALS protocol must satisfy regulatory needs and accepted standards. This article describes a validated FFF-MALS method aligned with technical specification ISO/TS 21362 for the analysis of lipid-based nanoparticles (LNPs) encapsulating siRNA and mRNA.

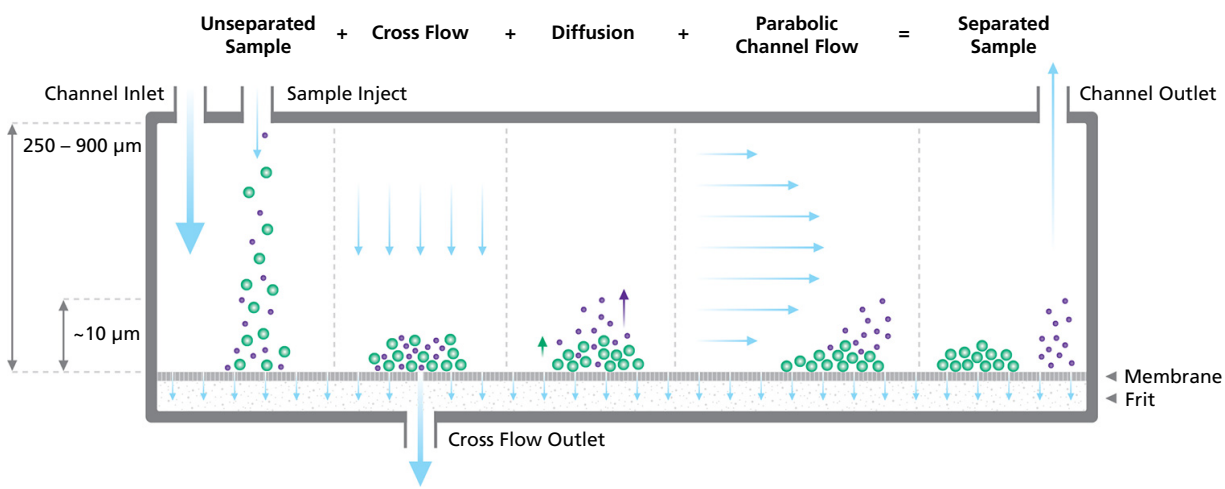
Lipid-based nanoparticles (LNPs) for nucleic acid delivery, especially for short interfering RNA (siRNA) and messenger RNA (mRNA), have recently attracted extraordinary attention, and are expected to revolutionize the medical field. At the end of 2020, a milestone was reached with two vaccines against the ongoing COVID-19 pandemic,

based on mRNA strands encapsulated in LNPs, approved by regulatory authorities in the USA and Europe: the BioNTech/Pfizer's tozinameran and Moderna's mRNA-1273. Apart from COVID-19 vaccines, further nucleic acid-based therapies are in development for a broad range of applications spanning immune-modulating agents, protein replacement therapies, regenerative medicine, and gene-editing complexes, amongst others. Nanocarriers such as LNPs can protect active pharmaceutical ingredients (APIs), enhance bioavailability, and thus improve safety and efficacy of novel therapies (1).

The use of lipid nanocarriers to deliver nucleic acid increases the complexity of the formulation and consequentially introduces the need for sophisticated analytics to ascertain a stable and safe drug product. Based on guidelines for drug products containing nanomaterials, including liposome characterization, the following parameters can be regarded as plausible critical quality attributes (CQAs): particle concentration, particle average size and polydispersity, nucleic acid loading levels, and chemical stability and physical stability (aggregation propensity). Clearly, the advancement of robust analytical methods for CQA determination that are compliant with regulatory requirements is essential to streamline development and quality control.

Field-flow fractionation (FFF) physically separates species of different sizes or, more precisely, hydrodynamic volumes (FIGURE 1). First, the unseparated sample is injected into the channel. In conventional channels, the sample attains equilibrium height above the membrane in a focusing step; this action tends to concentrate the sample in close proximity to the membrane. The dispersion inlet channel (also known as frit inlet channel) uses the "hydrodynamic relaxation" principle for which no focusing step is required,

FIGURE 1: The FFF principle resolves species of different sizes without a stationary phase.



diminishing unwanted effects of high local concentrations. A parabolic channel flow then transports the sample towards the channel outlet. Since small particles have a higher diffusion coefficient, they will on average be higher above the bottom membrane and hence exposed to a higher flow rate. Thus, small particles elute first, followed by larger species, inversely to the order in size-exclusion chromatography (SEC).

The particle retention time, t_r, is directly proportional to the hydrodynamic radius, R_h, and the tunable cross flow/channel flow ratio. Subsequent multidetector analysis including UV, multi-angle light scattering (MALS), online dynamic light scattering (DLS), and differential refractive index (dRI) detection elucidates several key sample parameters in the same measurement. This was recently demonstrated for pharmaceutical products including liposomal drug formulations (2,3). Based on the 2021 publication by Mildner et al. (4), we outline here the establishment of a robust FFF-MALS method for the characterization of lipid nanoparticles encapsulating RNA (RNA-LNP). Key parameters that can be measured with FFF-MALS are average particle size, polydispersity, morphology, physical stability, and particle concentration.

To fulfill regulatory requirements, the repeatability, reproducibility, and robustness of the analytical methods used to characterize a drug product need to be addressed according to the harmonized

guideline ICHQ2R1 (5). Even if ICHQ2R1 addresses mostly classical chromatographic methods and does not specify the requirements for sizing measurements such as FFF-MALS, ISO/TS 21362 (6) could be used as a suitable reference for the validation of FFF-MALS methods. The following criteria need to be met:

1. Recovery of the analyte ≥70% (performance separations, such as particle concentration, would require recovery ≥90%);
2. Relative standard uncertainty ≤5% for retention time, recovery, and particle size values.

This article describes FFF method development, including channel-type selection, and demonstrates the power and versatility of FFF-MALS for the characterization of siRNA-LNP and mRNA-LNP according to regulatory needs.

Experimental

The experimental procedure is described in depth in reference 3. In brief, the samples were analyzed using an Eclipse FFF instrument (Wyatt Technology) operated with an isocratic pump, degasser, and autosampler from the 1260 Infinity II series

(Agilent Technologies). Fractionation was performed in the Eclipse conventional long channel and dispersion-inlet channel. Sample recovery was calculated by integrating the main UV peak area at 260 nm for each sample, with and without the applied cross flow and focusing step.

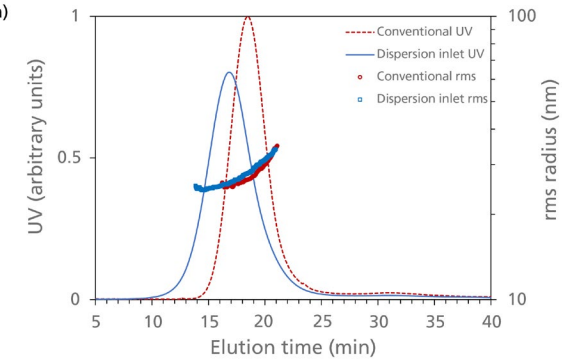
Detection was accomplished by a DAWN MALS instrument with embedded DLS module (Wyatt Technology). Data were collected and analyzed in ASTRA software (Wyatt Technology) to determine particle size and concentration. Further data processing calculated the particle size distribution in particles/mL/nm for 2 nm bin sizes (4).

High Recovery as Key Measurement Quality Indicator

Sample recovery (R%) is the key parameter to consider for initial method optimization. It is determined by main UV peak integration with and without cross flow and focusing step. During method development, the conventional setup with a standard FFF long channel was compared to a method using the dispersion inlet channel, where the sample is injected directly into the carrier stream. Here, no focusing is required, reducing the risk of sample destabilization and increasing recovery for delicate samples such as LNPs. FIGURE 2 shows the results obtained by measuring the same siRNA-LNP formulation by using the two channels. In both channels the sample is eluted and fractionated according to the particle size. Although in the tested configuration both channels fulfill the quality criteria in terms of R%, the dispersion inlet method was used for advanced characterization of LNPs due to the higher recovery and its benefits for sensitive samples.

FIGURE 2: Comparison of conventional and dispersion inlet channel separation and recovery of a siRNA formulation. (a) The UV fractograms of conventional long channel and dispersion channel are shown with rms radius as an overlay. The main species has a size of 26–27 nm. (b) Here, the results averaged over three measurements are reported including standard deviation of the mean. Spread: RMS max–RMS min across the full width half maximum (FWHM) of the peak.

(a)

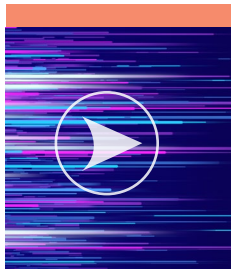


(b)

	R%	rms radius (nm)	spread (nm)
Conventional long channel	91 ± 1	27 ± 0.5	11 ± 2
Dispersion inlet channel	99 ± 1	26 ± 1	6.5 ± 0.5

Determination of Size, Morphology, and Particle Concentration by Using an Optimized Dispersion Inlet Method

Using the optimized fractionation method, with MALS and DLS detectors online, several



LNP key attributes can be determined, including: (i) average size and polydispersity, (ii) particle morphology, and (iii) particle concentration. As proof of concept, three formulations with variable mRNA payload were measured to determine both the sample size, polydispersity, and particle morphology: empty particles, particles with low (N/P = 8) RNA content, and particles with high (N/P = 3) RNA content. The particle size and polydispersity were higher for lower mRNA content, and even more pronounced for empty particles, indicating that a minimum amount of mRNA is needed to obtain a monodisperse formulation. In fact, it is known that in the absence of a sufficient amount of RNA, the LNP nanostructure where the mRNA is complexed by the ionizable lipids and the other particle components cannot be formed.

Differences in particle size and polydispersity are therefore associated with differences in particle morphology, another important parameter that can be determined by FFF-MALS. MALS with simultaneous online DLS to determine hydrodynamic radius (R_h) elucidates particle morphology (shape, core density) associated with the RNA loading. For a hollow sphere, ρ (the ratio of rms radius [determined by MALS] to R_h) is unity. For a sphere with a dense core, however, the scattering centers are distributed closer to the center of mass and ρ ≤ 0.77 is expected. FIGURE 3 shows ρ vs. retention time of mRNA-LNP samples with different RNA content, and of the empty LNP control sample. Interestingly, the empty particles possess ρ ≈ 1, typical of a hollow or irregular sphere, while increasing mRNA contents reduces the ratio. The value of the monodisperse mRNA particles match the ratio associated with a dense spherical particle. Note, the empty LNP and the mRNA particles loaded with a lower payload show a larger proportion of aggregates, and are less stable, showing the correlation between particle morphology, monodisperse size distribution, and the physical stability of the LNP formulations.

FIGURE 3: Comparison of mRNA-LNP samples with different mRNA content and empty particles. The LS fractograms at 90° MALS angle are shown overlaid with the ratio of rms radius and hydrodynamic radius, ρ. The ratio tracks the degree of encapsulated RNA.

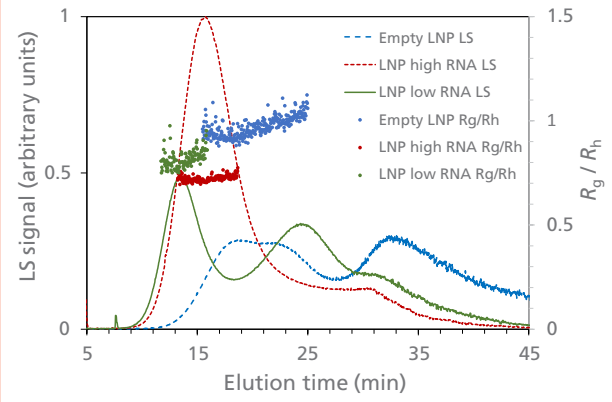
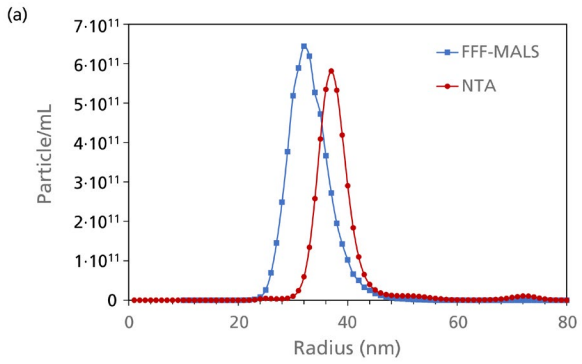


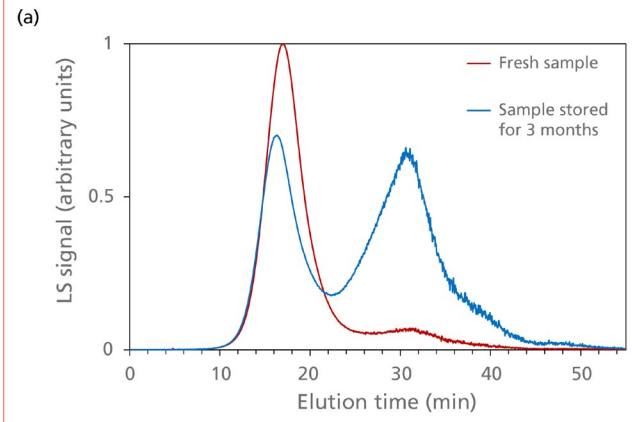
FIGURE 4: Particle size distribution by number and total particle concentration values obtained by FFF-MALS and NTA on a formulation of LNPs encapsulating siRNA.



(b)

Technique	Total particle/mL (SD)	Mode Radius nm (SD)
NTA	3.9E+12 (3E+11)	36 (2)
FFF-MALS	6.77E+12 (1.5E+10)	33 (2)

FIGURE 5: Aggregation content in a freshly prepared sample (red) and sample that was stored for three months (blue). (a) The LS fractograms at 90° MALS angle of both samples are shown. The relative size of the aggregate Peak 2 is significantly larger for the stored sample. (b) Numerical analysis of the particle concentration. Peak 2 contains 10 times more particles for the stored sample compared to the fresh sample.



(b)

	Peak 1 rms radius (nm)	Peak 1 total particle concentration (mL⁻¹)	Peak 2 total particle concentration (mL⁻¹)
Fresh sample	26.4	5.55 e12	2.24 e09
Stored sample	24.7	5.85 e12	2.01 e10

As demonstrated by Mildner et al. (4), the data obtained by MALS, in combination with knowledge of particle shape, structure, and refractive indices of the particles and solvent, determine particle concentration and thus derive the number-based particle size distribution. As a proof of concept, the results obtained by FFF-MALS were compared with particle concentration measured by nanoparticle tracking analysis (NTA) and showed remarkably comparable results (FIGURE 4). Notably, FFF-MALS detects and quantifies LNP particles below 30 nm in radius that are too small for NTA.

LNP Stability Assessment

A critical parameter for RNA-LNPs is their stability. As FFF-MALS can determine size, morphology, and concentration with high resolution, it is one of the methods of choice for evaluating the physical stability of the particles, and to determine their tendency to aggregate. Here, a freshly prepared LNP sample loaded with siRNA was compared to a sample prepared three months earlier and stored at 4 °C. The freshly prepared sample showed one main particle population (Peak 1: R_t = 17 min) and a small number of larger particles eluted as a second peak (Peak 2:

R_t = 31 min). After 3 months of storage, the size associated with the main population was slightly decreased, while the intensity of the peak associated with larger particles and/or aggregated was significantly higher (FIGURE 5). The change in the particle size distribution and substantial increase in sample polydispersity is called Ostwald ripening and is induced by the migration of lipids and particle components from smaller particles to larger ones. Higher sample polydispersity immediately following synthesis corresponds to faster Ostwald ripening and thus larger particle instability. This highlights the power of FFF-MALS to study changes in size, polydispersity, and concentration of LNP formulations over time.

Conclusion

Thorough method development and validation are crucial to move FFF-MALS into standard QC procedures in the nanomedicine field. Here, we demonstrated method development following the ISO/TS 21362 technical specification and showed results for RNA-LNP. We presented a robust FFF-MALS approach that can be used by the pharmaceutical industry to characterize nucleic acid-based nanotherapeutics during drug development and for quality control purposes. Importantly, FFF-MALS measures multiple CQAs of LNP nanoparticles encapsulating mRNA or siRNA, such as particle size distribution, particle morphology, and particle concentration, as well as physical stability and aggregation propensity.

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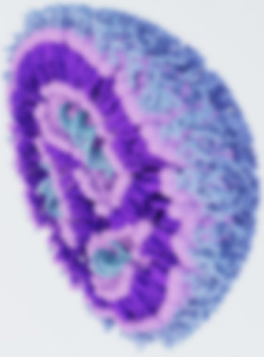
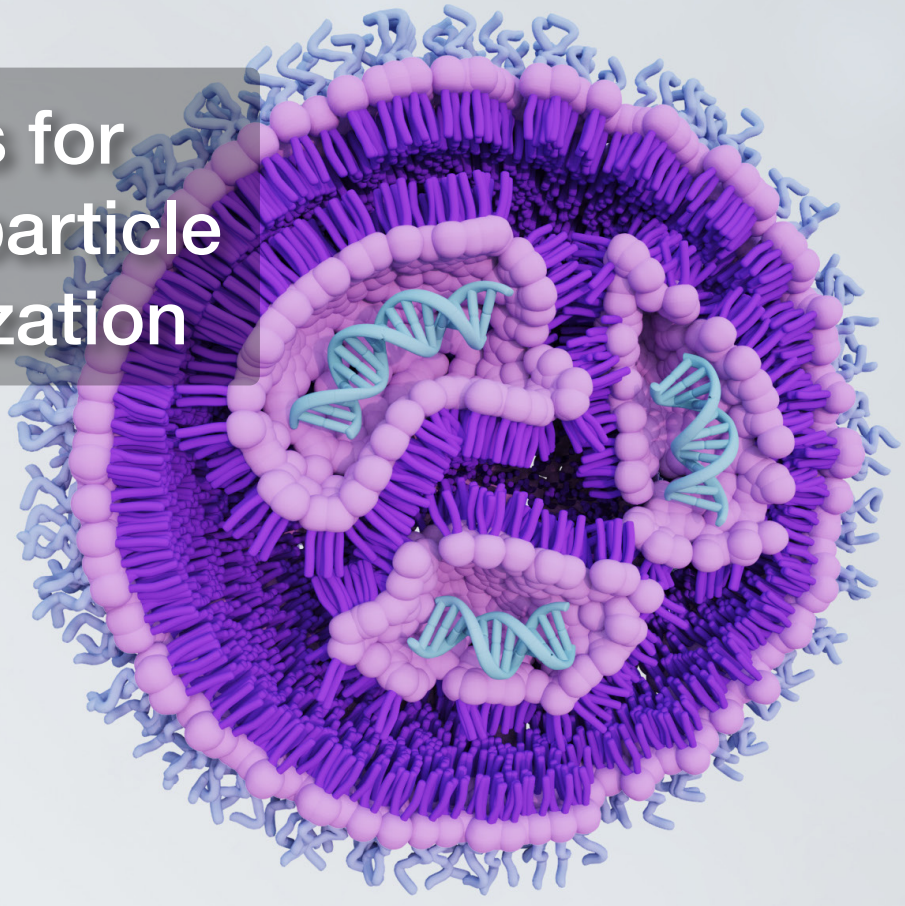
Solutions for Lipid Nanoparticle Characterization





Detailed LNP-RNA analysis with FFF-MALS-DLS

The size, size distribution and amount of RNA payload are critical to the efficacy and dosing of lipid nanoparticle vaccines and other therapeutics. Field-flow fractionation combined with multi-angle and dynamic light scattering (FFF-MALS-DLS) is perfectly suited to quantify these key attributes in a single automated assay.

Wyatt's FFF-MALS-DLS platform combines size-based separation using the Eclipse™ FFF instrument and standard pumps and autosamplers, with independent determination of size, shape and particle concentration by a DAWN® light scattering instrument. In combination with RI and UV absorbance data, the system quantifies the encapsulated payload at each size fraction.

To learn more about Wyatt's unique solutions for LNP-RNA characterization, visit www.wyatt.com/LNP

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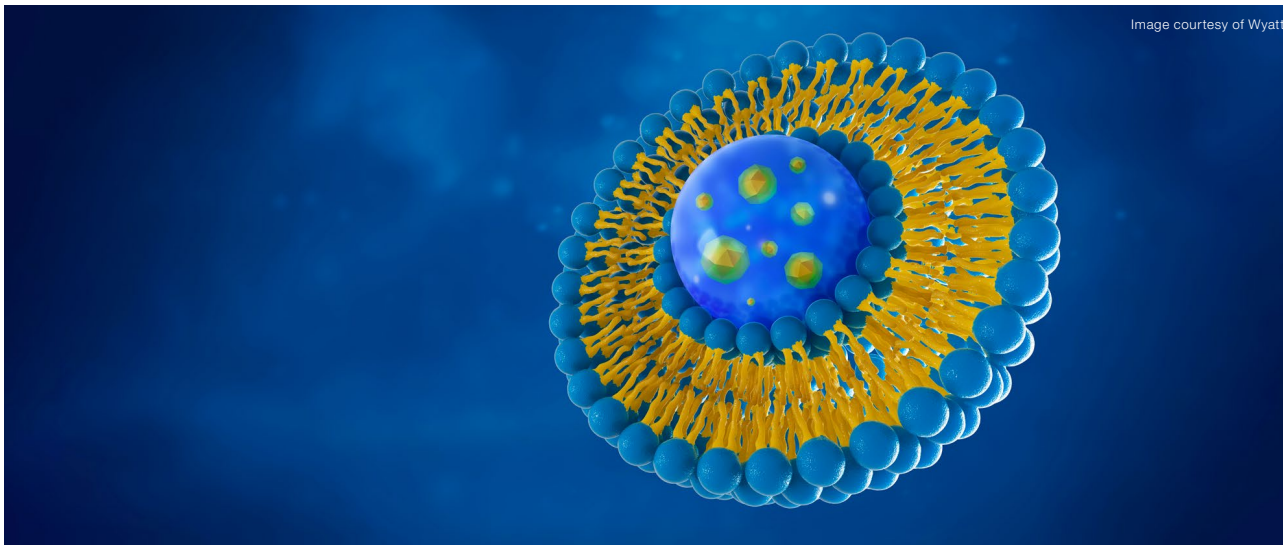


Measuring Physical Properties of Liposomes and LNPs for RNA Delivery With Multi-Detector Asymmetric-Flow Field-Flow Fractionation

Fanny Caputo and Christoph Johann

How to maximize the capabilities of a versatile separation technique to fully characterize modern nanopharmaceuticals.

Introduction

Regulatory requirements for the characterization of nanopharmaceuticals make it necessary to quantify key quality attributes of complex nanomedicine formulations such as particle size, polydispersity, drug loading, and stability. Multi-detector asymmetric-flow field-flow fractionation (MD-AF4) is among the most useful tools for this type of analysis, and it has evolved into a versatile, robust, reliable technique suitable for a wide variety of formulations. Recently, an international collaboration by the European Nanomedicine Characterization Laboratory (EUNCL) and the National Cancer Institute Nanotechnology Characterization Laboratory (NCI-NCL) successfully developed a standard operating procedure (SOP) using MD-AF4 for these measurements. With a few more standard tests under development, the adoption of MD-AF4 by the pharmaceutical industry is well underway. This presentation includes a step-by-step workflow for developing robust SOPs. In addition, it highlights several examples of how MD-AF4 can be used

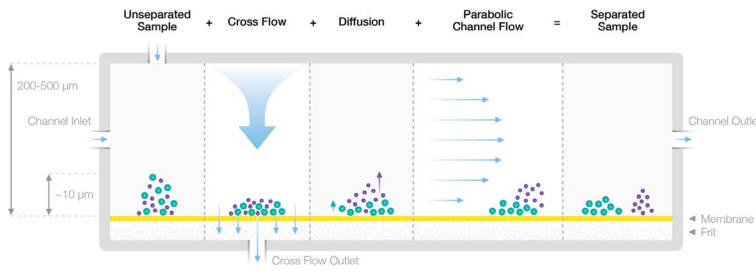
to simplify complex characterizations of key properties of liposomal products and lipid-based nanoparticles (LNPs) for RNA delivery and describes current work for developing ASTM standard methods.

Key Features of MD-AF4

MD-AF4 is the most versatile member of the field-flow fractionation (FFF) family of techniques. In FFF, particle separation is achieved without a stationary phase, by combining the parabolic channel-flow velocity profile of a carrier solvent with a perpendicular force that is selected according to the particle property. In AF4, cross flow, which is perpendicular to the carrier flow, concentrates the sample against a permeable membrane at the bottom of the channel. Diffusion of the particles works against this cross flow, forming a particle cloud with an average distance to the membrane that depends on the particles' diffusion coefficient. Smaller particles end up higher (away from the membrane) compared to the larger ones. The particles then migrate, carried by the channel flow, and the smaller particles migrate faster as they experience the higher velocities of the parabolic profile. This results in a very efficient separation, according to the diffusion coefficient of the particles, which is directly proportional to their hydrodynamic radius (FIGURE 1). Retention times depend on the square of the channel thickness, the ratio of the cross-flow rate to the channel-flow rate, and the viscosity of the solvent. Separation power may be varied in the course of AF4 fractionation by varying the cross-flow to channel-flow ratio. Since viscosity depends strongly on temperature, the temperature of the channel needs to be carefully controlled. Modern AF4 instruments contain several sensors to monitor all relevant parameters and ensure reproducibility, as well as diagnostic functions to assist in the system's maintenance. In addition, an optional dilution control module increases signal-to-noise ratios and improves quantification.

Typical detectors for MD-AF4 include multi-angle light scattering (MALS), dynamic light

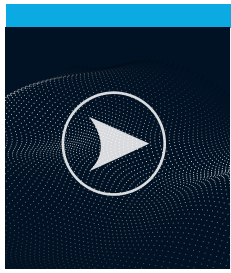
FIGURE 1: How traditional field-flow fractionation works.



Three essential components:

- Cross Flow concentrates the sample against the membrane
- Diffusion creates a concentration distribution depending on size
- Leading to migration speed which depends on the size

Measuring Physical Properties of Liposomes and LNPs for RNA Delivery With Multi-Detector Asymmetric-Flow Field-Flow Fractionation



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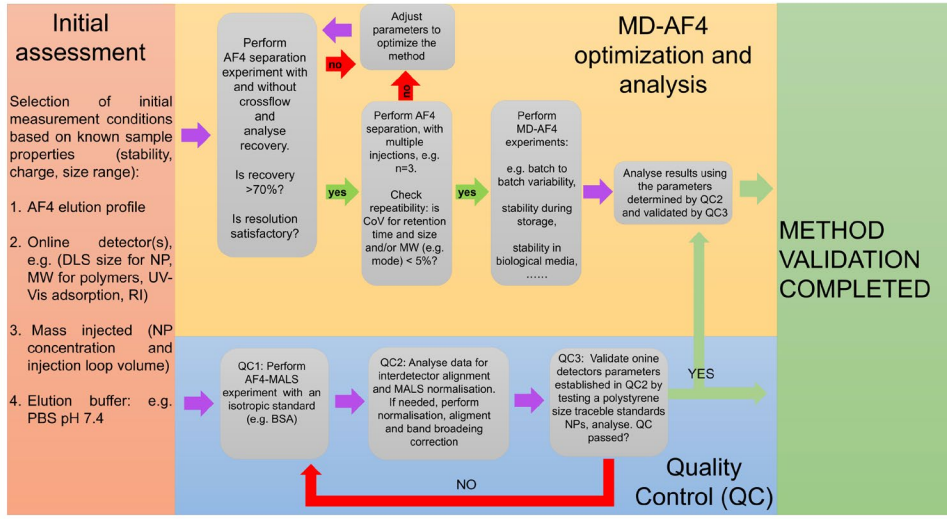
scattering (DLS), ultraviolet light (UV), and refractive index (RI). Combining MALS with DLS allows for determining the conformation of nanoparticles and differentiating between empty (hollow) and filled (solid) particles by measuring the ratio of their radius of gyration (R_g) to their hydrodynamic radius (R_h). In hollow spheres, the ratio is 1, as opposed to solid spheres, where the ratio is 0.77.

Developing Robust SOPs

More than 50% of the applications to the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for nanoparticle-based drugs, and more than 50% of clinical trials, are based on liposomal products, so the examples given here focus on liposomes and lipid nanoparticles (LNPs). For these applications, there are guidelines for method development provided by the National Cancer Institute Nanotechnology Characterization Laboratory (NCI-NCL) and the European Nanomedicine Characterization Laboratory (EUNCL), as well as technical specifications such as ISO/TS 21362:2018. Validation starts with the MALS detector, typically using bovine serum albumin (BSA).

In addition, the size range of the particles analyzed must be within the detector's range. This can be verified by using a size standard such as polystyrene (PS) particles. As far as the method robustness and precision criteria are concerned, the mass

FIGURE 2: MD-AF4 optimization method.



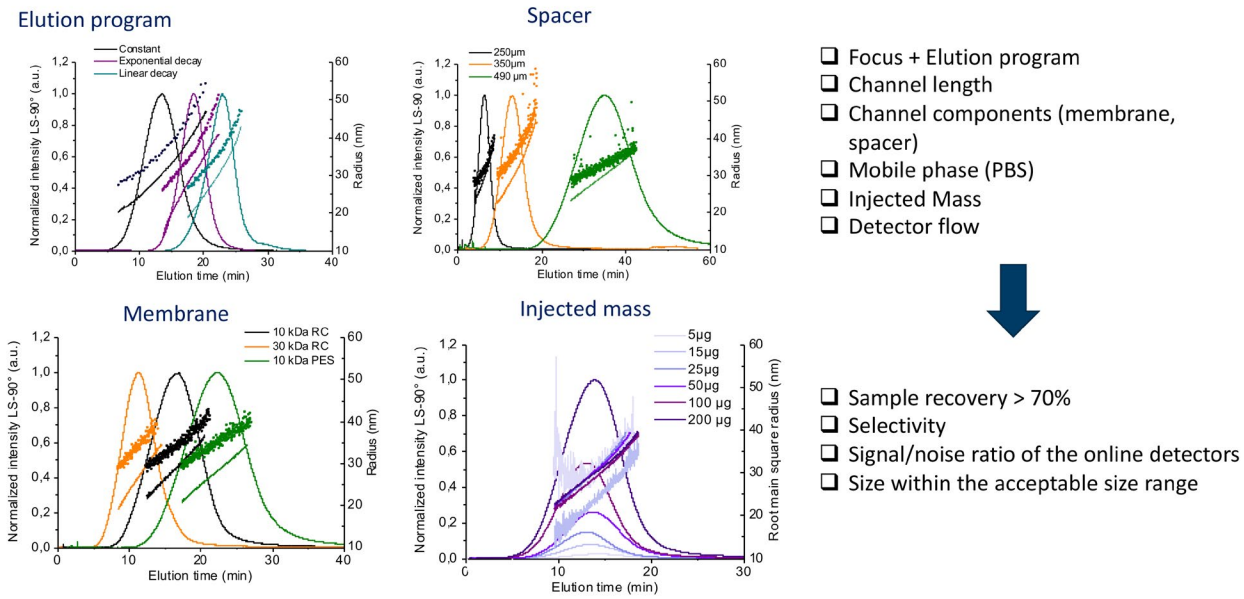
Caputo et al. Journal of Chromatography A, January 2021

Measuring Physical Properties of Liposomes and LNPs for RNA Delivery With Multi-Detector Asymmetric-Flow Field-Flow Fractionation

recovery rate has to be more than 70% and the relative uncertainty for retention times and molar mass measurements, less than 5%.

The diagram shown in FIGURE 2 details the specific steps in the workflow for MD-AF4 optimization and analysis.

FIGURE 3: Testing spacers, membrane materials and injection quantities.

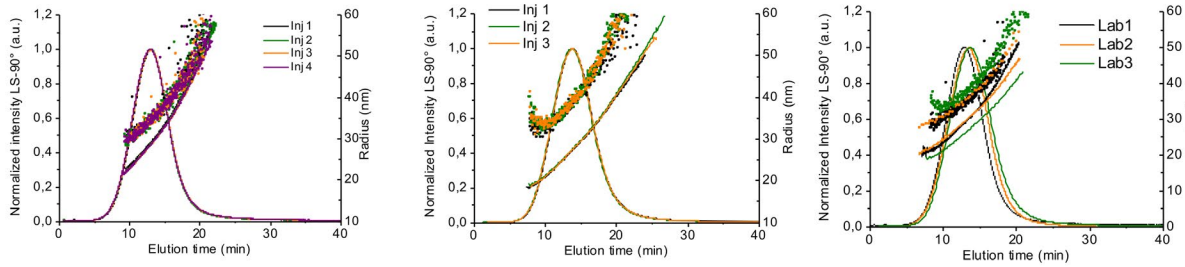


- ❑ Focus + Elution program
- ❑ Channel length
- ❑ Channel components (membrane, spacer)
- ❑ Mobile phase (PBS)
- ❑ Injected Mass
- ❑ Detector flow

- ❑ Sample recovery > 70%
- ❑ Selectivity
- ❑ Signal/noise ratio of the online detectors
- ❑ Size within the acceptable size range

FIGURE 4: Repeatability, reproducibility, and robustness verified for MD-AF4 optimization.

A) Repeatability Lab1 (Wyatt) B) Repeatability Lab3 (Postnova/Malvern) C) Reproducibility



Platform	Replicates	Recovery (%)	t_R (min)	R_g (nm)	R_g Spread (nm)	R_h (nm)	R_h Spread (nm)
Lab1	5	98 (1)	11.9 (0.3)	28.9 (0.3)	11.9 (0.6)	34.2 (0.4)	11.0 (1.4)
Lab2	3	95 (1)	13.1 (0.5)	30.2 (0.1)	11.8 (0.2)	35.9 (0.1)	11.3 (0.4)
Lab3	3	96 (1)	12.7 (0.1)	27.6 (0.1)	11.7 (0.4)	38.9 (0.1)	11.7 (0.6)
Mean (%SD)		96 (2%)	12.6 (4.8%)	28.9 (4.5%)	11.8 (1%)	36.3 (6.5%)	11.3 (3%)

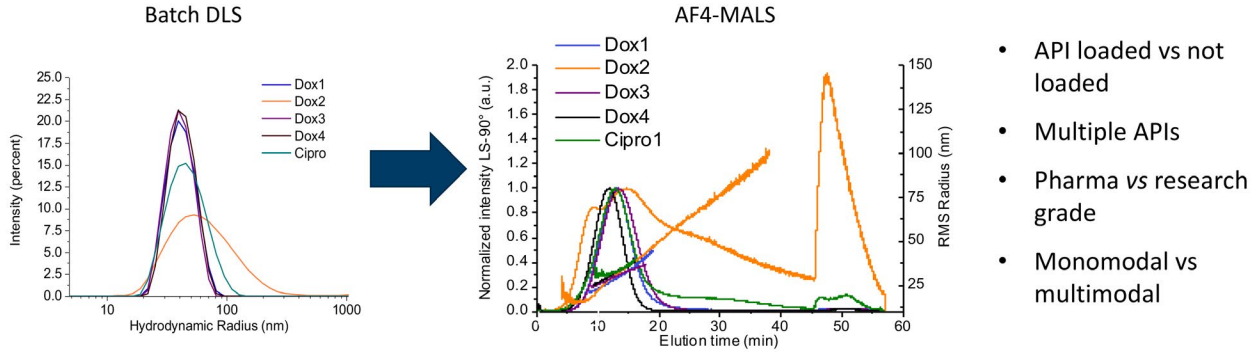
Bias: online DLS is the highest source of bias for sizing

A Practical Example: Liposomal Doxorubicin

The optimization of this method started by selecting the mobile phase in which the samples are stable. This is done by using DLS to monitor potential changes of the size distribution upon incubation with different solvents. In this case, a phosphate-buffered saline solution (PBS) worked best. In the first step, a series of experiments was performed with different elution profiles, including constant cross flow, exponential decay, and linear decay. Next, the researchers tested different spacers, ranging from 250 µm to 490 µm, membrane materials, and injection mass quantities to find the conditions that resulted in recoveries above the 70% target (FIGURE 3). To check for repeatability, reproducibility, and robustness, the researchers selected three laboratories that had different instrument configurations, two FFF instruments from Wyatt Technology® and one from Postnova Analytics®. Samples were prepared independently and provided to the labs, which analyzed 3–5 replicas to verify repeatability within each lab. The researchers then checked the method's reproducibility by comparing the results from the different labs using different configurations and operators. The results, shown in FIGURE 4, indicate a high level of reproducibility across the labs, with the only exception being the measurement of R_h by DLS, where the standard deviation of the measurement was 6.5%. The method was validated, but the researchers are conducting additional work to improve this result.

With a validated method in hand, the full power and versatility of MD-AF4 can be brought to bear. In addition, since size fractions of the samples can be collected, one can obtain off-line information regarding the size dependence of the shape or morphology of the particles, whether or not they contain the active pharmaceutical ingredient (API) of interest or if they have multiple APIs. An example of comparing DLS to MD-AF4 is given in FIGURE 5. DLS screening of four different samples of doxorubicin (Dox1 to Dox4) and a generic Cipro shows that Dox2 has a different

FIGURE 5: Batch-to-batch variability and biosimilarity.



particle size distribution (PSD) but it does not reveal the reason for this discrepancy, because of lack of resolution. MD-AF4 is able to fully characterize the PSDs (FIGURE 5).

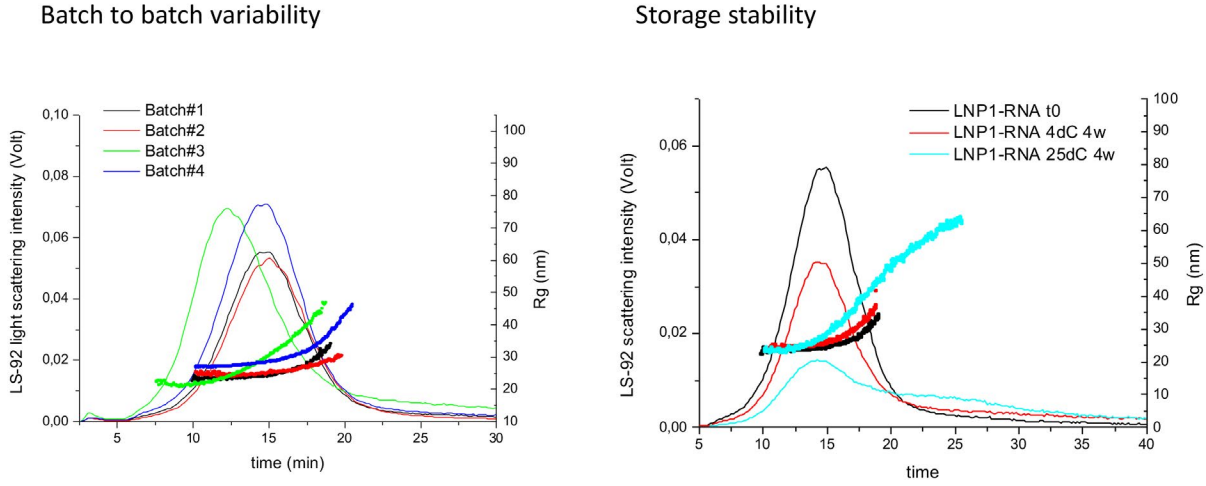
MD-AF4 is also a powerful technique for determining the stability of LNPs and LNP-RNA complexes in a variety of situations. For example, when LNPs are in the presence of serum proteins, a thin layer of protein corona can form if the proteins interact with the LNPs. This is easily observed by measuring their R_g and R_h by AF4-MALS, which can detect changes as small as 5%. In addition, peaks at the end of the elution would indicate the formation of aggregates. Wyatt's MALS analysis software reports particle concentration and particle size distributions, which can be manipulated to provide quantitative distributions in terms of number of particles per mL as a function of size. AF4-MALS results are very close to those obtained by common techniques,

such as nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS). It is important to note that for these measurements, the RI of the particles must be known with high precision: a small error in this value will generate a large error in the concentration results.

Analysis of Lipid-Based Nanoparticles for Nucleic Acid Delivery (LNPs-RNA)

Lipid-based nanoparticles for nucleic acid delivery is a very important area, as two of the approved COVID vaccines and one approved treatment for polyneuropathy are based on this technology. Because the LNPs-RNA have a different structure from

FIGURE 6: Batch-to-batch variability and storage stability.



the liposomes described earlier, the same SOP applied to liposomes cannot be used. In addition, the regulatory requirements for robustness are slightly different. These methods are currently under development and will be published soon. Nevertheless, some examples of the utility of MD-AF4 in this area were presented. Checking batch-to-batch variability is straightforward, very similar to how it is done for liposomes. In addition, it is easy to monitor storage stability at different temperatures, as the formation of aggregates upon heat-aging (an undesirable outcome) becomes quite obvious in the fractograms (FIGURE 6). These results confirm that the specific formulation analyzed here is not stable if stored at room temperature for four weeks. To verify stability of LNPs-RNA in biological media, such as PBS in combination with different sera (fetal bovine serum, human serum, cerebral spinal fluid, and others), researchers monitored the maximum and minimum values of R_g, and their spread. Again, increases in size of about 5 nm indicate the presence of a corona. Furthermore, by combining MALS and DLS detection, they calculated the ratio of R_g to R_h, to obtain information on the morphology and density of the particles. Because the LNPs-RNA have a dense structure that combines lipids and RNA closely packed together, the value of this ratio should be very close to the theoretical 0.77, and this is what they found. Further detail, such as distinguishing particle shapes, such as rods and ellipsoids, is possible by looking at their ratios of size by MALS to size by DLS.

Conclusions

MD-AF4 is a versatile technique for the analysis of nanopharmaceuticals, capable of delivering highly reliable measurements of their particle size, morphology, and concentration. In addition, MD-AF4 enables monitoring the stability of these important drugs in simple and complex media. Researchers can develop robust SOPs and test methods that comply with regulatory requirements and are extremely useful for formulation screening and quality control. Adoption of MD-AF4 will increase as specific methods for liposomal products and lipid-based nanoparticles, which are under development and expected to become available soon.

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