



## Live Webinar Q&A Sheet:

### Measuring physical properties of liposomes and LNPs for RNA delivery with multidetector asymmetric-flow field-flow fractionation

The recorded webinar may be viewed from the [FFF](#) webinars page. These questions were submitted by live viewers. Additional information on SEC-MALS, DLS, CG-MALS, RT-MALS and FFF may be found on the Wyatt web Library under [Webinars](#), [Application Notes](#), and [Bibliography](#), as well as on the corresponding [Product page](#) and [Solutions](#) page of our web site.

Please contact [info@wyatt.com](mailto:info@wyatt.com) with any additional questions.

#### Application-specific

*Q: Why is there a difference between liposomes and LNPs in characterization by MD-AF4? When do I expect to have differences in elution and characterization between samples?*

A: The lipid bilayer that forms the shell of a liposome is mechanically robust and chemically inert. Empty liposomes and those where the payload is fully contained within the structure will generally exhibit ideal elution in FFF regardless of the specific focus and cross-flow conditions, and will even pass through a size-exclusion column quite intact. If the payload, e.g. a drug nanocrystal, extends from the liposome envelope it may interact with the FFF membrane.

There are many types of lipid nanoparticles (LNPs), and some may be just as well behaved as liposomes. However, LNPs for RNA delivery are less stable and more prone to destabilization during focusing. This is why the elution method has to be optimized for each formulation. In the case of the LNPs, separation in a dispersion inlet channel (a.k.a. frit inlet channel) may reduce destabilization during focusing.

In addition, liposomes are generally quite spherical while LNPs may not be. Characterization by MALS and DLS, both of which are sensitive to shape and refractive index, will need to take into account these deviations from simple spheres and uniform content.

*Q: Will hollow liposomes collapse under pressure in FFF?*

A: Generally, they will not. Liposomes—even if hollow—are very stable and the pressures in FFF not so large. Please refer to the method described in [doi: 10.1016/j.jconrel.2020.01.049](https://doi.org/10.1016/j.jconrel.2020.01.049).



*Q: Is it possible to differentiate LNPs loaded with RNA or without RNA, and is it possible to calculate the encapsulation efficiency?*

A: FFF does not physically separate loaded from unloaded LNPs, unless they happen to have sufficiently different sizes. That would not generally be the case. However, the average RNA payload in an LNP may be quantified by combining FFF separation with MALS, UV and RI detection. This setup is commonly used to characterize the composition and relative content of conjugated macromolecules such as glycoproteins and adeno-associated viruses. The method, known as conjugate analysis, determines the molar mass of each component of the conjugate in each eluting fraction, with no additional experimental effort. In the past this method was generally not applicable to nanoparticles with radius above roughly 30 nm due to excessive scattering in the UV detector, which causes a large error in the absorption measurement. Recent algorithmic advances overcome the UV scattering issue and may be able to provide conjugate analysis of larger particles such as LNP-RNA, determining the relative fraction of RNA and the number of RNA molecules in the particle as a function of particle size. The degree of accuracy and reliability of this method is currently under evaluation, but so far it has been found to be quite accurate and precise.

In addition, fractions can be collected and analyzed offline to determine RNA payload as a function of size, e.g. by Ribogreen.

Encapsulation efficiency can be determined by quantifying the free RNA, which will elute in a separate peak and can be measured using UV absorption. Comparison of the free RNA mass with the amount used in sample preparation will provide a good indication of encapsulation efficiency, if sample recovery is shown to be sufficient (over 90%).

*Q: How much have coronas around liposomes impacted AF4-MALS characterization for size thus far?*

A: For information on protein corona measurements of liposomes please refer to the data published on <https://doi.org/10.1007/s00216-019-02252-9>. The amount of protein corona (and its thickness measured by FFF) depends on the degree of PEGylation and/or on the surface properties of the liposomal formulation tested. If a corona does form then AF4-MALS will certainly see it in the measured size.

*Q: How does the FFF method influence the shape of the liposome particles?*

A: An appropriate FFF method will not modify the particle shape during elution in order to prevent artifacts. If this does occur then different elution conditions should be tested. On the other hand, particle shape does influence the retention time, since elongated particles elute later than compact particles of equal volume.



*Q: Can AF4 be applied for analysis of micelles formed using surfactant?*

A: Surfactant micelles are not generally a good application for FFF because they are in dynamic equilibrium with the monomers and might dissociate to small monomers that escape through the membrane. SEC-MALS and batch DLS are better choices for analyzing micelles.

*Q: What is the best approach for Amyloid beta agglomerate separation with AF4 in terms of choice of condition or instrument membrane and carrier solution?*

A: Conditions for this and other applications may be found in the literature. Several publications regarding the use of FFF for separating amyloids may be found in Wyatt's online bibliography <https://www.wyatt.com/Bibliography>.

One very nice example is Rambaldi et al., *In vitro amyloid A beta1-42 peptide aggregation monitoring by asymmetrical flow field-flow fractionation with multi-angle light scattering detection*, Anal. Bioanal. Chem. <http://doi.org/10.1007/s00216-009-2899-1>

*Q: We are working on a protein nanoparticle and the amount of nanoparticle is around 5% relative to the total protein load. Can MD-AF4 be applied for such samples?*

A: In general, yes. AF4 can completely separate species that differ by just 15-20% in radius, enabling the characterization of each species independently of the other. Proteins and any aggregates are typically smaller than 10 nm, so if the nanoparticles are say 15 nm in size, they would be completely separated and measurable by MD-AF4.

#### **Applications of MD-AF4 in the pharmaceutical industry**

*Q: What information, in addition to size and shape, can I gain with multi-detector FFF, that is relevant for nanopharmaceuticals preclinical characterization?*

A: Multi-detection field-flow fractionation can be used to quantify:

- Particle concentration (with online MALS)
- Differential and cumulative size distributions (with online MALS and/or online DLS)
- Payload, such as the weight fraction of encapsulated peptides, nucleic acid or small molecule crystals (with UV-Vis and RI detectors)
- Molecular weight distributions of polymers such as PLGA that form nanopharmaceutical polyplexes (with MALS and RI detectors)
- Polymer chemical nature (with online Raman, see DOI: [10.1021/acs.analchem.9b05336](https://doi.org/10.1021/acs.analchem.9b05336))

In addition, size fractions can be collected for offline analysis of a large variety of properties, which can then be quantified and related to particle size.



Depending on the nano-formulation under consideration, all those parameters may be critical quality attributes, or important parameters to monitor when characterizing pristine batches (e.g. for batch-to-batch variability analysis) or investigating behavior and stability in complex biological media (e.g. human plasma).

For additional details please refer to the following reviews:

- Hu et al., The utility of asymmetric flow field-flow fractionation for preclinical characterization of nanomedicines (2020) <https://doi.org/10.1007/s00216-019-02252-9>
- Caputo et al., Asymmetric-flow field-flow fractionation for measuring particle size, drug loading and (in)stability of nanopharmaceuticals. The joint view of European Union Nanomedicine Characterization Laboratory and National Cancer Institute - Nanotechnology Characterization Laboratory (2021) <https://doi.org/10.1016/j.chroma.2020.461767>

Q: *What is missing to for FFF to be fully adopted by the pharmaceutical industry?*

A: These points are of key importance for the pharmaceutical industry:

1. **Expertise and guidance for developing robust methods** - Standard operating procedures for MD-AF4 have been developed by multiple experts and more are in the pipeline. See e.g. [https://ncl.cancer.gov/sites/default/files/NCL\\_Method\\_PCC-19.pdf](https://ncl.cancer.gov/sites/default/files/NCL_Method_PCC-19.pdf) and <http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL-PCC-022.pdf>
2. **The availability of standard test methods and guidelines that are recognized by regulatory agencies** - Standards are slowly being developed. The technical specification ISO/TS 21362:2018 *Nanotechnologies — Analysis of nano-objects using asymmetrical-flow and centrifugal field-flow fractionation* was a first milestone. More are coming, including a standard to measure liposomal products.
3. **Good laboratory procedures (GLP) compatible instrumentation and software** – ASTRA SP software meeting GLP requirements for MALS and DLS analysis is already available, and a 21CFR(11)-compliant version of VISION software for Eclipse is expected in early 2021. Together these render MD-AF4 suitable for the pharmaceutical industry setting. IQ/OQ procedures are available for all Wyatt instruments including Eclipse, DAWN and Optilab as well as the Agilent modules used with the Eclipse FFF system.

The combination of SOPs, standard test methods and guidelines and Wyatt's 21CFR(11)-compliant instrumentation, software and procedures fulfills the requirements of the pharmaceutical industry for adopting this platform. MD-AF4 is a versatile analytical platform that will answer regulatory needs for the characterization of nano-formulations. It will be invaluable to the field of lipid-based nucleic acid delivery, supporting regulators and other stakeholders in nanomedicine's imminent nucleic acid revolution.



*Q: Do you already have some experience in using AF4 as a validated method under GMP and what are the questions authorities ask when using this technique on bDP/DP specifications?*

A: At this time we do not have such direct experience, the SOP presented is generic and must be adapted by each company to the specific requirements of the drug product, the sensitivity of the clinical effect to the quality attributes measured, etc. The FDA and other regulatory agencies have published presentations and guidelines for doing so, and standard test documents are beginning to come online, so we expect to increasingly see GMP implementation. The best advice for now is to discuss the requirements with regulatory agencies prior to and during the adoption of MD-AF4 in a GMP environment in order to iron out wrinkles before they become entrenched.

### Using FFF

*Q: Is there any sample injection inlet or vials as it were for HPLC?*

A: Samples are loaded in vials and injected by a standard HPLC vial autosampler.

*Q: How can I find the best starting conditions separating an unknown sample?*

A: We suggest to first search the literature, to see if conditions have already been reported. If the particle size range of the sample is more or less known then the method may be optimized in-silico using [VISION DESIGN™ computer-aided FFF method development software](#). If it is not known, a quick analysis by batch dynamic light scattering, electron microscopy, nanoparticle tracking analysis or another sizing method will provide reasonable starting estimates for size which can be fed into VISION DESIGN.

Other tricks for method optimization are reported in ISO/TS 21362:2018, in the previously mentioned SOPs and in multiple papers, including:

- <https://doi.org/10.1016/j.chroma.2020.461767>
- <https://doi.org/10.1016/j.aca.2013.11.021>

*Q: How difficult is it to get started with FFF, given prior SEC experience?*

A: A user who is familiar with SEC will have a shallow learning curve with FFF. The instrumentation in terms of pumps, autosamplers and detectors is identical, and the software quite similar to standard chromatography software.

The main difference comes from the mechanism of FFF separation, which allows for method optimization by modifying flow rates and spacer thickness rather than swapping entire columns or mobile phase. VISION DESIGN software, provided with the Eclipse instrument, predicts the retention times of an FFF separation based on sample information and a planned flow program. The user can adjust flow-program parameters for immediate feedback on how they impact resolution and retention times. SEC users will also have to learn how to assemble/disassemble



the FFF channel in order to replace a membrane and how to install the spacer. Overall, new users should be up and running within a few weeks. Additional skills and confidence may be gained by attending Light Scattering University's FFF training course.

*Q: Does density and shape influence the separation in flow FFF?*

A: Flow FFF separation depends only on the diffusion coefficient, or the hydrodynamic radius, not on any other property of the sample. Density will not influence the separation at all, and shape only indirectly because the diffusion coefficient is influenced by shape. In most applications this is an advantage, because the shape is usually more or less uniform throughout the sample and resulting fractions from flow FFF are homogeneous in size.

*Q: Even the simplest samples seem to produce a peak after the cross flow is reduced to zero. How can we calculate percent recovery on cross-flow gradient condition?*

A: The authors suggest that recovery should be calculated without taking into account the particles coming out at cross flow of zero (retained peak). If a high retained peak is present, resulting in a low recovery, then the elution conditions are not optimal. However, no specific indications of what range to consider for recovery are specifically reported in ISO/TS 21362:2018 "Nanotechnologies — Analysis of nano-objects using asymmetrical-flow and centrifugal field-flow fractionation".

*Q: Any limitations of AF4?*

A: For sure, AF4 has limitations as does any other method. It works for a finite size range, a finite range of pH for aqueous solvents and a limited set of organic solvents. Other than those, the main potential limitations relate to:

1. Aggregation: During the focusing step the sample is concentrated close to the membrane and may aggregate either reversibly or irreversibly. This can be overcome using the dispersion inlet channel (formerly, the frit inlet channel), which does not require focusing.
2. Dilution: Once the sample exits from the channel, it is diluted by a factor ranging from 10 to 1000, which may lead to dissociation, escape from encapsulation, and/or poor signal to noise ratio. Dilution can be reduced by up to 10x with the new Eclipse Dilution Control Module.
3. Membrane interactions: certain analytes will stick to the membrane, precluding good recovery and separation. Only two membrane materials are provided, polyether sulfone and regenerated cellulose, but users may prepare membranes from other types of ultrafiltration membranes.

More information can be found at <https://www.wyatt.com/FFF-MALS>.



## Light scattering analysis

*Q: What is the advantage of adding DLS online to FFF versus batch DLS?*

A: The addition of a fractionation step greatly improves resolution, overcoming the rather low resolution of batch DLS. The resolution of FFF-DLS (and of FFF-MALS) is sufficient for reliable measure of batch-to-batch variability, which is generally not detected by batch DLS, and many other applications benefit from this capability. FFF will separate oligomers from monomers, enabling quantification of such small oligomers possible using light scattering intensity and/or concomitant UV absorption, while batch DLS cannot distinguish small oligomers from monomers and in fact the presence of small oligomers leads to errors in concentration measurements.

With FFF it is also possible to perform reliable measurements of size and polydispersity in complex biological media, since free proteins are fractionated and comes out before the particles to be measured. The ability to add multiple detection modes in FFF is of course a major benefit over batch DLS or NTA.

*Q: For particle concentration by MALS, the refractive index must be known. Where can I find the relevant values?*

A: There are databases for most common materials and literature values for various bionanoparticles. In case of multiple components, estimates can be made if the chemical composition is known, as the overall refractive index is the weight average of the refractive indices of the constituent materials. For example, the refractive index of empty liposomes can be derived as the weight average of the refractive indices of the lipids (1.45) and aqueous core of the liposome (1.331) assuming a known diameter and lipid layer thickness, and taking into account the density of lipids and of water for mass calculations.

In the case of LNPs encapsulating RNA, the measurement of mass of the total RNA and of the total lipids by reverse-phase chromatography can be used to calculate the weight percent (w-%) of RNA in order to determine overall particle refractive index, where

$$RI_{\text{LNP-RNA}} = RI_{\text{RNA}} \times w\text{-}\%_{\text{RNA}}/100 + RI_{\text{lipids}} \times (1 - w\text{-}\%_{\text{RNA}}/100),$$

with  $RI_{\text{RNA}, 660 \text{ nm}} = 1.62$  and  $RI_{\text{lipids}, 660 \text{ nm}} = 1.46$ .

Likewise, for enveloped viruses or extracellular vesicles, the overall refractive index is the weight-averaged refractive index of the lipids (1.46), proteins (1.58), nucleic acids (1.62) and any entrained solution (1.33). A reasonable guess for viruses and extracellular vesicles is  $\sim 1.50$ , though for small, non-enveloped viruses like AAV which may include a mixture of empty and full capsids, 1.45 may be a better estimate.



Q: *Does interpreting the numeric value of the  $R_g/R_h$  ratio require independent knowledge of the particle shape? For example, must we assume a sphere in order to interpret  $R_g/R_h = 0.77$  as a uniform particle?*

A: Yes, something about the particle shape or general structure need to be assumed for a reliable interpretation. For example, you could get a ratio of 0.77 for some combination of optically dense core and slightly elongated shape, though Occam's razor would tell you that it is most probably a uniform sphere. The  $R_g/R_h$  ratio is an indirect measurement of particle shape; independent confirmation with electron microscopy is always suggested by regulatory agencies as indicated here: <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/drug-products-including-biological-products-contain-nanomaterials-guidance-industry>

Q: *Can you distinguish rod-like lipid particles from spheres and other shapes?*

A: Yes, such shapes can be distinguished by MALS alone or by combining MALS and DLS data, depending on the size. For larger particles with at least one dimension in the range of 100 nm or more, the angular scattering profile detected by MALS can be fit to different shape models in order to determine which best fits the measurements. Long, narrow rods are well distinguished from spheres in the MALS data for such particles.

For smaller particles in particular, where the angular scattering profile is insufficiently differentiated between rods and spheres, the ratio of the rms radius from MALS ( $R_g$ ) to hydrodynamic radius from DLS ( $R_h$ ) is indicative of the shape. Uniform spheres have a characteristic ratio of 0.77, thin-walled hollow spheres have a ratio of 1.0, and rods have a ratio above unity. In principle this method works for the larger particles as well, but it is more difficult to make accurate DLS measurements on particles flowing through DLS detector than for the smaller particles, where DLS measurements are more accurate. More information can be found in <https://www.wyatt.com/library/application-notes/nanoparticle-drug-delivery-characterization-by-fff-mals-dls.html>.

Q: *Is the measurement angle for DLS the same between the labs (Malvern vs Wyatt) or do you think the DLS differences are based on the software algorithm?*

A: The DLS differences (coefficient of variation of the hydrodynamic diameter was 6.5%) that are reported in [doi: 10.1016/j.jconrel.2020.01.049](https://doi.org/10.1016/j.jconrel.2020.01.049) are due to two parameters:

- 1) Different hardware. For example, different flow cells which have different resolution and linear velocity within the cell; DLS is, in fact, measured at different angles in the two instruments and this can also induce some differences in the size measured.
- 2) Data analysis performed by different algorithms may also induce a small but significant variability. For example, the minimum and maximum correlation times used in the autocorrelation fit might differ, as well as the specific algorithm used to perform the fit.





More information on the expected coefficient of variation will be available with a larger interlab study involving multiple labs with different online DLS instrumentation when the ASTM standard under finalization will be tested to get a bias statement.