Live Webinar Q&A Sheet:

Size, concentration, payload and quality: Comprehensive characterization of LNP-RNA therapeutics by light scattering

The recorded webinar may be viewed from the FFF-MALS webinars page. These questions were submitted by live viewers. Additional information on SEC-MALS, DLS, CG-MALS, and FFF may be found in the Wyatt website Resources section 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 with any additional questions.

Q: *Which LNP attributes can be reliably determined with SEC-MALS? When is FFF MALS recommended?*

A: When SEC can provide ideal or close to ideal separation of the different sized lipid nanoparticles, then the data from the downstream detectors reliably quantify LNP quality attributes. To confirm an ideal SEC separation, check the overlay of radius from MALS on the chromatogram to make sure that the size decreases with elution volume for the entire peak region of interest. When a monotonic trend in radius vs. elution volume is not achieved by SEC, then consider trying FFF for improved separation.

Q: *What are the requirements on the empty LNP sample used to assess scattering correction?*

A: The MW range of the empty LNP sample (or multiple empty LNP samples combined) should be wider than that of the loaded LNP samples.

Q: *In the analysis plots, you showed the nucleic acid weight fraction. Is it also possible to plot the number of RNA molecules per LNP?*

A: ASTRA's LNP Analysis module provides a plot of the nucleic acid weight fraction versus elution volume, as well as a plot of RNA MW versus elution volume. The RNA MW can be easily converted to the number of RNA molecules by dividing by the MW of one RNA molecule.

Q: *Would the software be able to differentiate a mixture of loaded and empty LNPs?*

A: No. The software measures the average of payload content as a function of size, but it is not able to know the percentage of empty LNP in the sample.

Q: *Can the LNP payload analysis/determination be applied to liposome or LNP small molecule payload determination?*

A: Yes, as long as the small molecules have UV/Vis absorbance.

Q: *Can potential aggregation of empty LNP be differentiated from LNP with load?*

A: Yes, as they should have different payloads, which can be ascertained from the combined MALS-UV-dRI analysis.

Q: *In order to correct for UV scattering by lipid nanoparticles, one has to have particles of various sizes available. Is this correct?*

A: That is correct – the empty particles need to cover the entire size range of the loaded particles. LNPs are usually not uniform in size. Either one or multiple empty LNP samples may be used together to construct the UV scattering correction curve.

Q: *Which current LNP concentration are you working with for FFF? Which concentration should we work with to avoid UV scattering? Regarding the size at which UV correction is needed, 20 nm was mentioned. Is this radius or diameter?*

A: The typical LNP concentration is 0.1-1 mg/mL. UV scattering is inherent to nanoparticles from roughly 40 nm in diameter and above, and cannot be avoided by going to lower concentrations.

Q: *I have a DAWN coupled with HPLC, UV and dRI, using ASTRA 7.2. Can I do the LNP analysis using this setup?*

A: The instrumentation is suitable, but you would need to upgrade to ASTRA 8.1 or above with the LNP Analysis module

Q: *Since size of full and empty LNP are different, won't they scatter differently? If so, is it OK to use empty as a correction in your analysis?*

A: Scattering intensity is primarily related to molar mass rather than size. In any case, full and empty LNP samples often have a similar size range, but in any case multiple empty LNP samples can be used to cover the MW range of the loaded LNP samples in order to provide the scattering correction.

Q: *Does ASTRA need to be updated to obtain the new LNP analysis module? Can the same module be used for lipid particles with protein payloads?*

A: Yes, you would need ASTRA 8.1 with the additional LNP Analysis module for the new payload analysis. Yes, the same module could be used for protein payloads. For protein payloads it is preferable to measure UV at 280 nm instead of UV at 260 nm.

Q: *Do measurements need to be performed to obtain the inputs needed for the LNP analysis module?*

A: We need to know dn/dc of lipids, dn/dc of payload molecule, and extinction coefficient of the payload molecule. We do provide nominal values for all these parameters in the case of lipid

nanoparticles and nucleic acids, but it is preferable to measure them for specific molecules, and all the measurements can be performed with the instrumentation described in the webinar.

Q: Can RNA concentrations be measured when two different RNAs are encapsulated in LNP?

A: Yes, but this method only measures the total RNA weight concentration of the encapsulated RNAs.

Q: Does the LNP UV scattering correction requires that the empty LNP have no absorbance in any of its lipid components?

A: No, this correction also works for empty LNPs with some absorption.

Q: Does the analysis assume the chemical composition is the same for all LNP sizes for a given sample, as different compositions could result in different refractive index/UV properties?

A: No, this correction does not assume the chemical composition is the same for empty and loaded LNPs.

Q: Can other non-absorbing particles of similar size be used to measure UV scattering intensity?

A: It is possible, but it is better to use the empty LNPs whenever possible.

Q: Can RNA absorbed on the surface of NP be differentiated from encapsuled in NP?

A: I believe these two scenarios could be differentiated and we would be interested to conduct a feasibility study on these samples.

Q: Can the method also be used for other nano-conjugates?

A: Currently, the method is limited to LNP-encapsulated RNA or DNA; liposome or PLGA nanoparticle loaded with UV/Vis absorbing drugs; and HMW polysaccharide-protein conjugates. The general rule is, the carrier does not absorb light at the wavelength where the payload does absorb.

Q: Is UV wavelength at 260 or 280 nm supposed to be used for studying polysaccharides conjugates?

A: For polysaccharide-protein conjugates, I'd suggest to use UV at 280 nm for maximum sensitivity and resolution.

Q: Can LNP analysis be applied to more complex extracellular vesicles (EVs) if they contain both lipids and nucleic acids?

A: Not directly, because EVs also contain proteins. But we would be happy to work with you on EV characterization.

Dynamic light scattering

Q: *How does the DynaPro Plate Reader measure particle concentrations if it only measures backscatter at one angle?*

A: In order to determine particle concentration, you need to know the particle's volume, the scattered intensity at angle 0° and the refractive indices of the particle and solvent. Particle volume is determined by the DynaPro's DLS function, using the hydrodynamic radius and an assumption of spherical shape. The scattered intensity at angle 0 is calculated from the backscattered intensity (the DynaPro's SLS function), the form factor that corresponds to a sphere with the measured hydrodynamic radius, and knowledge of the scattering angle for light reaching the detector. In this manner, the DynaPro Plate Reader measures concentration of particles up to 160 nm in radius.

Q: *What is DLS precision (reproducibility & inter-assay) for RNA-LNP and what's the acceptable PDI index? How does sample prep affect size and PDI measurement?*

A: I'd say the DLS inter-assay repeatability could be as good as 1-2% and 5% for reproducibility. The acceptable PDI depends on many factors and is primarily determined by safety and efficacy concerns rather than by instrument performance. However, please be aware that sample preparation such as filtration or centrifugation, measurement settings, and analysis conditions could all affect size and PDI measurements.

DLS is often used to screen the RNA-LNP samples throughout its development or to detect big differences in size and PDI in QC. For more robust and in-depth analysis, SEC-MALS or FFF-MALS should be considered.

General light scattering

Q: *For SEC and FFF "peaks" of MW: how is the average MW based upon the "peak" defined?*

A: We often use weight-average MW as it is measured directly by MALS. When the separation of species with different MWs is adequate, number and z-average MWs can also be derived.

Q: *Slide 20: Is radius from MALS of the loaded and empty LNP samples, the spherical radius?*

A: Yes. These LNPs have structures close to spherical; as a result, the sphere or Lorentz-Mie model are generally used to fit the angular variation of the LS detector signals.

Q: *What range of angles and optical wavelengths are sampled in your static light scattering instrument?*

A: MALS angles are from 15 to 165 degree; nominal laser wavelength is 660 nm or 785 nm.

Q: *How are these light scattering instruments better than LC-MS?*

A: LS instruments characterize intact molecules in solution or particles in suspension without the risk of breaking down aggregates or otherwise disrupting complexes. In addition to molar mass, it provides size and can couple with additional detectors to evaluate composition. LS can also measures species with much larger sizes than MS, including LNP, liposomes, polymersomes, etc. For nanomedicines such as RNA-LNP, it can measure the payload of the sample in its intact form, in formulation buffer, as well as the concentration, size and shape of the particle.

Q: *Can light scattering instruments help identify compounds present in the samples?*

A: While light scattering does not provide a chemical analysis of the compounds, these instruments can help identify compounds in a sample by size, MW, shape, and extinction coefficient.

Q: *Is polarized light being measured in the Mie scattering measurements?*

A: Wyatt's MALS detector uses polarized light as the incident light, but does not in general analyze the polarization of the scattered light.

Q: *Mie scattering amplitudes are functions of particle shape as well as refractive index contrast with the surrounding medium. How are shapes interpreted from MALS?*

A: In some cases, especially when the particle size is in the vicinity of the MALS wavelength, shapes may be estimated from the light scattering form factor – the variation in scattered intensity with angle, measured by MALS. ASTRA offers basic shape models such as sphere and rod, so if one fits the angular data well and the other does not, you can be fairly certain of the particle shape. The data may be exported in order to try fitting shape models not provided by ASTRA.

Field-flow fractionation and size exclusion chromatography

Q: *Can empty and loaded LNPs be separated using AF4?*

A: Recall both SEC and AF4 separates based on hydrodynamic radius. Neither SEC nor AF4 separate empty from loaded LNPs, because they often have very similar sizes.

Q: *Where can I find the SEC and FFF conditions for the mRNA molecules?*

A: Please see this paper on SEC and FFF conditions by Merck scientists: "Polydispersity characterization of lipid nanoparticles for siRNA delivery using multiple detection size-exclusion chromatography", Zhang, J. et al., *Analytical Chemistry* **84**(14): 6088-6096 (2012). https://doi.org/10.1021/ac3007768.

and this one on FFF method development by the scientists from Wyatt and SINTEF: "Improved multidetector asymmetrical-flow field-flow fractionation method for particle sizing and

concentration measurements of lipid-based nanocarriers for RNA delivery", Mildner, R. et al., *European Journal of Pharmaceutics and Biopharmaceutics* **163**: 252-265 (2021). https://doi.org/10.1016/j.ejpb.2021.03.004.

Q: *Can you comment on nonspecific interactions of lipid particles with AF4 membranes?*

A: The nonspecific interactions between LNP and AF4 membranes need to be minimized for a successful AF4 separation. Various groups are currently collaborating to develop the standard operation procedures (SOP) for LNP and liposome separation, characterization, and quantitation. The following two publications have good discussions on AF4 methods for LNP separation.

"Improved multidetector asymmetrical-flow field-flow fractionation method for particle sizing and concentration measurements of lipid-based nanocarriers for RNA delivery", Mildner, R. et al., *Euro. J. Pharm. Biopharm*. **163**: 252-265 (2021). https://doi.org/10.1016/j.ejpb.2021.03.004

"Physical characterization of liposomal drug formulations using multi-detector asymmetrical-flow field flow fractionation", Parot, J. et al., *J. Controlled Release* **320**: 252-265 (2021). https://doi.org/10.1016/j.jconrel.2020.01.049

Q: *Could you suggest the matrix selection guide for SEC chromatography?*

A: I will the suggestion to the following publication: "Polydispersity characterization of lipid nanoparticles for siRNA delivery using multiple detection size-exclusion chromatography", Zhang, J. et al., *Anal. Chem*. **84**(14), 6088-6096 (2012). https://doi.org/10.1021/ac3007768