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APPLICATION NOTE

AN2615: Multi-attribute quantification of LNP-mRNA therapeutics by FFF-MALS and DLS

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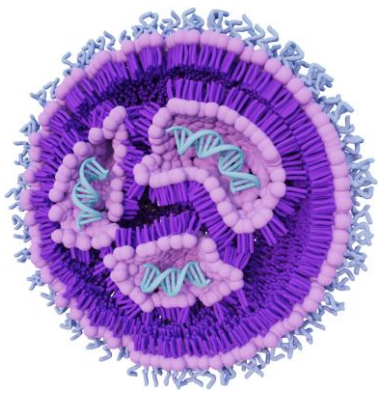
Summary

Bringing effective and safe lipid nanoparticle-formulated mRNA therapeutics to market requires quantitation of multiple quality attributes throughout development, manufacturing, and quality control. Multi-angle light scattering (MALS) coupled to separation by field-flow fractionation (FFF) offers an automatable, high-precision alternative to more cumbersome techniques used for LNP-mRNA characterization, such as dye-binding assays. Here, we use a single FFF-MALS to quantify multiple attributes for two LNP-mRNA vaccines against COVID-19:

- LNP size distribution
- LNP particle concentration
- Lipid and mRNA concentration
- Size-dependent mRNA payload distribution

We find that while the particles in the two vaccine samples span a similar size range, there are significant differences in the relative amounts of the different-sized particles and in how the mRNA drug substance is distributed across them. The approach is 21 CFR Part 11 compliant, does not require calibration curves for accurate quantitation, and can be used to evaluate batch-to-batch consistency and how drug substance and product attributes including stability depend on formulation, manufacturing, and storage conditions.



Introduction

Messenger RNA (mRNA) is a promising therapeutic agent against multiple diseases. To ensure effective and safe delivery, current mRNA therapeutics employ engineered mRNA molecules encapsulated in lipid nanoparticles (LNPs)[1]. LNP-mRNA drug products are manufactured by mixing the nucleic acid drug substance with multiple lipid components, e.g., phospholipids, cholesterol, PEG-lipids, and ionizable lipids, and the result is typically a polydisperse drug product with particles varying in size and nucleic acid content. Consequently, development, manufacturing, and quality control of stable, safe, and effective LNP-mRNA therapeutics requires quantification of multiple attributes including the LNP-mRNA size-distribution, mRNA integrity and concentration, and drug product stability.

Comprehensive quality attribute determination for LNP-mRNA therapeutics has typically demanded the use of multiple techniques, such as indirect RiboGreen™ dye-binding assays, cryo-electron microscopy, particle tracking analysis, mass spectrometry, UV/vis, and dynamic light scattering (DLS). Many of these techniques can be labor intensive or require analysts with highly specialized training. Batch measurements provide an average, low-resolution result for initial sample screening. Considering the entire sample a homogeneous solution may, however, lead to oversimplified or inaccurate drug product characterization, if the batch measurement fails to accurately resolve the properties of particle subpopulations or the lipid and mRNA subcomponents.

Here, we demonstrate how field-flow fractionation coupled to multi-angle light scattering (FFF-MALS) can be used for high-resolution multi-attribute quantification (MAQ) of two pioneering mRNA vaccines against COVID-19[2, 3]. FFF-MALS analysis physically separates the LNP-

mRNAs by size[4, 5, 6], followed by direct measurement of multiple attributes by combined MALS, differential refractive index (dRI), and UV absorbance detection. Despite the particles in the two vaccine samples spanning a similar size range, we measure notable differences in the particle size and concentration distributions, and in how the mRNA drug substance is dispersed across the LNPs.

Materials and Methods

Materials and reagents

Bivalent Comirnaty™ (Pfizer-BioNTech) and Spikevax™ (Moderna™) COVID-19 vaccines were thawed and transferred from the sealed vaccine vials using sterile syringes and needles before measurements. Samples were stored and handled following the manufacturers' guidelines over the course of the experiments.

Dynamic light scattering

Batch DLS measurements were carried out on a DynaPro™ NanoStar™ DLS instrument at 25 °C. Each sample was diluted 100-fold in phosphate-buffered saline (PBS) and loaded into a quartz cuvette. Data analysis was performed with DYNAMICS™ software. Reported uncertainties are the standard deviations from 3-6 replicate measurements.

Field-flow fractionation with UV-MALS-dRI detection

FFF separation was performed using neat samples with a separation method optimized for LNP-mRNAs on an Eclipse™ FFF instrument with a 350 µm fixed-height short channel connected to an HPLC pump and autosampler. PBS was used as the mobile phase. A DAWN™ MALS instrument, an Optilab™ differential refractometer, and a UV detector set to 260 nm wavelength were used for online detection. The FFF system was controlled by VISION™ software. Reported uncertainties are the standard deviations from triplicate measurements.

Data acquisition and analysis were performed using the ASTRA™ software. The LNPs are large enough to both absorb and scatter 260 nm UV light. To correct for UV scattering and obtain accurate concentrations from the measured 260 nm UV absorbance data we also measured empty LNP samples with the same FFF method as the vaccines. The empty LNP samples were prepared with lipid compositions and concentrations matching the vaccine manufacturer specifications. We used the results to generate experimentally derived UV scattering correction profiles with the ASTRA LNP Analysis Module software, according to the "Lipid Nanoparticle Payload Analysis" Guidance Manual embedded in the ASTRA software.

To calculate the average number of mRNA molecules per LNP, the expected molar masses of the mRNA drug substances were estimated from publicly available sequence data for BNT162b2 and mRNA-1273[7, 8].

Results and Discussion

To demonstrate LNP-mRNA MAQ by FFF-MALS we characterize the size, molar mass, particle concentration, and mRNA drug substance distributions of the Comirnaty and Spikevax bivalent vaccines against the original virus that causes COVID-19 and the Omicron variants BA.4 and BA.5.

DLS screening

We first performed batch DLS measurements to rapidly obtain an overview of the composition and size distributions of both samples. Batch DLS instruments like the DynaPro™ Plate Reader, ZetaStar™ instrument or NanoStar instrument are excellent solutions for quick walk-up measurements or automated high-throughput screening of LNP-mRNA samples, evaluating manufacturing, formulation and storage conditions[9], as well as freeze-thaw stability[10]. That said, batch DLS measurements yield comparably low-resolution size distributions, as the particles are not physically separated in the experiments.

By batch DLS, the Spikevax sample appears to contain larger particles than the Comirnaty sample. Specifically, the autocorrelation function (ACF) measured for the Spikevax sample decays more slowly than that from the Comirnaty sample (Figure 1, top panel), and fitting the ACFs using a standard cumulants model gives an average hydrodynamic radius (R_h) that is twice as large for the Spikevax sample compared to the Comirnaty sample. The DLS polydispersity, in contrast, appears to be effectively identical for the two samples (Table 1). Although this analysis can be helpful in quickly screening and flagging samples outside of specification, a cumulants model cannot capture detailed size distribution information for these highly polydisperse samples. Additional refinement via regularization analysis suggests that the samples are composed of two populations: small particles with ~15-30 nm in radius and larger particles with radii up to ~100-

200 nm (Figure 1, bottom panel). While DLS as a technique cannot directly measure the relative amounts of the different particles, the DYNAMICS software can estimate a coarse-grained % Mass distribution based on the regularization results (Figure 1, bottom panel). This low-resolution distribution suggests that the smaller particles account for ~95 % Mass for the Comirnaty sample, and ~80 % Mass for the Spikevax sample (Table 1). Regularization analysis thus uncovers that these products differ both in the size of their main species as well as in the size and content of larger particles.

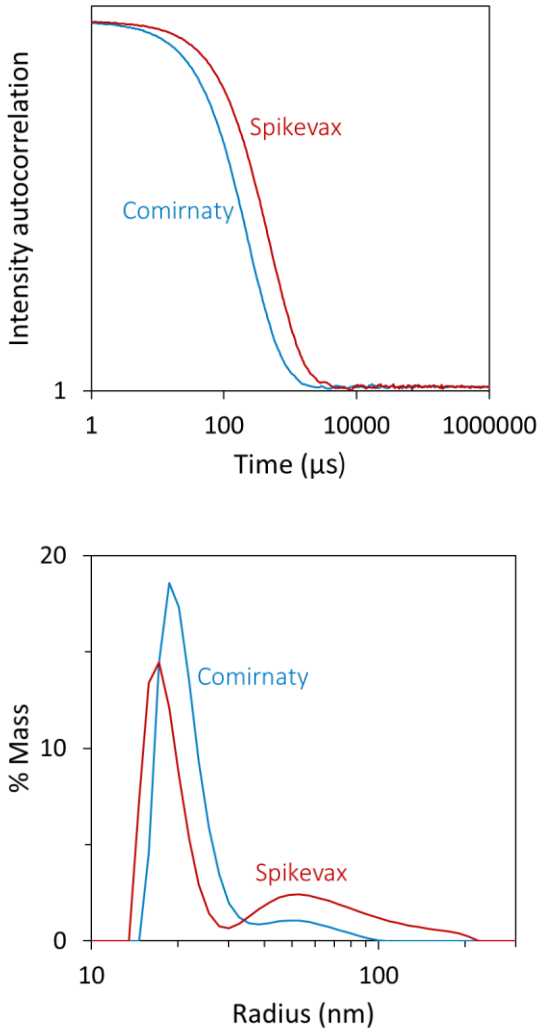


Figure 1. Batch DLS autocorrelation functions (top) and the corresponding size distributions from regularization analysis (bottom) of the Spikevax (red) and Comirnaty (blue) bivalent LNP-mRNA COVID-19 vaccines.

Table 1. Batch DLS results for the Spikevax and Comirnaty bivalent LNP-mRNA COVID-19 vaccines.

			Comirnaty	Spikevax
Cumulants		R_h (nm)	38.4 ± 1.1	75.4 ± 1.2
		DLS PDI	0.26 ± 0.02	0.24 ± 0.02
Regularization	**Main species**	R_h (nm)	25.0 ± 3.8	38.9 ± 16.7
		DLS PDI	0.37 ± 0.14	0.37 ± 0.17
		% Mass	95 ± 12 %	81 ± 8 %
	Large species	R_h (nm)	62 ± 32	183 ± 91
		DLS PDI	0.174 ± 0.004	0.27 ± 0.14

All values are average ± standard deviation of 3-6 replicate measurements.

High-resolution MAQ by FFF-MALS

Physical separation via FFF coupled with multiple detectors is required to reveal the high-resolution size and mass distributions of these samples. Specifically, the FFF method physically separates the mRNA-LNPs in both samples by size, and the online UV, MALS, and dRI detectors enable simultaneous quantitation of size distribution, molar mass distribution, and the payload distribution across the particles.

Detailed size distribution

FFF-MALS reveals significant differences in the size distributions of the two samples that are not evident by batch DLS alone. Figure 2 shows the FFF fractograms for each sample, overlaid with the geometric radius measured by MALS. As expected, both samples are polydisperse, with particle size spanning 20 nm to almost 150 nm geometric radius, in qualitative agreement with the batch DLS screening results.

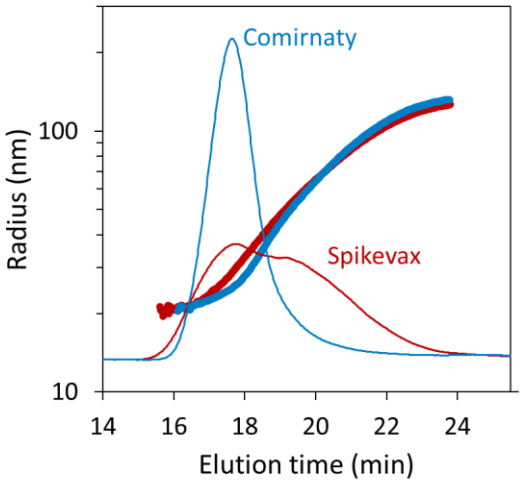


Figure 2. Size distributions for the Comirnaty (blue) and Spikevax (red) samples measured by FFF-MALS, overlaid on the dRI fractograms.

FFF-UV-MALS-dRI enables more in-depth quantitation of the size distribution within a single polydisperse peak. For example, inspecting the differential weight fraction as a function of size (Figure 3) reveals more significant differences among the samples, that would not otherwise be resolved by batch measurements. Both samples contain a species with mean radius around 25 nm and significant polydispersity. Overall, however, the Spikevax sample contains a greater weight fraction of large particles than the Comirnaty sample. Specifically, 50% (w/w) of the Spikevax particles have radii > 45 nm, while the corresponding number for the Comirnaty sample is only 12% (w/w).

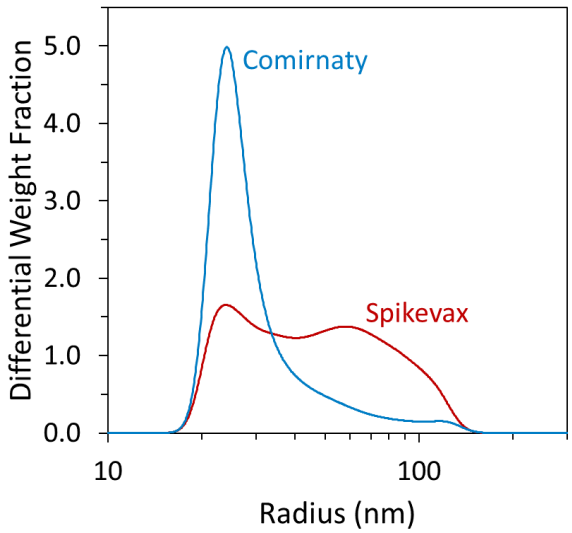


Figure 3. The differential weight fraction from FFF-MALS enables high-precision analysis of the LNP-mRNA size distribution.

Molar mass and composition

The LNP-mRNA molar mass ranges from ~10 MDa to 1 GDa, with a weight-average molar mass (M_w) of 95.4 ± 2.3 MDa for Comirnaty and 269.8 ± 5.1 MDa for Spikevax, reflecting the greater weight fraction of large particles in the latter sample. The experiment also provides the molar mass distributions of the lipid and mRNA components (Figure 4). In contrast to batch DLS measurements, FFF-MALS also measures the true dispersity ($Đ$, formerly called polydispersity index), defined as the ratio of the weight-average and number-average molar mass (M_w/M_n). The Spikevax sample has 2-fold higher dispersity (M_w/M_n = 5.01 ± 0.11) compared to the Comirnaty sample (M_w/M_n = 2.58 ± 0.08). Cumulants analysis of batch DLS data, in contrast, fails to capture this pronounced difference between the two samples (Table 1).

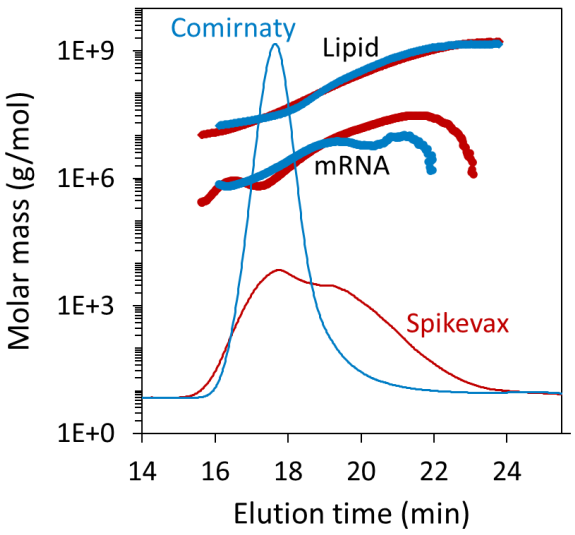


Figure 4. Measured mRNA and lipid molar masses from FFF-MALS, overlaid on the dRI fractograms.

Integrating the dRI and UV signals across the fractogram gives a total mRNA and lipid concentration for each product. We measure 0.106 ± 0.002 mg/mL mRNA for Comirnaty and 0.086 ± 0.001 mg/mL mRNA for Spikevax, both in excellent agreement with the manufacturers' specifications of 0.1 mg/mL[11, 12]. These concentrations correspond to mRNA drug substance doses of 31.8 ± 0.5 μg/dose for Comirnaty and 43.1 ± 0.8 μg/dose for Spikevax. The results are in good agreement with the manufacturers' product information (30 μg/dose for Comirnaty and 50 μg/dose for Spikevax, respectively[11, 12]). Simultaneously, we quantify a total lipid concentration of 2.06 ± 0.02 mg/mL for Comirnaty and 1.97 ± 0.01 mg/mL for Spikevax, in good agreement with the manufacturers' specifications (2.5 mg/mL and 2.0 mg/mL for Comirnaty and Spikevax, respectively) [11, 12]. The slight difference may be attributed to nonoptimized extinction coefficients or *dn/dc* values used for the analysis[13]. When available, samples of the free mRNA drug substance may be used to experimentally determine these constants with the analyses built into the ASTRA software, further improving accuracy.

Size-based payload distribution

FFF-MALS also enables high-resolution mRNA payload analysis for the intact LNP-mRNA drug product. Traditional measurements can only approximate how the mRNA is distributed as a function of LNP size by fractionating the sample, collecting fractions of different sizes, breaking the LNPs, and quantifying the amount of lipid and mRNA separately for each fraction. In addition,

techniques such as nanoparticle tracking analysis cannot measure the mRNA payload distribution across the particles, and reductionist analysts may be tempted to assume that the payload simply is evenly distributed across the particles. FFF-MALS, in contrast, can measure the true payload distribution in the sample.

On average, the mRNA drug substance accounts for (4.9 ± 0.1) % w/w of the Comirnaty sample and (4.2 ± 0.1) % w/w of the Spikevax sample, in agreement with the 4-5% (w/w) specified by the manufacturers. FFF-MALS reveals, however, that the mRNA-to-lipid ratio is not constant across the LNP size distribution. Although the weight fraction of mRNA never exceeds 10% of the total particle mass, certain LNP sizes appear to contain a higher weight fraction of mRNA than others for each sample. Specifically, particles with <40 nm radius in the Comirnaty sample have a higher mRNA weight fraction than the corresponding particles in the Spikevax sample, while the larger particles in the Spikevax sample have a higher mRNA weight fraction than in Comirnaty (Figure 5).

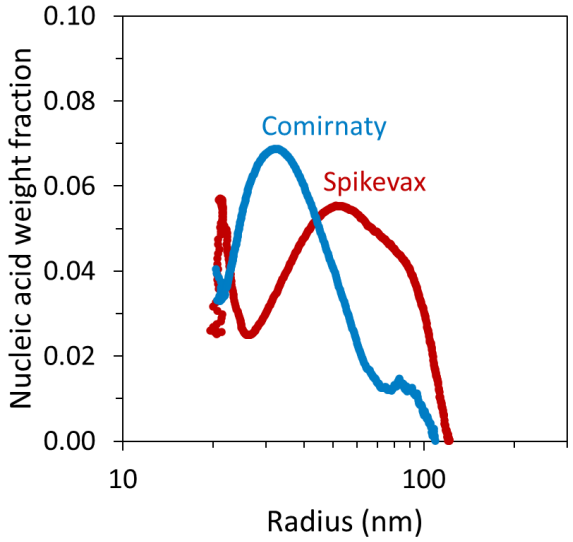


Figure 5. FFF-MALS can be used to measure the mRNA weight fraction as a function of LNP size.

By comparing the measured mRNA weight-average molar mass with the molar mass of the individual mRNA molecules in each vaccine[7, 8], the ASTRA software can calculate the average number of mRNA per LNP as a function of LNP size (Figure 6). We find that particles with ~20 nm radius contain a similar mRNA number in both vaccines, while the ~25-50 nm Comirnaty particles have a more mRNA molecules per LNP than the corresponding Spikevax particles. The results also suggest that the particles with >50 nm radius contain a significantly higher nucleic acid number in the Spikevax sample. This difference is also reflected in the average nucleic acid number across the entire fractogram peak of 1.79 ± 0.34 for the Comirnaty sample, and 7.50 ± 0.66 for the Spikevax sample.

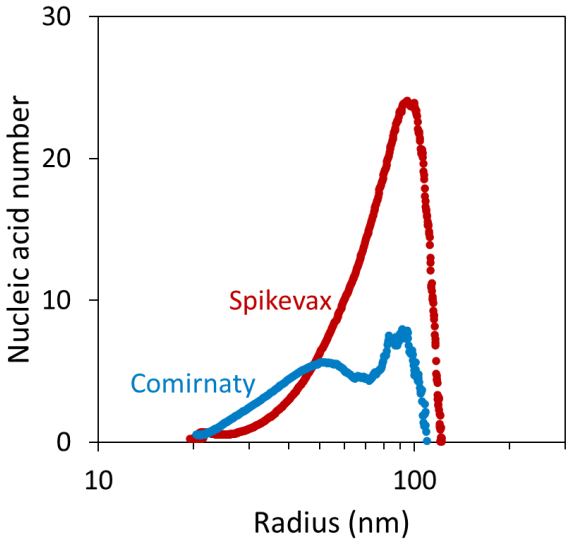


Figure 6. Average number of mRNA per LNP as a function of LNP size.

Although the Spikevax samples appear to have more mRNA per LNP, it is important to note that the largest particles with high nucleic acid number only constitute a small fraction of the total population. Using the combined FFF-UV-MALS-dRI data, we can quantify the particle concentration as a function of size and compare that with the mRNA number (Figure 7). Doing so, we see that the very large particles present in both samples account for < 1 % of the total number of particles in the sample. Understanding these distributions may help scientists identify and enrich LNP-mRNA with a desired size and payload content for optimal delivery or therapeutic effect.

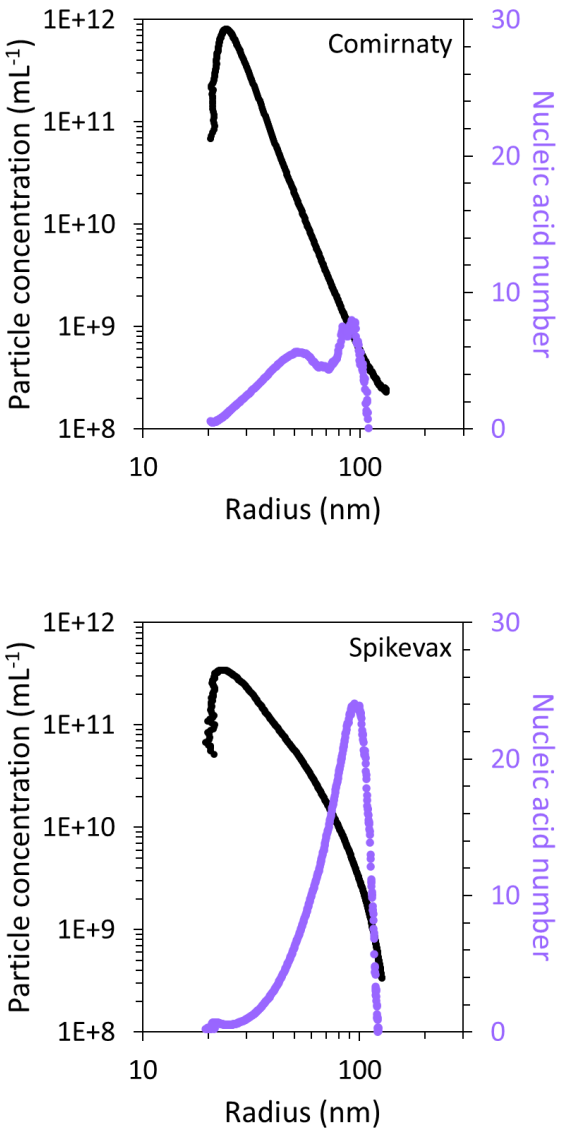


Figure 7. FFF-MALS can quantify the number and concentration of particles with a specific size and mRNA content in the Comirnaty (top) and Spikevax (bottom) samples.

Conclusions

Here, we have demonstrated that FFF-MALS can deliver multi-attribute quantification of LNP-mRNA therapeutics, using two vaccine products as examples. The method is 21 CFR Part 11 compliant, automatable, does not rely on calibration curves for accurate quantification, and has been validated against conventional offline measurements[13]. FFF-MALS goes beyond the capabilities of laborious or low-resolution methods like RiboGreen assays and particle tracking analysis. For example, the method not only quantifies the average quality attributes of an LNP-mRNA sample but also provides high-resolution insights into its lipid and mRNA subcomponents and how these are distributed across the particles within the sample, all in a single experiment. For the Comirnaty and Spikevax samples characterized here, we see clear differences in the high-resolution particle size and concentration distributions, and in how the mRNA drug substance is distributed across the LNPs. Such data are key to understanding structure activity relationships and may be leveraged to rationally optimize formulation, dosing, and in vivo efficacy of LNP-mRNA therapeutics.

Acknowledgements

The empty lipid nanoparticle samples were kindly provided by Dr. Jérémie Parot at SINTEF.

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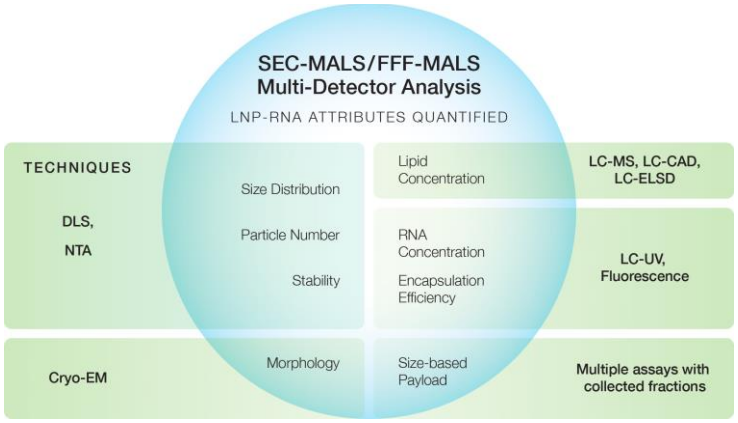

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