WHITE PAPER

WP2612: Meeting regulatory needs in the characterization of lipid nanoparticles (LNPs) for RNA delivery via FFF-MALS

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Abstract

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, no stationary phase is required during the fractionation process, and the optimal separation range can be fine-tuned to measurement parameters. This flexibility enables reliable measurement of complex pharmaceutical products such as nanomedicines, monoclonal antibodies and vaccines. However, for quality control purposes the FFF-MALS protocol must satisfy regulatory needs and accepted standards. Here, we describe a validated FFF-MALS method in line with technical specification ISO/TS 21362 for the analysis of lipid-based nanoparticles (LNPs) encapsulating siRNA and mRNA.

Analytical characterization of LNPs for drug delivery

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 technology encapsulated in LNPs, approved by regulatory authorities in USA and Europe: 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, 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, 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. As illustrated in Fig. 1, the unseparated sample is injected into the channel. In conventional channels, the sample is relaxed close to the membrane at approx. 10 % of the channel length in a 'focusing' step. Optionally, the 'dispersion inlet' (also known as 'frit inlet') channel uses the "hydrodynamic relaxation" principle wherein no focusing step is required, which can diminish unwanted effects due to 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 further distanced from the bottom membrane and hence exposed to a higher flow rate. Thus, small particles elute first followed by larger species, inversely to size-exclusion chromatography.

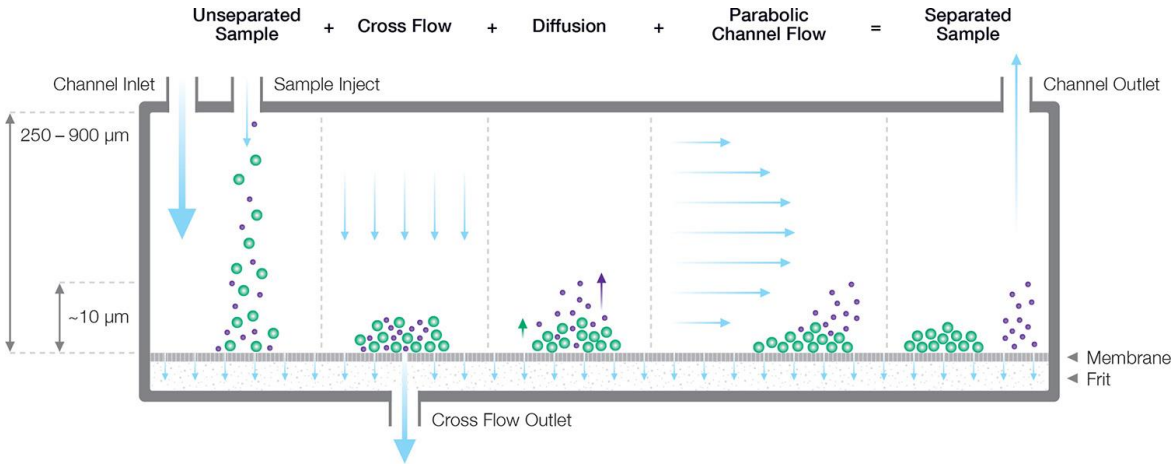


Figure 1. The FFF principle resolves species with different size without a stationary phase.

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

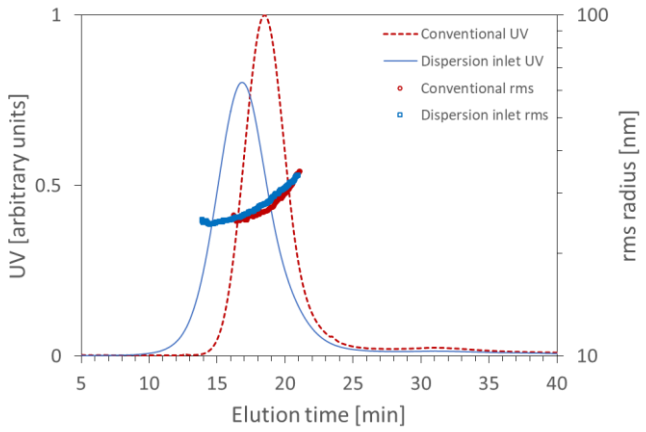
1. Recovery of the analyte ≥ 70 % (performance separations, such as particle concentration, would require recovery ≥ 90 %)
2. Relative standard uncertainty ≤ 5 % for retention time, recovery and particle size values

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.

High recovery as key measurement quality indicator

Sample recovery (*R*%) is the key parameter to consider for initial method optimization. It is determined by integrating the main UV peak and comparing results with cross flow and the focusing step, and without those. During method development, the conventional setup with a standard FFF long channel was compared to a similar method using the dispersion inlet channel, where the sample is injected directly into the carrier stream. In the latter configuration, no focusing is required, reducing the risk of sample destabilization and increasing recovery for

delicate samples such as LNPs. Fig. 2 shows the results obtained by measuring the same siRNA-LNP formulation using the two channels. In both channels the sample is eluted and fractionated according to the particle size. Although in the tested configurations 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.



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

Figure 2. Comparison of separation and recovery of an siRNA formulation using the conventional and dispersion inlet channels. Top: UV fractograms of conventional long channel and dispersion channel overlaid with rms radius. The main species has a radius of 26 - 27 nm. Bottom: recovery (*R*%), size (R_g) and spread averaged over three measurements, where spread = ($R_{g,\,max} - R_{g,\,min}$) calculated across the full width @ half maximum of the peak.

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 RNA content [N/P = 8] and high RNA content [N/P = 3]. 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, wherein mRNA is complexed by the ionizable lipids and the other particle components, cannot be formed.

Differences in particle size and polydispersity are associated with differences in particle payload and therefore morphology (shape, core density), another important parameter that can be evaluated by FFF-MALS via the shape factor ρ. The shape factor is defined as $R_g : R_h$, the ratio of rms radius to hydrodynamic radius. MALS with embedded DLS detection simultaneous determines both R_g and R_h. For a hollow sphere, ρ is unity. For a sphere with a dense core, however, the scattering centers are distributed more closely to the center of mass, and ρ is expected to be 0.77 or less.

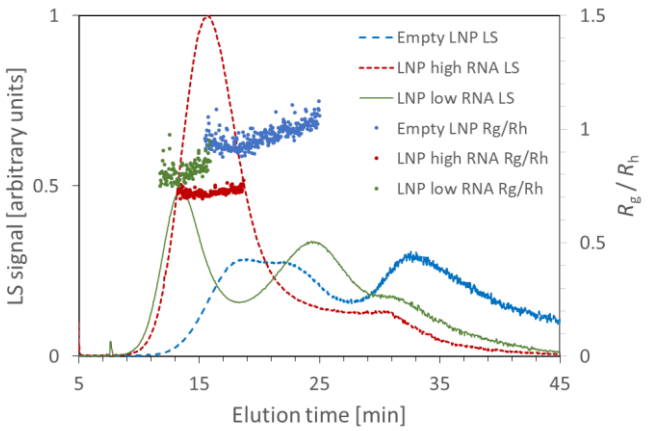
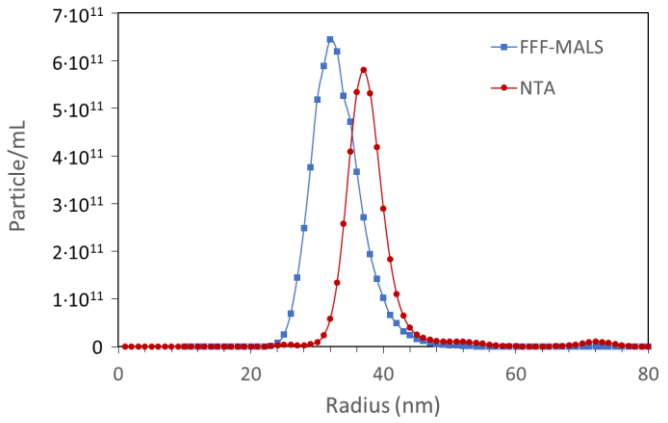


Figure 3. Comparison of mRNA-LNP samples with different mRNA content and empty particles. The LS fractograms at 90° MALS angle are shown overlaid with the ratio of rms radius and hydrodynamic radius, ρ. The radio tracks the degree of encapsulated RNA.

Fig. 3 shows ρ vs. retention time for mRNA-LNP samples with different RNA content, and for the empty LNP control sample. Interestingly, the empty particles possess $\rho \sim 1$, typical of a hollow or irregular sphere, while increasing mRNA content reduces the ratio. The value of ρ for the relatively monodisperse, high-mRNA-content particles matches that associated with a dense spherical particle. Note that the empty LNPs and those loaded with less mRNA show a larger proportion of aggregates and are less stable, further demonstrating the correlation

between particle morphology, monodisperse size distribution and the physical stability of the LNP formulations.

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 may be used to derive the number-based particle size distribution (PSD). As a proof of concept, the PSD obtained by FFF-MALS was compared with that measured by nanoparticle tracking analysis (NTA), showing remarkably comparable results even though the two techniques quantify different measures of size (Fig. 4). Notably, FFF-MALS detects and quantifies LNP particles below 30 nm in radius that are too small for detection by NTA.

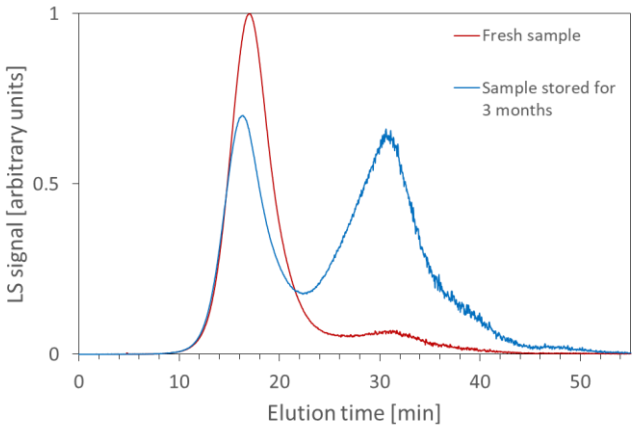


Technique	Total particle/mL (SD)	Mode Radius (SD)
NTA	(3.9 ± 0.3) x 10^{12}	36 ± 25
FFF-MALS	(6.77 ± 0.02) x 10^{12}	33 ± 2

Figure 4. Particle size distribution by number (top) and total particle concentration values (bottom) obtained by FFF-MALS and NTA, for a formulation of LNPs encapsulating siRNA. NTA determines hydrodynamic radius R_h while MALS determines a mass-weighted effective spherical radius.

LNP stability assessment

A critical parameter for RNA-LNPs is their stability. Due to the capability to determine size, morphology and concentration with high resolution, FFF-MALS is one of the methods of choice for evaluating physical stability of the particles and their tendency to aggregate. Here, a freshly prepared LNP sample loaded with siRNA was compared to a similar 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 (Fig. 5). The change in the particle size distribution accompanied by 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 one. 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 over time of LNP formulations.



	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 x 10^{12}	2.24 x 10^{9}
Stored Sample	24.7	5.85 x 10^{12}	2.01 x 10^{10}

Figure 5. Aggregate content in a freshly prepared sample (red) and one which was stored for three months (blue). Top: the light scattering fractograms at 90° scattering angle. Peak 1 is centered around 17 minutes, Peak 2 around 31 minutes. The relative size of the aggregate peak (Peak 2) is significantly larger for the stored sample. Bottom: total particle concentrations, where Peak 2 contains 10 times more particles for the stored sample than for the fresh sample.

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.

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, autosampler and UV detector of the 1260 Infinity II series (Agilent Technologies). Fractionation was performed in the Eclipse conventional long channel and in the dispersion-inlet channel. UV extinction was measured at 260 nm, and sample recovery was calculated by integrating the main UV peak area for each sample, with and without the applied cross flow and focusing step, and taking the ratio of the areas.

Detection was accomplished by a DAWN® MALS instrument with embedded WyattQELS™ 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).

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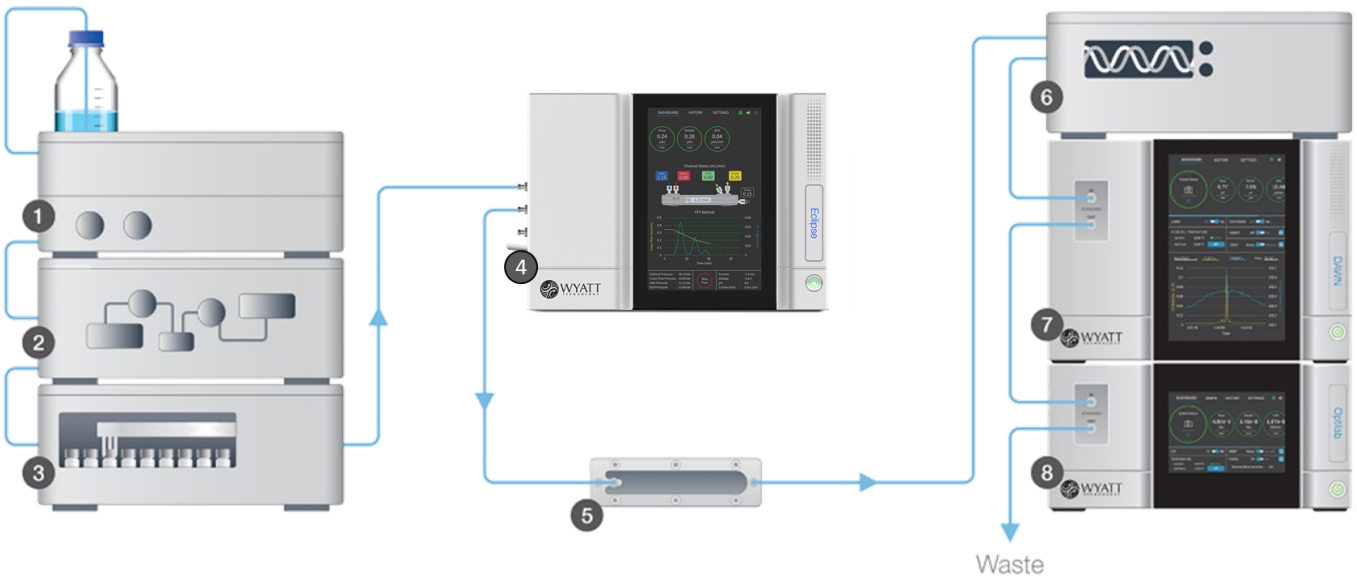

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4. Mildner, R. et al. Improved multidetector-asymmetrical flow field flow fractionation method for particle sizing and concentration measurements of lipid-based nanocarriers for RNA delivery. *Eur. J. Pharm. Biopharm.* **163**, 252-265 (2021).