mRNA/LNP Multiattribute Quantitation of Payload(s), Size and Heterogeneity With Size Exclusion Chromatography Coupled to Multiangle Light Scattering

Mateusz Imiołek[a], *Lavelay Kizekai*[b], *Bala Addepalli*[b], *Szabolcs Fekete*[a], *Matthew Lauber*[b]

a - Waters Corporation, Rue Michel Servet 1 Geneva, 1211, Switzerland

b - Waters Corporation, 34 Maple St. Milford, MA 01757, USA

INTRODUCTION

Size exclusion chromatography (SEC) is a separation technique that differentiates analytes according to their size and/or shape in solution.

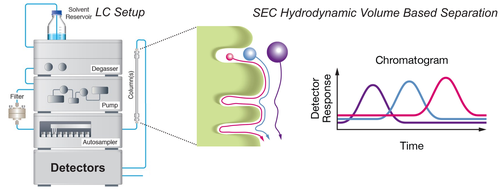


Figure 1. Schematics of LC setup for SEC-UV-MALS experiments and hydrodynamic radius separation principle of size exclusion chromatography.

Recent developments in column technology have enabled this type of analysis for lipid nanoparticles (LNPs) and their associated analytes like mRNAs. SEC coupled to multi angle light scattering (MALS) provides a straightforward way to characterize multiple critical quality attributes (size, MW, polydispersity) [1] and the potential of a modern widepore column for this type of analysis is presented.

While the payload quantification by the non-destructive SEC-MALS-UV-dRI method provides size-based payload analysis [2], its use is limited for rapid analyses. The method i) needs empty LNP reference to account for the light scattering contribution in the UV signals; ii) cannot differentiate between more than one payload. Similarly, techniques that allow payload separation require laborious nucleic acid extraction procedures and lack robustness (RPLC), while others may not be yet validated and suffer from low throughput (CGE) [3].

Here, SEC with strongly denaturing mobile phase is demonstrated to be an alternative for a higher throughput and more straightforward quantification of multiple payloads.

METHODS

System and samples

The LNP samples (Commercial COVID-19 vaccines: Spikevax™ COVID-19 Vaccine, mRNA, NDC 80777-279-99, monovalent, 0.1 mg/mL, Comirnaty™ COVID-19 Vaccine, mRNA, NDC 59267-1055-4, 0.1 mg/mL and in house produced LNPs loaded with 1:1 FLuc mRNA and gRNA, Acuitas Therapeutics, LNP7, LNP9, LNP13, 1 mg/mL) were transferred to low adsorption vials (186009186, Waters), and diluted where appropriate with detergent or water.

SEC-UV experiments were performed with an ACQUITY™ UPLC™ H-Class Bio QSM System equipped with the TUV detector (Titanium 5 mm flow cell, Waters). The SEC-MALS study was done with Arc™ Premier System with ACQUITY 2489 UV Detector, Wyatt Optilab™ dRI Detector and Wyatt DAWN™ Instrument with a WyattQELS™ Embedded Online Dynamic Light Scattering (DLS) Module.

SEC Columns and conditions

The SEC study for intact LNPs was performed with PEO bonded GTxResolve™ Premier SEC 1000 Å 3 µm Columns, while the denaturing SEC study used the diol bonded GTxResolve Premier BEH™ SEC 450 Å 2.5 µm Columns both in 4.6 x 150 mm format (Waters, 186010584 and 186010735, respectively). SEC-MALS was done with a larger, 7.8 x 300 mm column.

Optimized, universally denaturing SEC conditions use 1X PBS, 20% IPA and 0.2% SDS as the mobile phase flowed at 0.25 mL/min in a 10 min method or 0.5 mL/min in a 5 min method at 40 °C and 260 nm detection with 2 Hz data acquisition rate.

DISCUSSION

The advent of inert widepore SEC packing materials holds great potential for analysis of both intact and disrupted LNPs. Although certain formulations may still require method development, such native fractionations permit detailed MALS characterization of small/medium sized LNP populations.

On the other hand, using denaturing agents and a smaller pore size column achieves straightforward deformulation of LNPs, good separation and reliable quantification of multiple payloads, especially those containing guide RNA together with relevant mRNA.

References

[1] - D'Atri, V., Imiolek, M., (...), Lauber, M., Fekete, S., & Guillarme, D. (**2024**). Size exclusion chromatography of biopharmaceutical products: from current practices for proteins to emerging trends for viral vectors, nucleic acids and lipid nanoparticles. *J. Chromatogr. A*, 1722, 464862.

[2] - Jia, X., (...), Pennington, J. (**2021**). Enabling online determination of the size-dependent RNA content of lipid nanoparticle-based RNA formulations. *J. Chromatogr. B*, 1186, 123015.

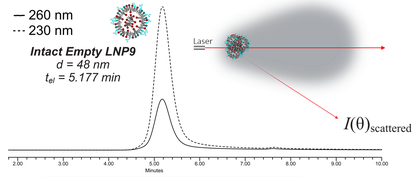
[3] - Lokras, A., (...), Foged, C. (**2022**). Simultaneous quantification of multiple RNA cargos co-loaded into nanoparticle-based delivery systems. *Int. J. Pharm.*, 626, 122171, 626, 122171.

EXPLORING THE POTENTIAL OF INTACT mRNA/LNPs 1000 Å SEC-MALS

We investigated novel non-ionic, hydrophilic widepore packing materials for SEC, which allows fractionation of some intact LNPs with good recoveries without complicated method development. However, certain formulations require standard method development so that residual hydrophobic interactions are mitigated.

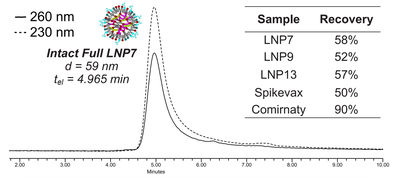
LNP dependent method development

Standard LNP: ***non-optimized conditions*** *1X PBS,* 25 °C
— recovery 52%



UV signal is observed due to the light scattering

Challenging LNP: ***semi-optimized conditions*** *0.1X PBS, 10% IPA,* 40 °C
— recovery 47%



Sample	Recovery
LNP7	58%
LNP9	52%
LNP13	57%
Spikevax	50%
Comirnaty	90%

UV signal is observed due to both absorption and light scattering

LNP7 can be analyzed under different conditions with minimal integrity loss

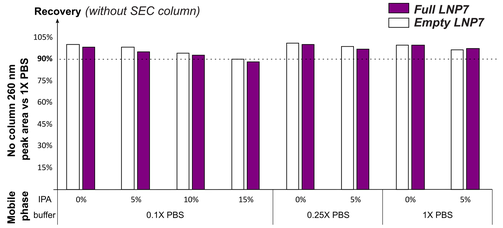


Figure 2. Investigation of the role of buffer strength (X dilution of PBS) and organic solvent additive (IPA—isopropanol) on 260 nm signal of LNP7 injected without SEC column at 0.25 mL/min and 40 °C.

Loss of peak area is caused by LNP deformulation/nucleic acid precipitation, which was minimal under most tested conditions (<10%).

LNP7 recovery may depend on buffer and organic solvent additive

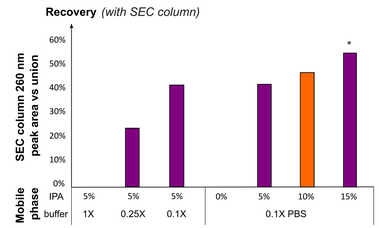
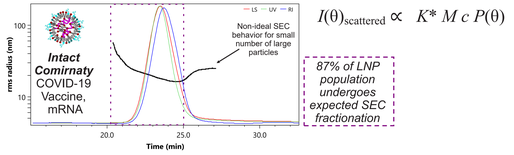


Figure 3. Investigation of LNP7 recovery as a function of buffer strength and organic solvent additive via 260 nm signal of the sample injected on a SEC column.

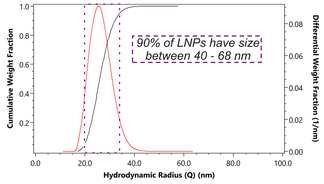
** The integrity of LNP was controlled via A_{260}/A_{230} ratio, which suggests this LNP becomes destabilized in the presence of 15% IPA.*

MALS provides information on size and polydispersity



$$I(\theta)_{scattered} \propto K^* M c P(\theta)$$

87% of LNP population undergoes expected SEC fractionation



Figures 4&5. SEC-MALS chromatogram with 90° light scattering (LS), 260 nm UV and refractive index (RI) traces showing fractionation of intact Comirnaty COVID-19 Vaccine, mRNA sample with determined average radius of gyration at each point. Data analysis software (ASTRA™ Software) allows results fitting and distribution analysis of DLS data (right), which revealed that majority of LNPs have hydrodynamic radius in a narrow range.

SEC fractionation used 1000 Å, 7.8 x 300 mm column with 95% 2X PBS, 0.001% pluronic-F68, 5% IPA, pH 7.4 mobile phase flowed at 0.3 mL/min.

CONCLUSIONS

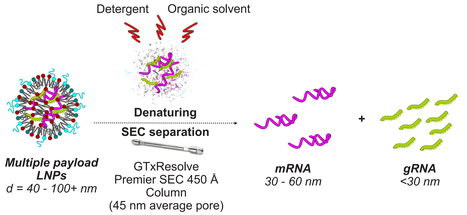
New generation of GTxResolve SEC Columns allow detailed characterization of LNPs

- 1000 Å column is well suited for fractionation of small to medium sized intact LNPs with MALS detection
- 450 Å column can be used for denaturing separations and quantification of the payloads

DENATURING SEC 450 Å PAYLOAD QUANTIFICATION

Accurate quantification of multiple payloads requires complete LNP disruption and efficient separation of its contents. Evaluation of offline sample denaturation protocols revealed incomplete release of payloads for more stable formulations. However, we found that SEC offers the possibility for online disruption of intact LNPs using denaturing mobile phase without any prior sample preparation.

Idea of denaturing SEC separations



Establishing universal denaturing conditions

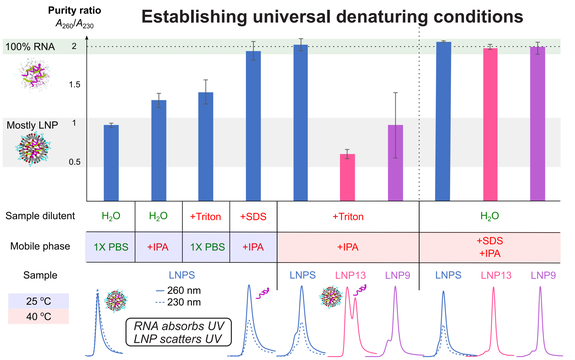


Figure 6. Schematics of denaturing SEC experiment that upon the use of disruptive agents (surfactants and non-aqueous solvents) achieves online deformulation of the LNP, releasing and separating of the nucleic acid payloads, which can be quantified via UV detection at 260 nm. The denaturation conditions were optimized for model LNP (right)- LNPS = Spikewax COVID-19 Vaccine, mRNA based on the fact that RNA has a defined absorbance ratio at specific wavelengths A_{260} /A_{230} nm ~ 2, which can be used to verify to completeness of denaturation (ie absence of LNP scattering component).

Sample dilution with detergent or use of organic solvent additive alone in mobile phase provided only partial disruption for other formulations. Inclusion of both denaturing agents in the mobile phase allowed full deformulation of all tested LNPs.

! *denaturation needs both organic solvent & detergent*

⊘ *non-universal conditions*

✓ *complete deformulation of all tested LNPs*

Comparison of native and denaturing SEC

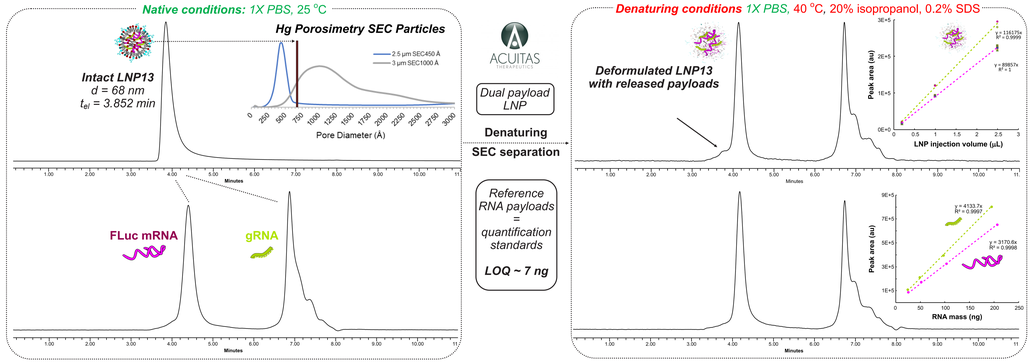


Figure 7. Standard SEC separations allow fractionation of LNPs and separation of reference RNAs. Note the pore size distribution, which suggests that intact LNPs are largely excluded from SEC 450Å column pores. However, when run in denaturing conditions the LNP is deformulated and releases the payloads, while the reference nucleic acid separation does not change significantly.

High throughput and specific quantification of payloads

Sample	Ratio mRNA / gRNA	gRNA [mg/mL]	Fluc mRNA [mg/mL]	Total [mg/mL]	Expected [mg/mL]
LNP9	0.987 ± 0.065	0.544 ± 0.033	0.537 ± 0.014	1.082 ± 0.036	1.08
LNP13	1.016 ± 0.108	0.526 ± 0.049	0.535 ± 0.028	1.061 ± 0.056	1.07

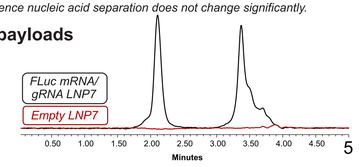


Figure 8. The assay could be further shortened to a total analysis time of 5 mins while maintaining excellent payload separation. The conditions were tested for several LNP formulations and led to reproducible results (±<5%) and empty LNPs were found not to influence the results.

We would like to thank ***Acuitas Therapeutics*** *for providing dual payload LNPs and reference materials.*

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