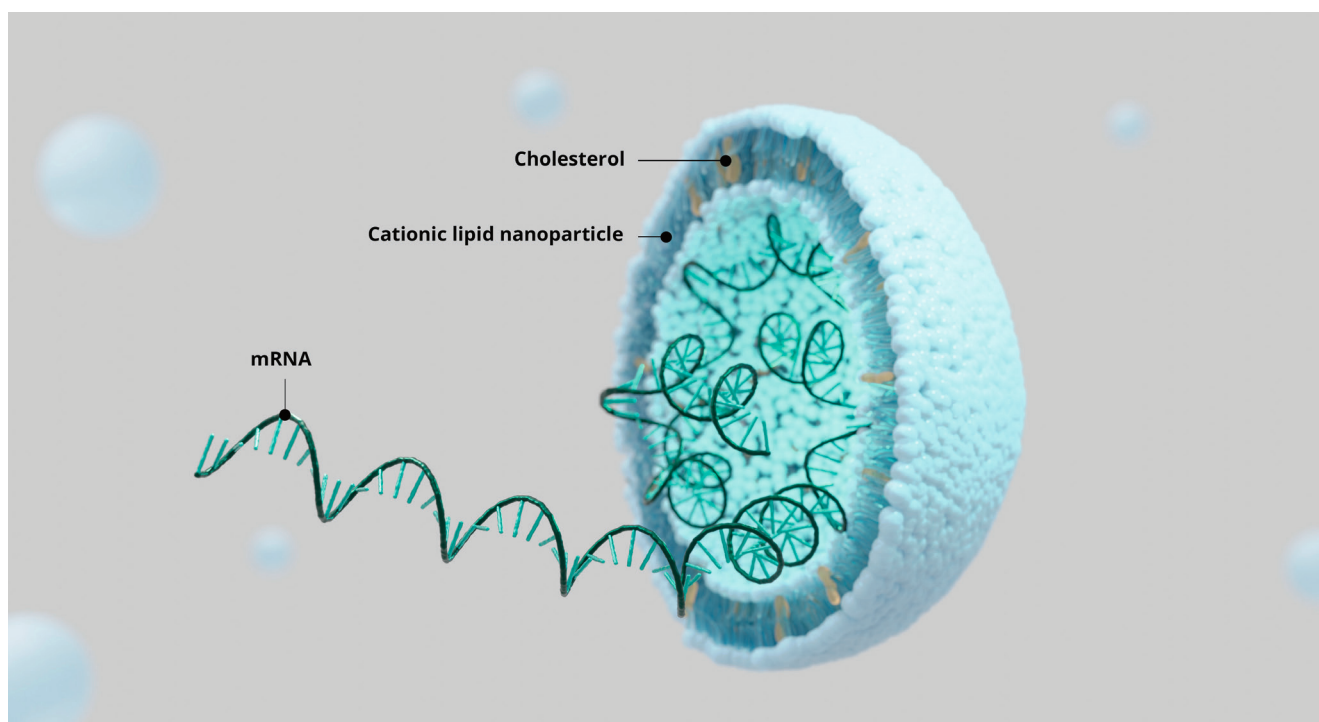


An alternative approach for determining the encapsulation efficiency of mRNA in LNPs via AEX

To enhance intracellular delivery and preserve mRNA integrity against RNase-mediated degradation, therapeutic mRNA can be encapsulated in lipid nanoparticles (LNPs). These LNPs transport the payload directly into the cytoplasm, enabling efficient translation. Particle size, composition, and reproducibility are now tightly controlled. However, defining the critical quality attributes of mRNA and LNPs remains challenging. The encapsulation efficiency (EE) of the mRNA in the LNP is one key element of the quality control. The encapsulation efficiency defines the proportion of mRNA that will eventually be delivered into the cells and is therefore directly linked to the therapeutic potential of the drug product. Current workflows often rely

on spectrofluorimetric detection using fluorescent dyes such as RiboGreen (Thermo Fisher Scientific), which bind to mRNA. However, this method has several drawbacks such as it is prone to matrix effects, the binding of the dye to mRNA is highly sensitive to environmental factors or the structure of the LNP can be affected by dilution. While it works well at low mRNA concentrations it becomes more inaccurate at high concentrations because of optical saturation effects. As LNPs exhibit neutral charge, anion exchange chromatography (AEX) offers a targeted strategy to separate free mRNA from unretained drug product. Moreover, AEX provides native conditions, which suit the physicochemical fragility of LNPs.



This Technical Note based on the publication and data of the University of Geneva and Sanofi's mRNA Center of Excellence in France demonstrates that the AEX method provides a refined and more robust alternative to commonly

applied analytical workflows [1]. During the evaluation of Tsalmpouris et al., specific technical limitations emerged and were partly addressed through methodical adjustments.

Table 1: Chromatographic conditions [1].

Columns:	YMC Accura BioPro IEX QF (3µm) 100 x 4.6 mm ID (bioinert coated SUS)
Part Nos.:	BioPro IEX QF (3µm) 100 x 4.6 mm ID (PEEK) QF00S03-1046PTC QF00S03-1046WP
Eluents:	A) 25 mM glycine (pH 10.1) B) 25 mM glycine + 1.5 M NaCl (pH 10.1) C) 25 mM glycine + 3 M NaCl + 0.05 % Triton X-100 reduced (pH 11.0)
Gradient:	50 %B (0–2 min), 50–53 %B (2–2.25 min), 53 %B (2.25–4 min), 53–100 %B (4–6 min), 100 %B (6–8 min) Washing step: 100 %C (8.01–12 min) Re-equilibration: 50 %B (12.01–22 min)
Flow rate:	0.2 mL/min
Temperature:	25°C
Injection:	2 µL
Detection:	UV at 230, 260 nm
Sample:	mRNA (in-house) LNP (in-house) Disrupted LNP with 4 % reduced Triton X-100 (T4TE20x)

The principle of EE determination

To determine the EE the sample has to be analysed twice. One time undiluted and under native conditions to determine the concentration of free mRNA. And for the second determination the sample has to be disrupted with a

surfactant to evaluate the total mRNA concentration. The EE can be calculated with the following equation, considering the dilution factor of 10 of the second analysis. The peak area can be representatively used for the concentration.

$$EE = 1 - \frac{\text{free mRNA}}{\text{total mRNA}} = 1 - \frac{\text{area undiluted sample}}{\text{area disrupted sample} \times 10}$$

Optimum column hardware

Tests of bioinert coated column hardware compared with PEEK column hardware show that using PEEK hardware leads to non-specific adsorption negatively affecting the EE

to smaller values. Using the bioinert coated YMC Accura BioPro IEX QF column with the same stationary phase provides high sensitivity with stable peak areas.

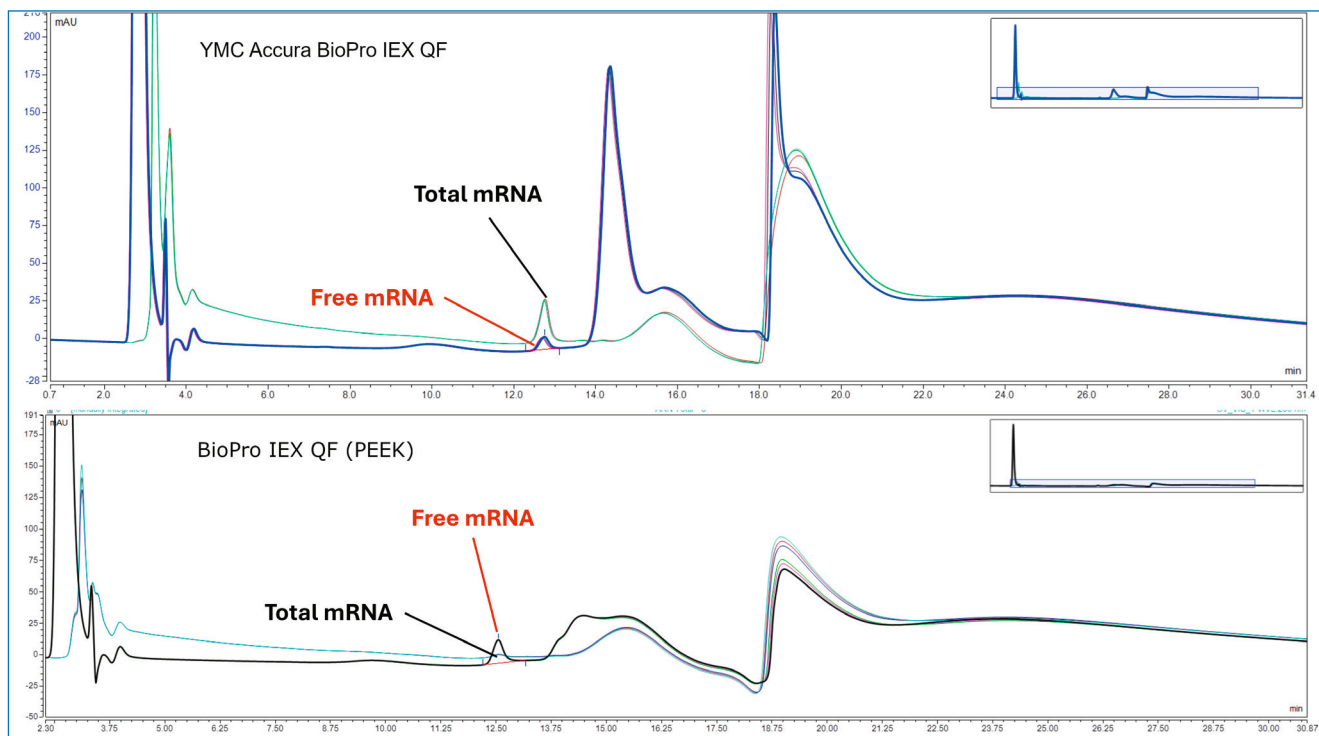


Figure 1: Analysis of the intact drug product and the disrupted LNP using bioinert coated YMC Accura column hardware and regular PEEK column hardware.*

*Courtesy of University of Geneva, Institute of Pharmaceutical Sciences of Western Switzerland (ISPSO)

Carry-over studies

To eliminate carry-over observed during injection of the drug substance (DS), the workflow requires an optimised washing step with validated effectiveness. Therefore, washing cycles of 0.5-5 min as well as a high salt concentration of 3 or 5 M NaCl and high pH (10.5, 11.0) were tested. Carry-

over was decreased with larger washing cycles, but a high pH or salt concentration alone was not sufficient. Only the combination of both reduced the carry-over below 0.1% in just 0.5 min. The ideal washing solution contains 3 M NaCl at pH 11 (see Figure 2).

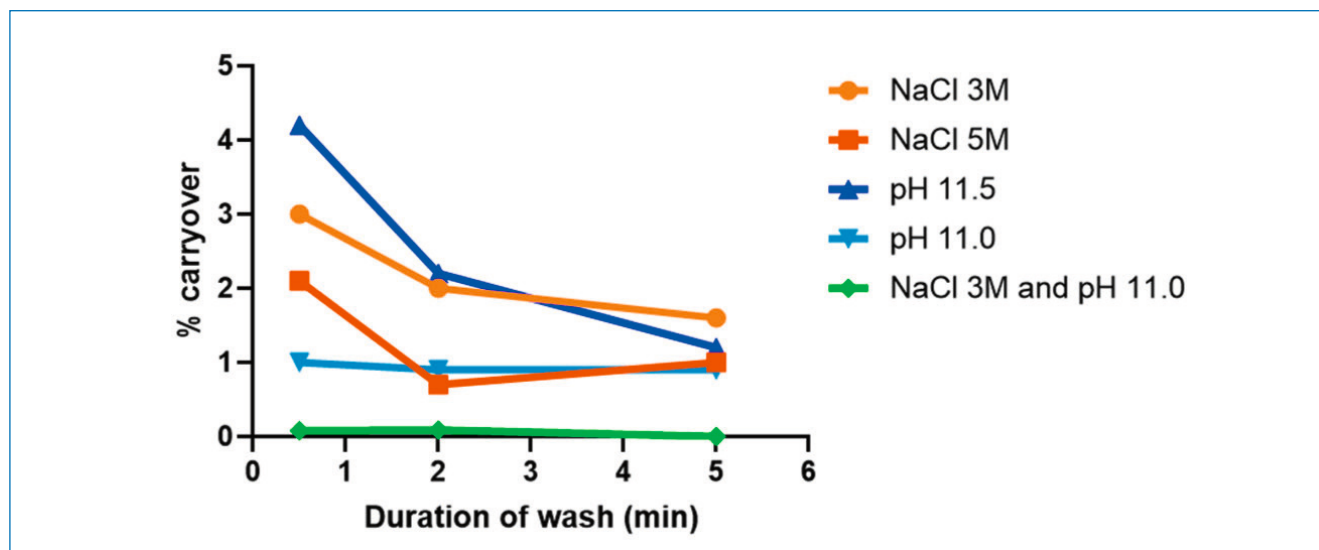


Figure 2: Carry-over (%) obtained in the Triton X-100 blank following injection of 1 mg/mL DS, under different conditions of wash composition and duration [1].

Carry-over effects caused by adsorbed LNPs

LNPs can be adsorbed onto different surfaces in the system. When injecting the disrupted sample containing surfactant, the adsorbed LNPs can be disrupted in turn. This effect distorts the results by reducing the calculated EE, as the total mRNA concentration appears artificially

elevated. A blank injection containing surfactant (T4TE20x) demonstrates (Figure 3) that a significant amount of mRNA is released. Therefore, an optimised wash with 0.05% Triton X-100 reduced was implemented (4 min) to disrupt all adsorbed LNPs.

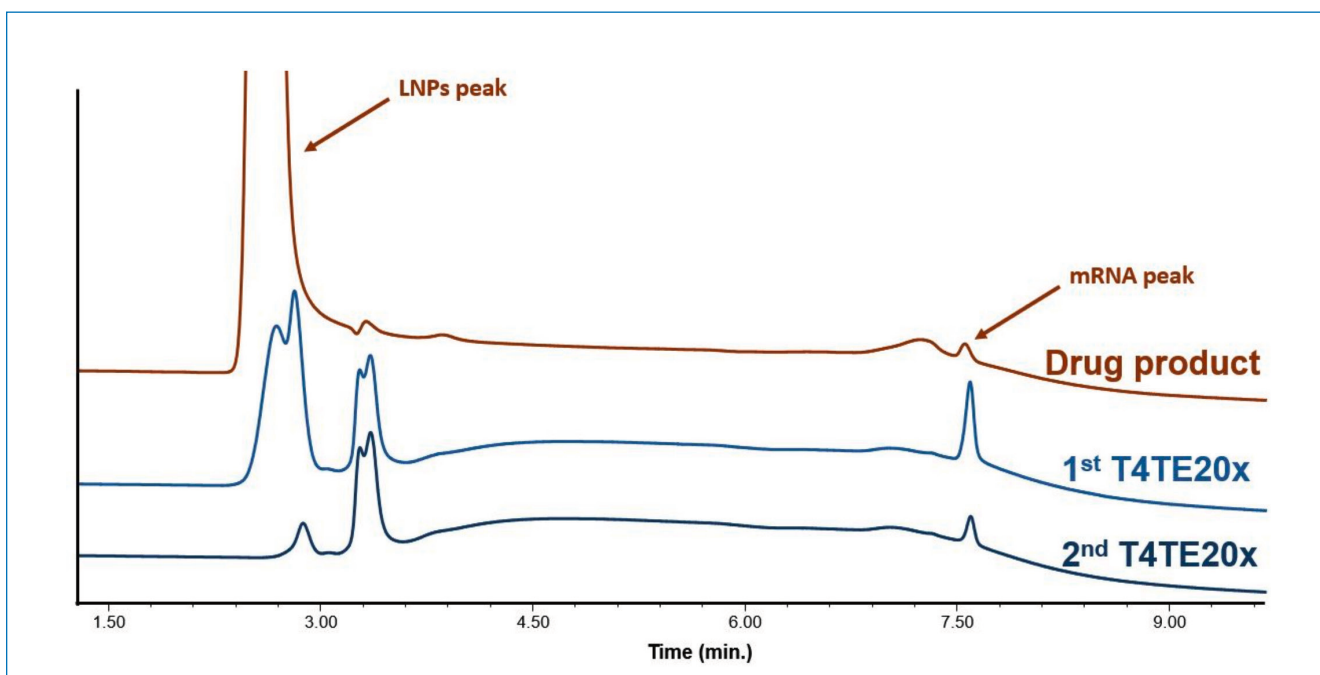


Figure 3: mRNA recovered from the injection of a drug product, followed by two T4TE20x blanks [1].

Separation of surface-associated mRNA

In some drug product (DP) samples a second pre-mRNA peak was observed which absorbed across all UV-wavelengths. The absorbance ratio of 260/230 nm confirms the assumption of an mRNA-LNP within the analysed sample. This phenomenon does not appear when the empty LNP or the empty together with a DS spike is injected. But it appears when injecting the empty LNP spiked with DS at an acidic pH. The data suggest that mRNA binds to the positively charged surface of the LNP.

To improve the separation of the free mRNA and the pre-mRNA peak an isocratic step was implemented. Due to the on/off- mechanism no peak broadening appeared here. Light-scattering effects currently prevent the quantification of the pre-mRNA peak using standard detection methods. However, structural information about the LNP can still be derived on a qualitative level. In selected cases, the peak behaves in a stability-indicating manner but lacks the consistency required for a validated degradation marker [1].

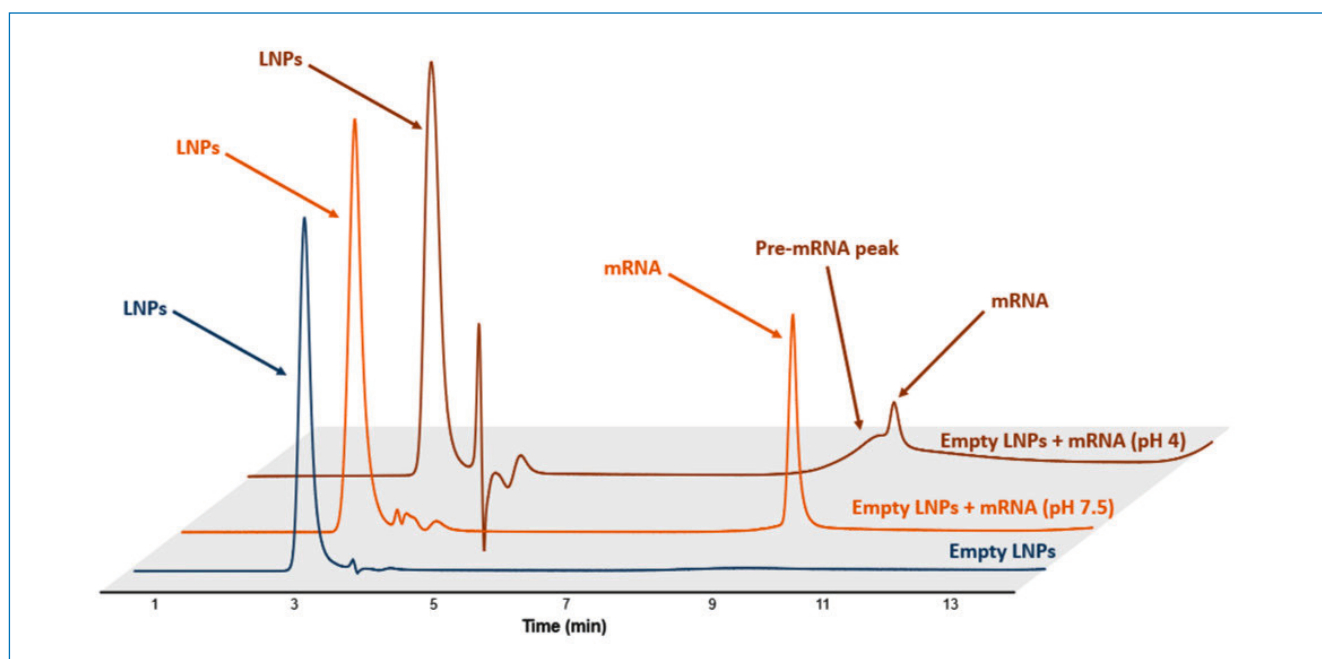


Figure 4: Analysis of empty LNPs spiked with mRNA at two different pH conditions: pH 4 and pH 7.5 [1].

AEX method vs. commonly used RiboGreen approach

The comparison of both analytical approaches reveals consistent results in many cases, while the RiboGreen method occasionally reports a notably lower EE. Figure 5 illustrates the correlation between the RiboGreen and the

AEX results across selected samples. The blue square marks a sample with aligned outcomes, whereas the orange star indicates a data point where both methods diverge significantly.

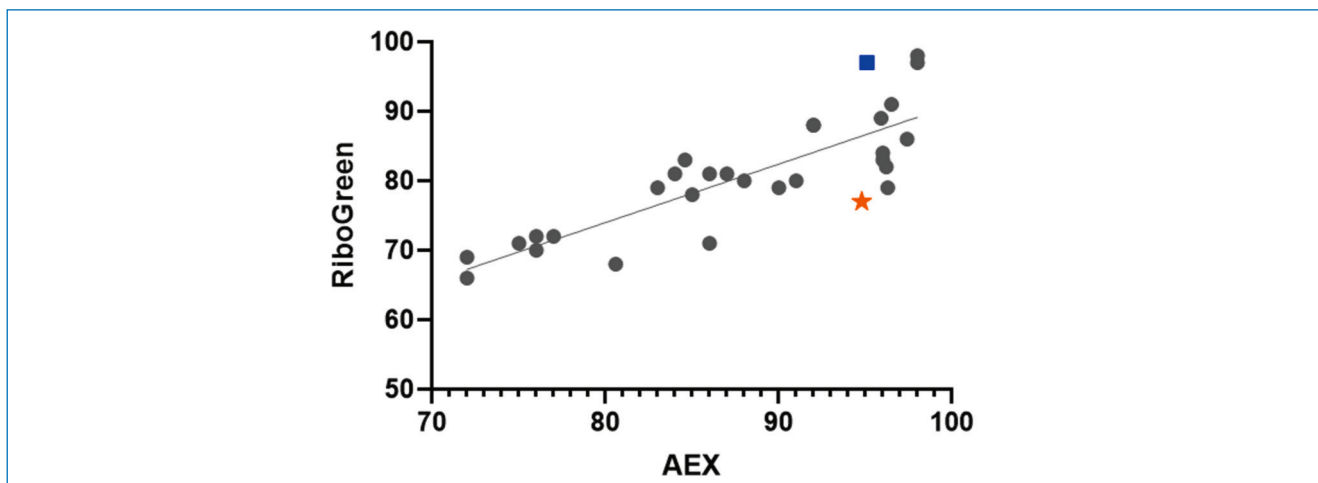


Figure 5: Correlation between EE values obtained with the RiboGreen assay and the AEX method on 30 samples [1].

Figure 6 displays the corresponding chromatograms. The blue sample produces a peak exclusively for free mRNA, while the orange sample additionally reveals a pre-mRNA

signal. The data suggest that the RiboGreen dye binds to surface-associated mRNA, which falsely contributes to the free mRNA signal and results in a reduced EE.

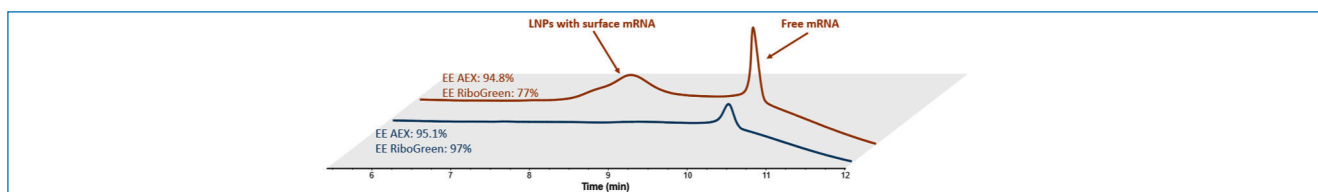


Figure 6: Chromatograms obtained for the samples highlighted in Figure 5, with the corresponding colours [1].

Conclusion

The AEX method accelerates analysis, reduces cost, and yields additional structural insights into the LNP, positioning it as a viable alternative for determining EE. The study provides evidence for the presence of surface-associated or

transmembrane mRNA within specific formulations. Further research must establish whether this mRNA fraction retains therapeutic functionality.

References

[1] Athanasios Tsalmpouris, Sofiane Mahjoubi, Camille Malburet, Chamsan Daher-Hassan, Marc François-Heude, Jean-François Cotte, Davy Guillaume, and Jonathan Maurer *Analytical Chemistry* 2025 97 (35), 19275-19282;
DOI: 10.1021/acs.analchem.5c03299