Application Note

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Determination of mRNA Encapsulation Efficiency with the Agilent 1290 Infinity II Bio LC System

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Abstract

Encapsulation is an effective way of delivering pharmacologically active compounds to a specific site of action in the body. This study demonstrates the determination of encapsulation efficiency of F-luciferase mRNA-loaded lipid nanoparticle (LNP) using an alternative HPLC method other than the usual RiboGreen assay method. An ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) method was developed using the Agilent 1290 Infinity II bio LC system with a diode array detector (DAD). Extracted mRNA was separated on the Agilent PLRP-S column with triethylammonium acetate (TEAA) as the ion-pairing reagent. This enabled the determination of the encapsulation efficiency of mRNA LNPs using IP-RP-HPLC.

Introduction

The encapsulation of in vitro transcription (IVT) mRNA with LNPs is a promising therapeutic modality with potential to treat a variety of diseases. The development of mRNA LNP therapeutics requires the implementation of robust quality control methods throughout the development process. The Agilent Fragment Analyzer system has been widely adopted to measure purity and size to ensure success throughout the IVT mRNA development workflow.[1] LNPs are a nonviral delivery vector that protect and ensure the delivery of the delicate RNA therapeutic to the site of action. They are synthesized by co-assembly of five distinct components: ionizable lipid, helper phospholipids, polyethylene glycol (PEG)-lipid, sterol, and nucleic acid.[2] High-flow-rate microfluidic mixing involves the fast mixing of aqueous (containing nucleic acid cargo) and organic (containing lipid mixture) phases, which results in the formation of LNPs with low polydispersity and high encapsulation efficiency.[3]

The encapsulation of the mRNA into LNPs is routinely assessed using the RiboGreen assay[4] early in development, in part because of the high-throughput functionality of this method using a detection platform such as the Agilent BioTek Synergy multimode reader or Agilent BioTek Cytation multimode microplate reader.[5] One of the limitations of this method is that it cannot distinguish between intact and other RNA impurities.[6] Characterization of nucleic acid encapsulation efficiency is one of the critical quality attributes to determine the success of an mRNA-LNP formation.[2] The ability to distinguish between full-length and fragment-encapsulated improves mRNA-LNP characterization. IP-RP-HPLC is a versatile analytical method that is used to separate and quantify RNA molecules[7,8], distinguishing RNA impurities from intact RNA. This method uses the separation principle based on the complex formation between the polyanionic nucleic acid and positively charged ion-pairing reagent.[6] The neutral and relatively hydrophobic complexes were then separated using RP-HPLC.

In this study, the LNP-extracted F-luciferase mRNA by IP-RP-HPLC was analyzed using the 1290 Infinity II bio LC system with a DAD. The concentration of the extracted mRNA was estimated using the calibration curve of F-luciferase mRNA and subsequently, the encapsulation efficiency was determined.

Experimental

Equipment

Analysis of F-luciferase mRNA was performed using 1290 Infinity II bio LC system with the following components. The LC system was operated using Agilent OpenLab CDS version 2.7 or later versions.

- Agilent 1290 Infinity II bio high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (with variable slit) (G7117B)
- Agilent Max-Light cartridge cell, 10 mm, 1.0 µL, 60 bar (G4212-60008)

Reagents and materials

Chemicals: All solvents used were LC grade. Acetonitrile and isopropanol were purchased from JT Baker (Phillipsburg, NJ, U.S.), and methanol and TEAA were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). Fresh ultrapure water was obtained from a Milli-Q Integral system (Millipak, Merck-Millipore, Billerica, MA, U.S.) equipped with a 0.22 µm membrane point-of-use cartridge.

Preparation of mRNA-LNPs: mRNA-LNPs were produced using the same lipid composition as Spikevax, the COVID-19 vaccine pioneered by Moderna. mRNA was in vitro transcribed from a PCR-amplified dsDNA template, purified using spin columns, and then dissolved in 1 mM sodium acetate buffer (pH 4.7) for the stock solution. For the Spikevax formulation, heptadecan-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy)hexyl)amino) octanoate (SM-102), 1,2-distearoyl-sn-glycero[1]3-phosphocholine (DSPC), 1,2-dimyristoyl-rac-glycero[1]3-methoxypolyethylene glycol-2000 (DMG-PEG 2K), and cholesterol, which were supplied from MedChemExpress, were dissolved in ethanol at the molar ratio of 50:1.5:10:38.5 to form the organic phase. The mRNA was dispersed in 25 mM sodium acetate to form the aqueous phase. These two phases were mixed using the benchtop microfluidic device at the volume ratio 3:1, and the total flow rate was 12 mL/min. The N:P ratio was 5.67:1. The formed mRNA LNPs were then buffer exchanged with 20 mM Tris (pH 7.4) and concentrated in ultracentrifuge tubes with a molecular weight cutoff of 30 kDa at 4 °C × 2,500 g × 60 minutes to a total lipid concentration of approximately 4 mg/mL. The formed mRNA-LNPs were subjected to lyophilization with or without cryoprotectants such as mannitol and trehalose.

Preparation of F-luciferase mRNA: F-luciferase mRNA was synthesized using the HiScribe T7 High Yield RNA synthesis kit for mRNA synthesis from New England BioLabs (Ipswich, Massachusetts, U.S.).

Concentration of F-luciferase mRNA: The concentration of the synthesized F-luciferase mRNA was determined using the Agilent Cary 60 UV-Vis spectrophotometer (part number G6860A). The concentration of the mRNA was calculated using Beer-Lambert's law. The calibration curve for F-luciferase mRNA was prepared for concentrations of 4.35, 8.7, 13, 34.8, and 43.5 µg/mL.

Extraction of mRNA from mRNA LNP: mRNA was extracted from mRNA LNP using the isopropanol precipitation method.[9] For the nonlyophilized samples, 20 µL of the sample was diluted 10-fold in 180 µL ammonium acetate (60 mM) in isopropanol. For lyophilized samples, 30 µL of nuclease-free water was added before taking 20 µL of the dissolved samples and diluting 10-fold in 180 µL ammonium acetate (60 mM) in isopropanol. The samples were vortexed briefly and centrifuged at 14,000 x g for 15 minutes at 4 °C. The supernatant was discarded, and the pellet was washed with 1 mL isopropanol, vortexed, and centrifuged at 4 °C. The pellet was dried and resuspended in 100 µL nuclease-free water at room temperature.

LC analysis

Table 1. IP-RP-HPLC method for analysis of mRNA.

Parameter	Value
Column	Agilent PLRP-S column, 4000Å, 2.1 × 150 mm, 8 µm (p/n PL1912-3803)
Mobile Phase	A) 100 mM TEAA, pH 7 B) 100 mM TEAA, pH 7 in acetonitrile
Gradient	Time (min) %B 0.0 10 1.5 10 7.5 15 9.5 15 16 25 20 25 25 90 27 90 27.1 10 Stop time: 30 min Post time: 3 min
Flow Rate	0.3 mL/min
Temperature	50 °C
Detection (DAD)	260 nm, 4 nm bandwidth, and reference wavelength 360 nm, 40 nm bandwidth
Peak Width	> 0.013 min (0.25 s response time) (20 Hz)
Injection	5 µL, use vial/well bottom sensing Draw speed 100 µL/min; Ejection speed 400 µL/min
Needle Wash	Flush port, 5 s 50% Methanol (50:50; v:v)

Calculation of encapsulation efficiency

The concentration of LNP-extracted mRNA was measured against the calibration curve generated with the F-luciferase mRNA. Using Equation 1, the encapsulation efficiency of the mRNA loaded LNP samples was calculated.[4]

$$\text{Encapsulation Efficiency} = \frac{\text{Concentration of LNP} - \text{extracted mRNA}}{\text{Concentration of mRNA used in encapsulation}} \times 100\%$$

Equation 1.

Results and discussion

An IP-RP-HPLC method was developed on the PLRP-S column. Gradient separation conditions were optimized for separation of the F-luciferase mRNA and minor impurity. Figure 1 shows the chromatogram of F-luciferase mRNA at 43.5 µg/mL . The chromatogram clearly indicates the successful elution of the F-luciferase mRNA peak at a retention time of 13.2 minutes. Additionally, the presence of a smaller peak, eluted at 10.6 minutes, suggests the possible presence of an mRNA fragment.

The calibration curve for F-luciferase mRNA described indicates a robust analytical method with high precision and accuracy. The concentration range of 4.35 to 43.5 µg/mL covers a suitable span for quantification, and the limit of quantification being less than 5 µg/mL is quite sensitive for most experimental needs. The precision of the analysis (n = 6) is excellent, with retention time %RSD being less than 1% and area %RSD less than 10%, ensuring reproducibility and reliability of the results. Moreover, the linearity of the calibration curve with an R^2 value greater than 0.997 demonstrates a strong direct relationship between the concentration of F-luciferase mRNA and the detector response. This level of linearity is essential for accurate quantification across the specified range. The calibration curve, as shown in Figure 2A, is a critical component for the validation of the analytical method and supports the integrity of the data. Figure 2B shows the overlaid chromatograms of F-luciferase mRNA at different calibration levels.

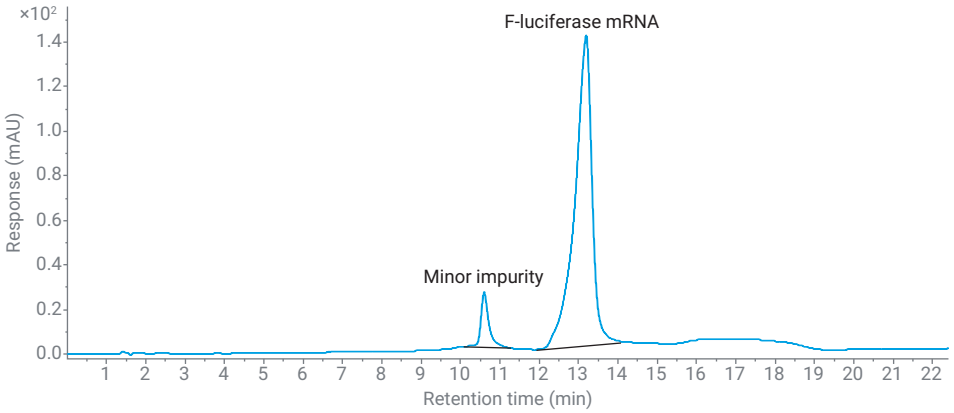

Figure 1. Representative HPLC chromatogram of F-luciferase mRNA (43.5 µg/mL) analyzed on the Agilent PLRP-S column at 50 °C.

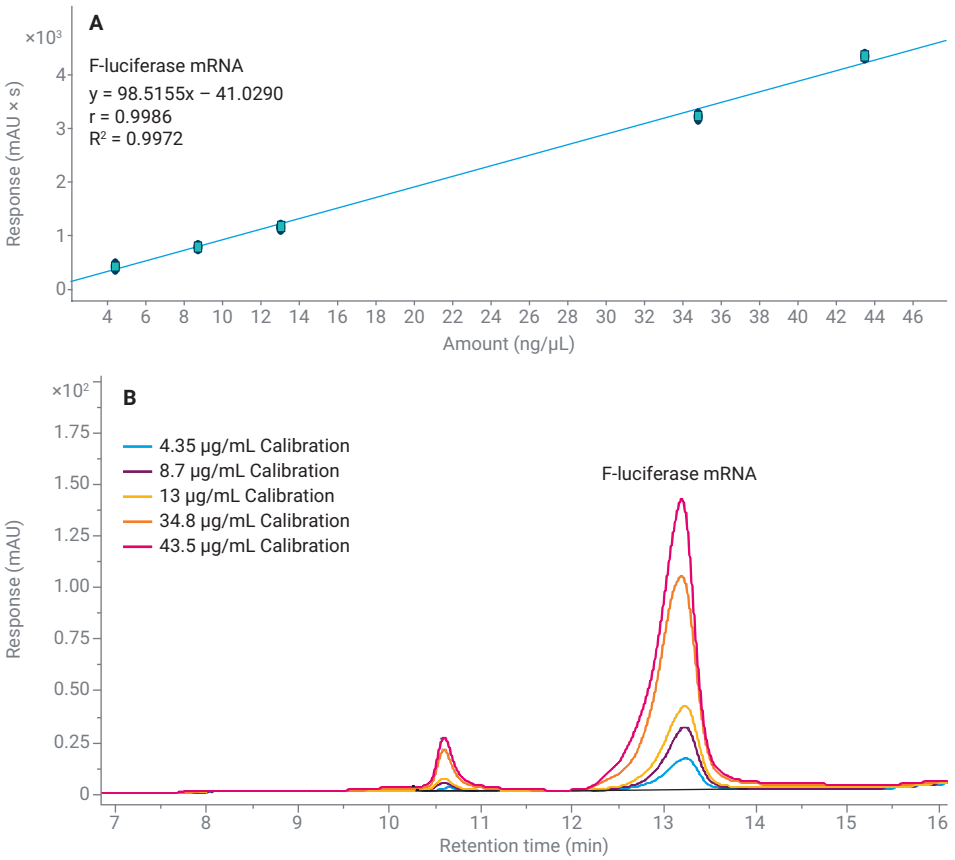

Figure 2. (A) Calibration curve of free F-luciferase mRNA from 4.35 to 43.5 µg/mL. (B) Overlaid chromatograms showing the peaks at different calibration levels.

Figure 3 shows the overlaid chromatograms of extracted mRNA from lyophilized mRNA LNP without cryoprotectant (NIL) and free F-luciferase mRNA. The LNP-extracted F-luciferase mRNA peak elutes at the same retention time as the free mRNA, confirming the size of F-luciferase mRNA and demonstrating the applicability of the method for measuring the concentration of mRNA.

To estimate the encapsulation efficiency for the F-luciferase mRNA-loaded LNP, the concentration of the LNP-extracted mRNA was required to be expressed as a percentage of concentration of mRNA used in the encapsulation. The concentration of the LNP-extracted mRNA was estimated from the calibration curve. The encapsulation efficiency for the LNP, with or without lyophilization, and with or without cryoprotectants, was calculated.

The encapsulation efficiency for the respective cryoprotectants, lyophilized or nonlyophilized, is plotted in a bar graph in Figure 4. Lyophilized LNPs show higher encapsulation efficiency than nonlyophilized LNPs. The encapsulation efficiency is also higher with cryoprotectant than without cryoprotectant. mRNA of lyophilized LNPs with cryoprotectant may be more stable during storage compared to nonlyophilized LNPs without cryoprotectant.

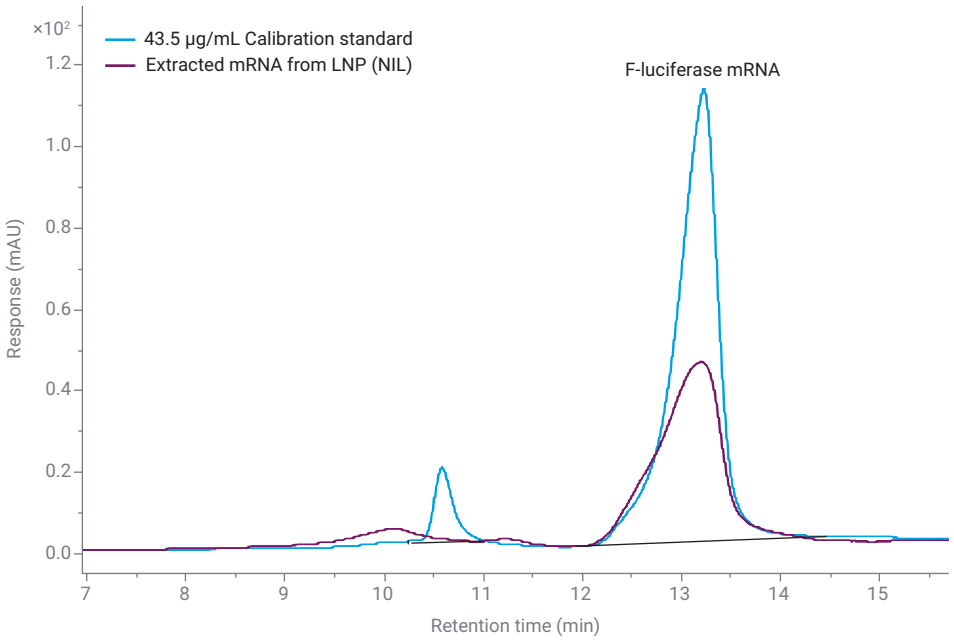

Figure 3. Overlaid chromatograms of 43.5 µg/mL calibration standard and extracted F-luciferase mRNA from lyophilized mRNA LNP without cryoprotectant (NIL).

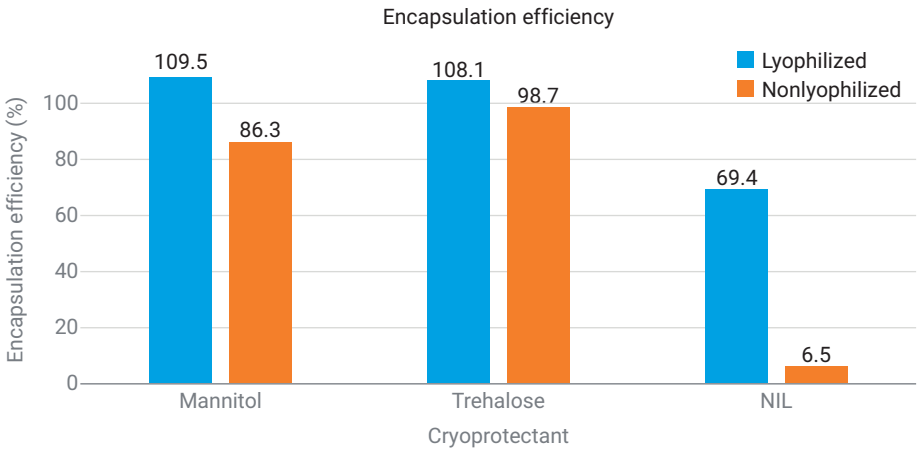

Figure 4. Encapsulation efficiency of lyophilized and nonlyophilized mRNA-LNPs with or without cryoprotectants.

Conclusion

This study presents an IP-RP-HPLC method for detecting F-luciferase mRNA and highlights the use of isopropanol extraction of lipid nanoparticles (LNPs) to analyze mRNA. This method represents an alternative approach to the standard RiboGreen assay to determine encapsulation efficiency, using the Agilent 1290 Infinity II bio LC system and the Agilent PLRP-S column. This method has added advantages for identifying impurities formed during mRNA-lipid reactions or mRNA synthesis. Such advancements in analytical techniques are crucial for progress in nanomedicine, particularly for the optimization of mRNA delivery systems in therapeutic applications.

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