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Lipid nanoparticles for local delivery of mRNA to the respiratory tract: Effect of PEG-lipid content and administration route

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ABSTRACT

Design of inhalable mRNA therapeutics is promising because local administration in the respiratory tract is minimally invasive and induces a local response. However, several challenges related to administration via inhalation and respiratory tract barriers have so far prevented the progress of inhaled mRNA therapeutics. Here, we investigated factors of importance for lipid nanoparticle (LNP)-mediated delivery of mRNA to the respiratory tract. We hypothesized that: (i) the PEG-lipid content is important for providing colloidal stability during aerosolization and for mucosal delivery, (ii) the PEG-lipid content influences the expression of mRNA-encoded protein in the lungs, and (iii) the route of administration (nasal versus pulmonary) affects mRNA delivery in the lungs. In this study, we aimed to optimize the PEG-lipid content for mucosal delivery and to investigate the effect of administration route on the kinetics of protein expression. Our results show that increasing the PEG-lipid content improves the colloidal stability during the aerosolization process, but has a negative impact on the transfection efficiency in vitro. The kinetics of protein expression in vivo is dependent on the route of administration, and we found that pulmonary administration of mRNA-LNPs to mice results in more durable protein expression than nasal administration. These results demonstrate that the design of the delivery system and the route of administration are important for achieving high mRNA transfection efficiency in the respiratory tract.

1. Introduction

Messenger RNA (mRNA) has emerged as a potent and promising drug class for the prevention and treatment of infectious diseases, cancer, and genetic abnormalities [1,2]. Vaccines based on mRNA offer several advantages with respect to safety, efficacy, and specificity, compared to traditional vaccines based on live attenuated pathogens, which have safety concerns due to the risk of reversion to virulence [3]. In particular, the mRNA-based vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have demonstrated the remarkable potential of the mRNA vaccine technology in the fight against infectious diseases due to their rapid manufacturability as well as high efficacy and safety [4].

The majority of pathogens, including SARS-CoV-2, enter the host through the respiratory tract mucosa, and they remain to be prominent causes of global mortality and morbidity associated with infectious diseases [5–8]. During the coronavirus disease 2019 (COVID-19) pandemic, it became apparent that the COVID-19 mRNA vaccines administered parenterally (e.g., via intramuscular injection) offer only very limited, if any, protection against virus transmission [9,10]. The reason is that parenterally administered COVID-19 mRNA vaccines induce robust systemic immunity that prevents severe disease, but are not able to induce protective immunity at mucosal surfaces [11,12]. To induce mucosal immunity, mucosal vaccine administration is required, which enables the induction of both mucosal and systemic immunity that can efficiently neutralize pathogens at the mucosa and block transmission [13,14].

The mucosa comprises a highly organized immune system, making it
an important target for vaccination [15]. In contrast to systemic immunity, antigens that induce mucosal immunity originate from the mucosal surface of various cavities. They are taken up by microfold (M) cells and dendritic cells (DCs) at the mucosal inductive sites, and they induce immune responses at the mucosal effector sites [16]. Induction of mucosal immunity leads to proliferation of effector T cells and production of IgG and IgA antibodies that can effectively block and neutralize invading pathogens at an early stage of microbial infection as a frontline defense against infection [17].

Although respiratory vaccination is highly attractive, successful nasal or pulmonary delivery of mRNA vaccines is challenging due to the presence of a number of physical and biological barriers at the respiratory mucosal site [18]. The mecano-physical barrier formed by a layer of epithelial cells on the surface of the respiratory tract prevents inhaled pathogens from passing through. Among these epithelial cells, goblet cells secrete protective mucus that traps foreign particles and pathogens. Foreign particles trapped in the mucus are continuously expelled from the upper airways by mucociliary clearance [19,20]. The airway and alveolar fluids constitute another barrier containing enzymes (e.g., proteases and nucleases), opsonins, and antimicrobial proteins (e.g., lactoferrins and lysozyme) [21]. Therefore, there is a need to design advanced delivery systems that can protect mRNA, and overcome these physical and biological barriers to induce an immune response in the airway mucosa.

Lipid nanoparticles (LNPs) represent the most clinically advanced mRNA delivery system with two marketed products, i.e., the COVID-19 mRNA vaccines Comirnaty® and Spikevax® from Pfizer/BioNTech and Moderna, respectively. In general, the LNPs used for mRNA delivery typically include four components: (i) an ionizable lipid, (ii) cholesterol, (iii) a helper phospholipid, and (iv) a PEGylated lipid (i.e., PEG-lipid). Ionizable lipids, which are neutral at physiological pH, but positively charged at slightly acidic pH, are responsible for packaging the negatively charged mRNA in the LNPs [22]. Helper lipids provide structural stability to the LNPs by supporting the bilayer and improving mRNA delivery. Cholesterol affects the packing of lipid membranes and stabilizes lipid membranes by filling in gaps between membrane lipids [23]. PEG-lipids are used to coat the LNPs to enhance the colloidal stability in biological environments, because PEG forms a hydrated layer on the surface of the nanoparticles that reduces the adsorption of plasma proteins, thereby improving the biodistribution and reducing recognition by the immune system [24,25]. The PEG layer is also important for preventing particle aggregation during manufacturing and aerosolization of nanoparticles. In addition to the effect on the colloidal stability of LNPs, the PEG-lipid density determines the mobility of nanoparticles in the mucus layer in the lungs by affecting the Brownian diffusion of the particles [26,27].

Inhalable mRNA therapeutics represent a promising approach for achieving localized protein expression in the lungs [28–31]. Parenteral administration of mRNA-LNPs is used in most clinical trials, and this route of administration provides in most cases very limited access to the lungs [32]. Therefore, administration via the inhalation route is of interest for delivery of mRNA to the lungs. Pulmonary delivery of mRNA has advantages such as needle-free dosing, and increased patient compliance. However, the necessity of devices for administration of inhalable dosage forms raises stability concerns for LNPs [33]. The stress caused by shear forces and the air-water interface during nebulization or spraying of mRNA-LNPs may compromise the LNP structure and mRNA stability, leading to ineffective mRNA delivery in the lungs. However, it has been shown that mRNA-LNPs can be stabilized against nebulization-induced stress to improve mRNA delivery to the lungs [34].

In the present study, we used the ionizable lipid C12-200 as a model ionizable lipid [35]. We hypothesized that: (i) the PEG-lipid content of C12-200 LNPs is important for the colloidal stability of mRNA-LNPs during dosing and for mucosal delivery of mRNA, and (ii) the content of PEG lipids in LNPs may affect expression of protein encoded by the delivered mRNA in the lungs following pulmonary or nasal administration. Hence, the specific purpose of the study was to examine the effect of PEG-lipid content on: (i) the colloidal stability of mRNA-LNPs during spraying, (ii) the in vitro transfection efficiency in the human lung epithelial cell line A549, and (iii) the in vivo expression of the model protein Firefly luciferase (FLuc), which was evaluated by bioluminescence imaging. Furthermore, we investigated the role of the administration route on the kinetics of protein expression in the lungs by comparing the pulmonary and nasal administration routes.

2. Materials and methods

2.1. Materials

CleanCap® mRNA encoding the reporter protein FLuc, fully substituted with 5-methoxyuridine (1 mg/ml in 1 mM sodium citrate buffer, pH 6.4), was acquired from TriLink BioTechnologies (San Diego, CA, USA). Triton™ X-100, citric acid, sodium citrate tribasic dihydrate, cholesterol, and Dulbecco’s Modified Eagle’s Medium-high glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gibco™ Dulbecco’s phosphate-buffered saline powder (D-PBS, pH 7.2), UltraPura™ distilled water (DNase/RNase free), Quant-IT® RiboGreen® RNA Reagent, Lipofectamine® MessengerMAX™ reagent, Slide-A-Lyzer™ G3 dialysis cassettes (3,500 MWCO), and Nunclon™ delta surface 96-well plates (white bottom) were obtained from Thermo Fisher (Waltham, MA, USA). C12-200 was synthesized, purified, and characterized as reported previously [35]. The lipids 1,2-dioleoyl-sn-glycero-3-phosphothanolamine (DOPE), and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DMPE-PEG2000) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Absolute ethanol was procured from VWR Chemicals (Radnor, PA, USA). Tris-EDTA (TE) buffer solution 100X (TE Buffer), and Amicon® Ultra-4 (10,000 MWCO) centrifugal tubes were purchased from EMD Millipore (Bedford, MA, USA). RNase-free water was used for all procedures pertaining to mRNA-LNP preparation. All other chemicals and reagents were either analytical or molecular biology grade, and were acquired from commercial suppliers.

2.2. Preparation and physicochemical characterization of mRNA-LNPs

The mRNA-LNPs were manufactured using the NanoAssemblr® Ignite microfluidics mixing system (Precision Nanosystems, Vancouver, Canada) equipped with a toroidal microchip. The organic phase comprised of C12-200 (35.0 mol%), DOPE (16.0 mol%), cholesterol (48.4/75.4/46/41.4/45 mol%, respectively, depending on the PEG-lipid content), and DMPE-PEG2000 (1.1/5.3/4/3 mol%, respectively), which were weighed before the addition of absolute ethanol (VWR Chemicals). The lipid solution was heated and bath-sonicated at 37 °C for 20–30 s until completely soluble, and briefly vortexed before addition to a vial prefilled with absolute ethanol. The aqueous phase was prepared by diluting the mRNA in sterile-filtered citrate buffer (50 mM, pH 4). The two phases were mixed at a flow rate ratio (FRR) of 3:1 (aqueous:organic) using a total flow rate (TFR) of 12 mL/min. The weight ratio of C12-200 to mRNA was 10:1. The mRNA-LNPs were then dialyzed for 3.5 h against RNase-free D-PBS using Slide-A-Lyzer™ G3 dialysis cassettes (3,500 MWCO). Dialezed mRNA-LNP dispersions were concentrated using Amicon® Ultra-4 centrifugal filter units with 10,000 MWCO by centrifugation for 45 min at 2500 × g at 4 °C. The mean hydrodynamic diameter (Z-average), and polydispersity index (PDI) were determined by dynamic light scattering (DLS) by using the photon correlation spectrometry technique. The surface charge of the particles was estimated by analysis of the zeta potential (by laser-Doppler electrophoresis). For determination of the size distribution and PDI, the mRNA-LNP dispersions were diluted in D-PBS to a lipid concentration of approximately 0.1 mg/mL. For determination of the zeta potential, the mRNA-LNP dispersions were diluted in D-PBS to a lipid concentration in the range of 0.5–1 mg/mL. The measurements were performed in triplicate.
using a Zetasizer Nano SZ (Malvern Instruments, Malvern, UK) at 25 °C. The mRNA entrainment efficiency was quantified by using the QuantiTm RiboGreen® RNA assay kit (Thermo Fisher) and a FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany), essentially as described previously, but with minor modifications: the formulations were diluted with 2 % (w/v) Triton™ X-100 instead of 1 % (w/v) [36]. To measure the influence of the administration procedure used for pulmonary dosing of mice (intratracheal instillation) on the physicochemical properties of the mRNA-LNPs, the mRNA-LNP dispersions were sprayed into a tube using a PennCentury MicroSprayer (Penn-Century Inc., Wyndmoor, PA, USA). The suspensions were kept below

and after spraying the dispersions by lab-on-a-chip RNA electrophoresis

0.0310

fitting. For all samples, the data were fitted over the q range of

subtracted using BioXTAS RAW. SasView (version 5.0.4) was used for

the scattering vector

K in 60 s exposures for 10 cycles with a sample

flux at the sample position was of the order of 3

pipetting robot), and a temperature-controlled flow-through cell. The

instrument is equipped with a 250 W liquid gallium alloy X-ray

source (Xolver L instrument (Xenocs, Grenoble, France) accessible at the

CPHSAXS Facility (University of Copenhagen, Copenhagen, Denmark).

Small angle x-ray scattering (SAXS) measurements of the mRNA-LNP

dispersions prepared with different PEG-lipid content (Table 1) at a

density of 4,000 cells/well. The cells were incubated at 37 °C for

additional 24 h. The ONE-Glo™ + Tox Luciferase Reporter and Cell

Viability Assay kit (Promega, Madison, WI, USA) was used to quantify

the Fluc expression and measure the cell viability. The first part of the assay consisted of a non-lytic fluorescence assay [CellTiter-Fluor™ (CTF) Cell Viability Assay]. For the ONE-Glo™ + Tox assay, 50 μL 2× CTF reagent was added to each well in triplicates and incubated at 37 °C for 30 min. The plate was shielded from ambient light, and the relative fluorescence units (RFUs) was measured using a fluorescence plate reader (FLUOstar OPTIMA) at an excitation wavelength of 380–400 nm and an emission wavelength of 505 nm. The relative cell viability was calculated using Eq. (1). Live cells show a high fluorescence due to peptide (substrate) cleavage activity, resulting in fluorescence. In the second part of the assay, the ONE-Glo™ Luciferase Assay System was

2.4. Cell culture and in vitro Fluc mRNA transfection assay

The human lung epithelial cell line A549 was purchased from the American Type Culture Collection (cat no. CCL-185, Manassas, VA, USA). The A549 cell line was grown in F-12 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 % (v/v) fetal bovine serum. The cells were kept in a 5 % CO2 – 95 % atmospheric air incubator at 37 °C. The medium was changed every second day, and the cells were passaged at a confluency of approx. 70 %. Briefly, cells were rinsed in D-PBS, and detached using TrypLE™ Express enzyme at 37 °C. Tryptsin was neutralized by the addition of fresh medium, and the cells were pelleted by centrifugation (700×g for 5 min). The cells were counted using a NucleoCounter® (ChemoMetec, Allerød, Denmark). For transfection, A549 cells were seeded in a 96-well white-walled plate at a density of 4,000 cells/well. The cells were allowed to adhere for 24 h at 37 °C, and the Fluc mRNA-LNP dispersions were added to each well in triplicates at different mRNA doses (from 3.125 ng to 200 ng). Cells transfected with Lipofectamine® MessengerMAX™ reagent were used as positive transfection control, and untreated cells incubated with D-PBS served as negative control. The cells were incubated at 37 °C for additional 24 h. The ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay kit was used to quantify the Fluc expression and measure the cell viability. The first part of the assay consisted of a non-lytic fluorescence assay [CellTiter-Fluor™ (CTF) Cell Viability Assay]. The second part of the assay, the ONE-Glo™ Luciferase Assay System was

2.3. Lab-on-a-chip RNA electrophoresis

The integrity of Fluc mRNA loaded into LNPs was assessed before and after spraying the dispersions by lab-on-a-chip RNA electrophoresis

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Lipid composition of mRNA-loaded LNPs with different PEG-lipid content (LNP1-5).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation</td>
<td>Lipid composition (mol %)</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>LNP1</td>
<td>35.0</td>
</tr>
<tr>
<td>LNP2</td>
<td>35.0</td>
</tr>
<tr>
<td>LNP3</td>
<td>35.0</td>
</tr>
<tr>
<td>LNP4</td>
<td>35.0</td>
</tr>
<tr>
<td>LNP5</td>
<td>35.0</td>
</tr>
</tbody>
</table>

Table 2 | Physicochemical properties of Fluc mRNA-loaded LNPs with different PEG-lipid content (LNP1-5), prepared at a C12-200:Fluc mRNA weight ratio of 10:1, dispersed in D-PBS (pH = 7.4).
<table>
<thead>
<tr>
<th>Formulation</th>
<th>Z- average (nm)</th>
<th>Polydispersity index (PDI)</th>
<th>Zeta potential (mV)</th>
<th>Fluc mRNA entrapment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNP1</td>
<td>172.9</td>
<td>0.060</td>
<td>−2.84</td>
<td>98.2</td>
</tr>
<tr>
<td>LNP2</td>
<td>157.9 ± 5.2</td>
<td>0.075 ± 0.020</td>
<td>−3.19</td>
<td>97.6 ± 0.52</td>
</tr>
<tr>
<td>LNP3</td>
<td>127.1</td>
<td>0.106</td>
<td>−3.35</td>
<td>95.3</td>
</tr>
<tr>
<td>LNP4</td>
<td>120.9</td>
<td>0.142</td>
<td>−5.27</td>
<td>98.0</td>
</tr>
<tr>
<td>LNP5</td>
<td>109.1 ± 5.9</td>
<td>0.146 ± 0.010</td>
<td>−4.11</td>
<td>94.8 ± 0.61</td>
</tr>
</tbody>
</table>

Data represent mean values of biological duplicates, except for the Z-average, PDI and mRNA entrapment of LNP2 and LNP5, for which data represent mean values ± SD (n = 9).
used to quantify FLuc expression. At 24 h, 25 µL ONE-Glo™ reagent was added to the wells and incubated at 22 °C for 3 min to lyse the cells. The data was normalized to the luminescence of untreated cells and cell viability, using Eq. (2).

\[
\text{Relative cell viability (RFU\%)} = \frac{\text{Fluorescence intensity of test cells (LNPs)}}{\text{Fluorescence intensity of untreated cells (D – PBS)}} \times 100\%
\]

\[
\text{Relative luminescence units (RLU\%)} = \frac{\text{Luminescence intensity of test cells (LNPs) – Untreated cells (D – PBS)}}{\text{Relative cell viability}} \times 100\%
\]

2.5. In vivo bioluminescence imaging of mice

In vivo imaging was used to quantify the protein expression after administration of FLuc mRNA-loaded LNPs to mice. All experimental work was approved by the Danish National Experiment Inspectorate under permit 2022-15-0201-01221, and was performed in accordance with the directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Female BALB/cOlaHsd mice aged 7–9 weeks were obtained from ENVIGO (Limburg, Netherlands). To evaluate protein expression in the respiratory tract, mice were dosed by nasal (N = 3 per group) and pulmonary (N = 4 per group) administration, respectively, of FLuc mRNA-loaded LNPs. Mice were anesthetized with 2 % isoflurane prior to an intranasal dispensation of 15 µL mRNA-LNP dispersion by pipetting into each nostril (0.5 mg/kg). Prior to pulmonary administration by intratracheal instillation, the mice were anesthetized with a mixture of xylazine and ketamine (9 mg/kg and 100 mg/kg, respectively, Rompun®, Leverkusen, Germany and Ketaminol®, Boixmeer, Netherlands) by intraperitoneal injection. In brief, for pulmonary administration, the mice were placed in the supine position on the bench, the incisor loop was hooked around the incisors, and the tongue was rolled out of the oral cavity. The speculum was slowly advanced into the oral cavity, past the hard and soft palates. Once the tracheal opening was visible, the PennCentury MicroSprayer needle was inserted with the tip over the tracheal opening, and the mRNA-LNP dispersion (0.25 mg/kg in dispersion of 50 µL mRNA-LNP) was sprayed using the PennCentury MicroSprayer. Whole body and organ imaging were performed at different time points (2, 4, 6, 24, and 48 h) post administration by using an IVIS Lumina XRMS imaging system (PerkinElmer, Waltham, MA, USA). First, whole body imaging was performed. The substrate of FLuc (D-luciferin, Thermo Fisher Scientific) was injected intraperitoneally 20–22 min before whole body imaging at a dose of 150 mg D-luciferin potassium salt/kg body weight, and the mice were anesthetized prior to imaging using isoflurane and oxygen from a precision vaporizer. For organ imaging, the mice were euthanized, and the lungs, the spleen, and the liver were isolated. Prior to organ imaging, isolated organs were immersed in a solution of D-luciferin (15 mg/mL) for 2 min. The bioluminescence emission was measured, and the intensity of the emitted light was quantified as radiance (number of photons/s/cm²/sr) using the Living Image® Software v4.3.1 (Perkin Elmer).

2.6. Statistics

Graphing and statistical analysis were performed using GraphPad Prism v10 (Dotmatics, Boston, MA, USA). The in vitro transfection data and the in vivo imaging data were analyzed by unpaired, two-tailed t-test at a 0.05 significance level, and a value of p < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Physicochemical properties and morphology of mRNA-LNPs with different PEG-lipid contents

To investigate the effect of PEG-lipid content on the physicochemical properties and transfection efficiency of LNPs loaded with FLuc mRNA, five different mRNA-LNP formulations (mRNA-LNPs containing 1, 1.5, 3, 4, and 5 mol% PEG-lipid, respectively) were prepared (Table 1). As model ionizable lipid, C12-200 was chosen due to the ease of synthesis [35], as compared to the ionizable lipids ALC-0315 and SM-102 used in Comirnaty® and Spikevax®, respectively. In addition, C12-200-based LNPs have been shown to mediate efficient RNA delivery in vivo [37,38]. As helper lipids, DOPE and cholesterol were used, and DMPE-PEG2000 was applied as stabilizing PEG-lipid. The LNPs were prepared by using a microfluidics mixing system based on the nanoprecipitation method. The lipid mixtures were solubilized in ethanol, followed by microfluidic mixing with an aqueous mRNA solution (C12-200:mRNA weight ratio 10:1) at an FRR of 3:1 (aqueous to organic solvent phase) and a TFR of 12 mL/min. Increasing the PEG-lipid content from 1 mol% to 5 mol% led to a decrease in LNP size from approx. 174 nm to 109 nm (Table 2). The hydration of the headgroup as a result of an increasing PEG-lipid concentration causes lateral repulsion of the surface of the lipid bilayers. To reduce the high lateral repulsion, the particle size will be reduced. This is believed to be the reason for the decrease in particle size with increasing PEG-lipid content. In addition, there was an increase in the PDI value from approx. 0.06 to approximately 0.15 when the PEG-lipid content was increased from 1 mol% to 5 mol% (Table 2). This increase in PDI may be linked to the decreased particle size. In addition, a study reported that the type and content of helper lipids is important for structural integrity, resulting in more compacted and stable particles. DOPE-containing LNPs have been reported to form uniformly spherical particles in comparison to DSPC-containing LNPs [39].

Increasing the PEG-lipid content from 1 mol% to 5 mol% led to a very small decrease in zeta potential from ~2.84 mV to ~4.11 mV (Table 2). A high PEG-lipid content may reduce the propensity for aggregation of LNPs under high ionic strength conditions, such as in blood. The correlation between physicochemical properties and intracellular protein expression of LNPs in HEK-293 cells has previously been studied, and it was found that a large particle size resulted in higher cellular uptake and protein expression levels [40].

In general, mRNA-LNPs with different PEG-lipid contents displayed a high mRNA entrainment efficiency in the range of 95–98 % (Table 2). The PEG-lipid content affects the entrainment efficiency of mRNA in LNPs, and it was reported that LNPs can be made more stable and smaller by increasing the content of PEG-lipid, while a higher PEG-lipid
content can negatively affect the mRNA entrapment efficiency of the LNPs. This was explained by the fact that the thicker PEG-lipid layer leads to a smaller size of the vesicles for encapsulation of the mRNA. As a result, mRNA-LNPs with a relatively high entrapment efficiency (above 75 %) are obtained when the PEG-lipid molar ratio is below than 5 %. In addition, it was noted that a low PEG-lipid content can result in an increased size of the LNPs during nebulization, which can negatively affect endocytosis [41].

To measure the influence of the administration procedure, used for subsequent pulmonary dosing of mice (intratracheal instillation with the PennCentury MicroSprayer), on the physicochemical properties of the mRNA-LNPs, the mRNA-LNP dispersions were compared before and after spraying using the PennCentury MicroSprayer in terms of size, PDI, and mRNA entrapment efficiency. After spraying, mRNA-LNPs displayed similar values with respect to size and PDI (Table S1), and spraying did not have an impact on the mRNA entrapment efficiency either. This suggests that the stability of the mRNA-LNPs is not affected by the spraying process with the MicroSprayer (Table S1).

Next, we investigated the morphology of the mRNA-LNPs using cryo-TEM and the internal structure using SAXS to further characterize the structure of mRNA-LNPs with different PEG-lipid contents (Table 1). Cryo-TEM images (Fig. 1A) show a decreasing size of LNPs with increasing PEG-lipid content, which is in agreement with the DLS results. Electron-dense spheres were observed for all the mRNA-LNPs with different PEG-lipid contents, and a multilamellar structure was visible for all compositions, except for mRNA-LNPs containing 1 mol% PEG-lipid. While mRNA-LNPs with 1 mol% PEG-lipid appear as aggregates without a clear internal structure, the mRNA-LNPs containing 1.5 mol% PEG-lipid present a thick shell, composed of several lamellar repeats, and a structured core that resembles a disordered hexagonal phase, as described in the literature [42,43]. Increasing the PEG-lipid content did not affect the morphology of the LNPs, and their images appear similar to those obtained for mRNA-LNPs with 1.5 mol% PEG-lipid. The mRNA-LNP dispersions display a clear peak in the SAXS curves (Fig. 1B), which suggests the presence of a repeat distance in the particles. The distance (d) can be derived from the position of the peak, q, using the equation d = 2πr/q. The curve was fitted with a broad peak model, and the position of the peak was found to be similar for the different formulations, except for mRNA-LNPs with 5 mol% PEG-lipid (Table S2). However, this formulation displays the least defined peak, i.e., the lowest intensity and the highest full width half maximum, which is likely due to the smaller size of the mRNA-LNPs and the lower number of repeat units per particle. The intensity of the peak decreased as the PEG-lipid content was increased (see insert in Fig. 1B), while the peak of mRNA-LNPs with 1 mol% PEG-lipid does not follow this trend and shows a lower intensity than observed for mRNA-LNPs with 1.5 mol% PEG-lipid, suggesting less internal order. This result is in accordance with the lack of internal structure found in the cryoTEM images (Fig. 1A).

The cryoTEM images, together with the SAXS results, suggest that the morphology and internal structure of these mRNA-LNPs do not display major differences within the PEG-lipid content range studied, except for the mRNA-LNPs with 1 mol% PEG-lipid, which shows a less ordered structure.

3.2. Integrity of FLuc mRNA in LNPs after spraying with the PennCentury MicroSprayer

The integrity of FLuc mRNA loaded into LNPs was measured before and after spraying the formulations using the PennCentury MicroSprayer. Charged RNA molecules are driven by a voltage gradient, and a fluorescent dye intercalates only intact RNA molecules, allowing their migration to be tracked in the analysis. Electropherograms therefore display fluorescence units (FU) as a function of migration time (Fig. 1C). The retention time of the intact FLuc mRNA peak in all samples, which appears between 36 and 38 s, is proportional to the content of intact mRNA that the dye is able to bind. The small peak appearing at a migration time of 19 s corresponds to the internal standard used by the software to correct for drift effects. There was no difference in migration times and FU of the formulations before and after spraying. The peak of intact FLuc mRNA did not shift or disappear after spraying the formulations.

3.3. Effect of PEG-lipid content of mRNA-LNPs on in vitro protein expression

The transfection efficiency of FLuc mRNA-LNPs with different molar contents of PEG-lipid was evaluated. The A549 cells were treated with LNPs loaded with FLuc mRNA and assayed 24 h after transfection for FLuc expression and effect on cell viability. Increasing the PEG-lipid content of LNPs did not affect the viability of A549 cells (Fig. 2A) but significantly inhibited the ability of LNPs to transfect cells (Fig. 2B). The LNPs containing 1.5 mol % PEG-lipid mediated the highest FLuc expression, while a higher content of PEG-lipid led to a significant (p < 0.0001, unpaired, two-tailed, t-test) decrease in FLuc expression. The FLuc expression mediated by mRNA-LNPs with 1.5 mol% PEG-lipid was four times higher than the FLuc expression mediated by mRNA-LNPs with 5 mol% PEG-lipid. These results show that a high PEG-lipid density on the LNP surface reduces the ability of LNPs to transfect cells. This may be because a high PEG-lipid density inhibits receptor-mediated endocytosis by reducing the adsorption of serum proteins [44] and interfering with cellular uptake of LNPs [45,46], which limits the intracellular delivery of mRNA. A recent study has also shown that increasing the content of PEG-lipid before and after nebulization reduces the transfection efficiency in HEK-293 cells and NuLi-1 cells [40].

In addition, using the PennCentury Microsprayer, the effect of spraying on the expression of FLuc was compared for all formulations. Notably, the FLuc expression levels in A549 cells transfected with mRNA-LNPs containing 1 mol% and 1.5 mol% PEG-lipid decreased significantly after spraying (p < 0.0001, unpaired, two-tailed, t-test), but there was no significant difference after spraying for mRNA-LNPs with higher PEG-lipid content (3 mol%, 4 mol% and 5 mol%) (Fig. 1B). Interestingly, although no significant changes in the physicochemical properties were detected for any of the formulations before and after spraying (Table S1), it was observed that FLuc expression upon in vitro transfection remained similar when the PEG-lipid content was increased (3 mol%, 4 mol%, and 5 mol%). A similar trend was also found in a recent study, where increasing the PEG-lipid content reduced the transfection efficiency. However, a higher PEG-lipid content was also shown to improve the transfection efficiency after nebulization. Hence, it is likely that mRNA-LNPs with a higher PEG-lipid content are more stable and not affected by the nebulization process, as compared to mRNA-LNPs with a lower PEG-lipid content [34].

3.4. Kinetics of FLuc expression after nasal and pulmonary administration of mRNA-LNPs

Based on the in vitro protein expression results, FLuc mRNA-loaded LNPs modified with 1.5 mol% PEG-lipid were selected for in vivo studies. We evaluated the kinetics of FLuc expression mediated by mRNA-LNPs in mice after nasal and pulmonary administration, respectively. The formulations were administered intranasally (0.5 mg/kg FLuc mRNA encapsulated in LNPs) and pulmonary (0.25 mg/kg FLuc mRNA encapsulated in LNPs), respectively, into the lungs of mice, and whole-body imaging of the animals was performed at different time points by using the IVIS system. In addition, bioluminescence imaging of isolated organs (lungs, spleen, and liver) was performed after euthanization, also using the IVIS system.

After nasal administration, a luminescence signal was observed exclusively in the nasal cavity (Fig. 3A). The signal was approx. 100 times higher than the background at 2 and 4 h, and the signal was 1000 times higher than the background at 6 and 24 h (Fig. 3B). Following pulmonary administration by intratracheal instillation, a
Fig. 1. Physicochemical properties and morphology of mRNA-LNPs containing different PEG-lipid contents. (A) Cryogenic transmission electron microscopy (cryo-TEM) imaging and (B) small angle x-ray scattering (SAXS) measurement of dispersions of mRNA-LNPs with different PEG-lipid content, the black lines are fitted models to the data. Data have been shifted for clarity; the intensity of the peaks is plotted against the PEG-lipid content in the insert. (C) Electropherograms of FLuc mRNA before and after spraying dispersions of mRNA-LNPs containing different PEG-lipid contents. The scale bars in the images represent 100 nm.
bioluminescence signal was observed in the thoracic region of the mice by whole-body imaging (Fig. 3C), but organ imaging of excised lung tissue provided more robust data when assessing FLuc expression in the lungs. This may be due to the motion incurred by ventilation and cardiac contractions, which can affect the bioluminescence scatter signal during whole-body imaging.

Isolated organs (lungs, spleen, and liver) were subsequently imaged. A bioluminescence signal was observed in the lungs at the earliest time point of 2 h in mice dosed via both administration routes (Fig. 3D and E). Furthermore, the absence of a bioluminescence signal in other organs showed that localized protein expression in the lungs is achieved after nasal and pulmonary administration, respectively (Fig. S1).

Following pulmonary administration, the bioluminescence signal in the lungs continued to increase beyond 4 h, whereas the FLuc expression decreased in mice dosed via nasal administration. While the highest protein expression in the lungs was 100 times higher than the background at 24 h after pulmonary administration by intratracheal instillation, the highest response after nasal administration was 100 times higher than the background at 4 h (Fig. 3F). In a recent study, a comparable protein expression profile was found, and the bioluminescence response in the thoracic region was maximal at the 24 h time point following pulmonary administration of Cy5-Luc mRNA-LNPs by intratracheal instillation [31]. In another study, it was found that LNPs loaded with AncNanoLuc mRNA (dose of 20 μg per mouse) gave rise to higher luciferase expression in the lungs 48 h after nebulization [30].

In the present study, we observed a rapid decline in FLuc expression in the lungs 4 h after nasal dosing, albeit these mice received a two-fold higher dose of mRNA, as compared to the pulmonary dose. This may be due to the rapid clearance of LNPs from the administration site after nasal dosing, and different cellular uptake kinetics or release kinetics of mRNA at the site of deposition. Several previous studies have shown that the administration route of mRNA-LNPs influences the kinetics of antigen expression [48,49]. In a recent biodistribution study conducted with self-amplifying mRNA encoding the rabies virus glycoprotein loaded in nanoparticles, it was found that nasal administration to mice resulted in poor retention of nanoparticles, as compared to intradermal and intramuscular administration, respectively, and this suggests that a dose fraction is cleared and/or swallowed during the first few hours after nasal dosing [49]. Moreover, it was demonstrated that the route of administration influences the magnitude of the immune responses that mRNA-LNPs induce.

3.5. Effect of PEG-lipid content in mRNA-LNPs on FLuc expression in mice

PEGylation is a well-known strategy to increase the diffusion of nanoparticles across mucosal surfaces, and it also confers an increased resistance to shear stress during aerosolization of the LPN formulations [40]. Therefore, although the *in vitro* transfection efficiency of mRNA-LNPs with higher PEG-lipid content was reduced, we also tested this hypothesis *in vivo* by comparing the luminescence signal after 24 h in the lungs of mice dosed with FLuc mRNA-LNPs containing 1.5 mol% PEG-lipid and 5 mol% PEG-lipid, respectively. After pulmonary administration by intratracheal instillation, the FLuc expression in the lungs was significantly higher (*p* < 0.05) for mice dosed with mRNA-LNPs containing 1.5 mol % PEG-lipid than for mice dosed with mRNA-LNPs modified with 5 mol % PEG-lipid (Fig. 3H). These *in vivo* results support our *in vitro* transfection findings and show that a content of 1.5 mol % PEG-lipid is sufficient to stabilize LNPs against the shear stress imposed by aerosolization, and to successfully deliver mRNA to the lungs. In addition, a higher PEG-lipid content (5 mol%) in LNPs resulted in reduced cellular uptake of LNPs, which may explain the lower protein expression in the lungs, as compared to the expression mediated by LNPs with 1.5 mol% PEG-lipid. Design rules for the combination of PEG-lipid content and helper lipids to improve the delivery of mRNA to the lungs have been shown in several studies [34,50]. For example, a combination of a low PEG-lipid content and neutral helper lipids improves the performance of LNPs, while a high PEG-lipid content is required to improve the performance of LNPs when cationic helper lipids are used [30].

4. Conclusions

The present study investigated the design criteria of LNPs for local delivery of mRNA LNPs. The composition of LNPs plays an important
role in ensuring effective delivery of mRNA to the lungs. Our findings comprise: (i) Modification of mRNA-LNPs with 1.5 mol% PEG-lipid is optimal to ensure colloidal stability of LNPs during the aerosolization process and to achieve mRNA delivery to the lungs, (ii) LNPs can be designed as effective delivery systems for mRNA transfection in a lung epithelium cell line, and (iii) pulmonary delivery by intratracheal
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Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2024.114266.

References


