

Stabilising Nebulised mRNA-Lipid Nanoparticles Using PEG-Lipid Alternatives

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Summary Inhaled mRNA-lipid nanoparticle (LNP) therapeutics hold transformative promise for treating pulmonary diseases by enabling targeted delivery and minimising systemic exposure. However, the stability of mRNA-LNPs during nebulisation remains a critical barrier, particularly due to the destabilising effects of aerosol shear forces and the immunogenicity associated with conventional PEG-lipids. This study systematically compares the performance of traditional PEG-lipids (DSPE-PEG, DMPE-PEG, DMG-PEG) and novel polymer-lipid alternatives (DMG-pSar, DMG-PX, DMG-PY, DMG-PZ) in stabilising mRNA-LNPs during and after nebulisation. LNPs were formulated with two clinically relevant ionisable lipids (ALC-0315 and SM102) and characterised for hydrodynamic diameter, polydispersity index (PDI), and in vitro transfection efficiency before and after nebulisation. Results demonstrate that DSPE-PEG provides the most robust stabilisation, maintaining small particle size, low PDI, and high transfection efficiency post-nebulisation. In contrast, increased hydrophilicity in alternatives such as DMPE-PEG, DMG-PEG, and especially DMG-pSar and DMG-PZ, leads to greater LNP destabilisation and reduced mRNA delivery. Notably, among the DMG polymer conjugates, DMG-PY (least hydrophilic) outperforms DMG-PX and DMG-PZ, highlighting the importance of balancing hydrophilicity and membrane anchoring. Lower molar percentages (1.5%) of all polymers consistently yield better outcomes. These findings provide critical guidance for the rational design of LNP surface chemistry, supporting the development of safer and more effective inhaled mRNA therapies by optimising stability and delivery efficiency for clinical translation.

Key Message Among all tested PEG-lipids and polymer-lipid alternatives, DSPE-PEG consistently provides the best stabilisation and transfection efficiency after nebulisation. In contrast, increased hydrophilicity in alternatives such as DMPE-PEG, DMG-PEG, and especially DMG-pSar (PX, PY, PZ) leads to reduced nanoparticle stability and mRNA delivery, guiding optimal LNP design for inhaled mRNA therapies.

Introduction Inhaled messenger ribonucleic acid (mRNA) therapy offers transformative potential for lung diseases by enabling localised delivery, maximising therapeutic efficacy, and minimising systemic side effects. This strategy is especially promising for conditions such as cystic fibrosis (CF), primary ciliary dyskinesia (PCD), asthma, and idiopathic pulmonary fibrosis (IPF), where direct lung administration targets diseased tissue with precision. For instance, inhaled mRNA encoding cystic fibrosis transmembrane conductance regulator (CFTR) protein (ARCT-032) is in Phase 2 trials for CF [1], while mRNA therapies for PCD aim to restore ciliary function [2]. Preclinical studies show that nebulised mRNA-lipid nanoparticles (LNPs) can suppress fibrotic pathways in IPF more effectively than systemic administration by delivering anti-interleukin-11 fragments directly to lung fibroblasts [3]. A key component of these LNP formulations is poly(ethylene glycol)-lipid (PEG-lipid), which stabilises LNPs during nebulisation and extends circulation. However, PEG-lipids are increasingly linked to immunogenic risks, including anti-PEG antibody production that accelerates clearance, triggers anaphylaxis, and reduces efficacy, problems worsened by repeated dosing in chronic respiratory diseases. These challenges are heightened in inhalation therapies, where aerosolisation subjects LNPs to shear forces that destabilise PEG-based formulations, risking mRNA leakage and reduced transfection efficiency. Overcoming these limitations is crucial for the success of inhaled mRNA therapeutics in lifelong conditions where safe and sustained dosing is essential. The immunogenicity and variable biological responses to PEG-lipids underscore the urgent need for alternative stabilising polymers, especially for aerosolised applications where LNPs face additional physical stress. This study aims to develop and optimise PEG-lipid alternatives to stabilise mRNA-LNPs during nebulisation and enhance mucus penetration. The goal is to maximise post-nebulisation stability and transfection efficiency for pulmonary mRNA delivery. A library of candidate PEG alternatives is generated to formulate LNPs with diverse ionisable lipids, helper lipids, and novel stealth lipids for optimisation. By leveraging advances in polymer chemistry and nanomedicine, this research seeks to address the central challenge of maintaining LNP stability and efficacy during aerosolisation.

Materials 6-((2-hexyldecanoyl)oxy)-N-(6-((2-hexyldecanoyl)oxy)hexyl)-N-(4-hydroxybutyl)hexan-1-aminium (ALC-0315) and firefly luciferase mRNA (FLuc mRNA, R1005-APE) were purchased from Stratech (E0139-SEL). Polyadenylic Acid (PolyA, 10108626001), DMEM, heat-inactivated FBS,

Penicillin/Streptomycin (100X, Gibco), and molecular grade ethanol (64-17-5) were from Sigma Aldrich, St. Louis, MO, USA. DMG-PEG2000 (7944/250) was from Bio-Techne, Minnesota, USA. DSPE-PEG2000 (HY-142979-50mg) was from Cambridge Biosciences, England. Cholesterol (700100P), DSPC (816-94-4), and DMG-polysarcosine25 (pSar25) (870340P) were from Avanti Lipids AL, USA. Quant-iT™ RiboGreen® RNA Kit (Invitrogen) and Opti-MEM™ Reduced Serum Media (Gibco) were from ThermoFisher Scientific, Waltham, MA, USA. A549 (CCL-185) cells were from ATCC, Manassas, VA, USA. DMEM (Gibco, 10741574) was from Sigma Aldrich. Folded capillary zeta cell (DTS1070) and Brand Semi-micro cuvette (BR759015) were from Thermo Fisher Scientific, New Hampshire, USA.

Experimental Methods

Preparation of LNP Formulations: LNPs encapsulating mRNA were formulated by manually mixing a lipid-ethanol phase and an aqueous phase, which included the mRNA. The aqueous phase consisted of 50 mM sodium acetate (pH 4.8). The ethanol-lipid phase involved the lipids dissolved in ethanol solution. The lipid mixture comprised either ALC-0315: DSPC: Cholesterol: PEG/pSar lipid or SM102: DSPC: Cholesterol: PEG/pSar lipid at a molar ratio of 50: 10: X: Y (where Y was 1.5, 5 or 10 molar ratio and cholesterol molar ratio adjusted accordingly). The N/P ratio was fixed at 6:1, and the volume ratio of aqueous phase to organic phase was set at 3:1. Stock solution of FLuc mRNA or PolyA (1000µg/mL) was diluted such that the final nucleic acid concentration was at 25 µg/mL in aqueous phase, while the stock solutions of lipids were dissolved in ethanol at 10 mg/mL. The lipid-ethanol mixture was then added to the aqueous nucleic acid solution and mixed gently using the pipette 30 times. The resultant mixture was left at room temperature for 30 minutes to allow for self-assembly. The formulations were nebulised using Aerogen Solo Vibrating Mesh Nebuliser.

Characterisation of LNPs: LNPs were characterised by Dynamic Light Scattering (Malvern Zetasizer Ultra, UK) for Z-average size, polydispersity index (PDI), and zeta potential. LNPs (25 µg/mL) were diluted to 2.5 µg/mL in 50 mM Tris buffer for size/PDI and in ultrapure water for zeta potential. Encapsulation efficiency (EE) was determined using the Quant-iT™ RiboGreen RNA Assay Kit. Calibration standards were prepared by serial dilution of the mRNA stock (100 µg/mL) to 1 µg/mL in 1X TE buffer. Controls included free mRNA/PolyA and buffer. RiboGreen reagent (200-fold) was mixed 1:1 with samples (100 µL each), incubated for 5 min at room temperature, and fluorescence measured (excitation 485 nm, emission 535 nm; SpectroMax M5). All measurements were performed in triplicate. EE(%) was calculated as: $\%EE = [(Total\ mRNA - Unencapsulated\ mRNA) / Total\ mRNA] \times 100$.

Cell Culture: Human adenocarcinoma alveolar basal epithelial (A549) cells were maintained using DMEM medium supplemented with 10% FBS and 100U/mL penicillin and 100µg/mL streptomycin. The cells were incubated at 37 °C in a 5% CO₂ humidified atmosphere.

Transfection Assay: A549 cells (20,000 cells/well in a 96-well plate) were treated with LNPs (1 µg/mL mRNA) or Lipofectamine® complexes (positive control) for 24 hours. Luciferase activity was quantified post-lysis according to the manufacturer's protocol (Promega GloMax Explorer), with relative light units (RLUs) normalised to controls. Data represent triplicates (mean ± SD). Two-way ANOVA was used for statistical analysis.

Results and Discussion To understand the effect of different PEG-lipid and PEG-lipid alternatives on the stability of the formulated LNPs, two ionisable lipids, SM102, which was used as the ionisable lipid in Moderna's Spikevax Covid-19 vaccination and ALC-0315, which was used in Pfizer/BioNTech's vaccine, Comirnaty, were chosen. DSPE-PEG, DMPE-PEG, DMG-PEG, and DMG-pSar differ in their lipid anchor length, saturation, and hydrophilic polymer block, which strongly influence LNP stability and delivery. DSPE-PEG, with its long saturated C18 chain, anchors firmly in the lipid layer of LNP, providing prolonged surface protection and enhanced stability, especially in biological fluids [4]. In contrast, DMPE-PEG and DMG-PEG, both with shorter C14 chains, desorb more rapidly, leading to less durable protection but potentially facilitating faster cellular uptake [4]. DMG-pSar, which replaces PEG with highly hydrophilic polysarcosine, offers excellent steric stabilisation and resistance to protein adsorption, but its extreme hydrophilicity can overly shield the LNP and reduce transfection efficiency [5]. While increased hydrophilicity generally improves colloidal stability and prevents aggregation, excessive shielding (as with DMG-pSar) can hinder cellular uptake and endosomal escape, ultimately reducing mRNA delivery efficacy [6].

The encapsulation efficiency of all given formulations was above 90%. The data clearly demonstrate that the structural and hydrophilic differences among DSPE-PEG, DMPE-PEG, DMG-PEG, and DMG-pSar critically influence the stability and transfection efficiency of mRNA-loaded LNPs, both before and

after nebulisation. DSPE-PEG consistently yields the highest transfection efficiency at lower molar percentages (1.5%) and retains superior performance post-nebulisation for both SM102 (Figure 1c) and ALC-0315 LNPs (Figure 1d). This is attributed to its strong anchoring and moderate hydrophilicity, which together maintain LNP integrity under mechanical stress, as reflected by relatively small increases in particle size and PDI after nebulisation. In contrast, DMPE-PEG and DMG-PEG, both with shorter C14 chains, show lower transfection and greater increases in size and PDI post-nebulisation, suggesting that their increased hydrophilicity and faster desorption from the LNP surface compromise stability, especially under stress. DMG-pSar, which features a highly hydrophilic polysarcosine block, exhibits the lowest transfection efficiency and the largest increases in size and PDI after nebulisation, particularly at higher molar percentages, indicating that excessive hydrophilic shielding can hinder cellular uptake and endosomal escape despite preventing aggregation. These findings are consistent with the literature, which shows that PEG-lipids with longer, saturated anchors (like DSPE-PEG) provide more durable surface protection and better stability during aerosolisation, while shorter-chain PEG-lipids or highly hydrophilic alternatives (like DMG-pSar) can lead to rapid desorption, reduced functional delivery, and instability under stress [7]. The observation that lower PEG or polysarcosine molar percentages (1.5%) yield better transfection and more uniform particle sizes further aligns with the established understanding that excessive surface shielding impairs delivery efficiency [8].

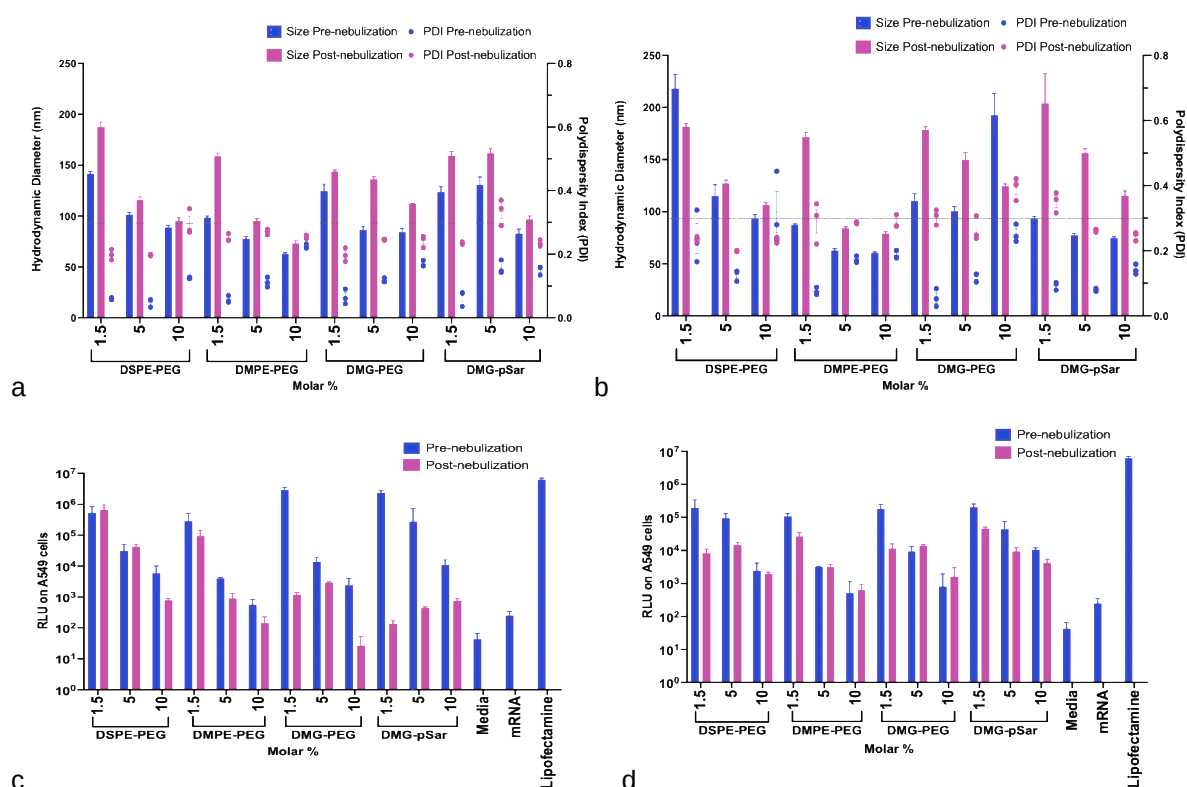


Figure 1. (a,b) Hydrodynamic diameter and PDI of mRNA-loaded LNPs formulated with varying molar percentages (1.5%, 5%, 10%) of DSPE-PEG, DMPE-PEG, DMG-PEG, or DMG-pSar before and after nebulisation with (a) ionisable lipid SM102 and (b) ALC-0315. Blue bars and dots represent size and PDI before nebulisation, while pink bars and dots represent post-nebulisation values. Data are shown as mean $\pm$ SD ($n = 3-5$). (c,d) Transfection efficiency of mRNA-LNPs formulated with different PEG-lipid or polysarcosine-lipid types and molar percentages, measured as RLU in A549 cells, before (blue) and after (pink) nebulisation using ionisable lipid (c) SM102 and (d) ALC0315. Controls include media only, free mRNA, and Lipofectamine. Data are mean $\pm$ SD ($n = 3$).

With the DMG polymer conjugates, the names have been anonymised due to a current patent application. The current data show that among the DMG polymer conjugates, DMG-PY (the least hydrophilic) consistently offers the best stabilisation of nebulised mRNA LNPs, as evidenced by the smallest increase in particle size and lowest PDI after nebulisation, along with higher post-nebulisation transfection efficiency compared to DMG-PX and especially DMG-PZ. DMG-PX, with intermediate hydrophilicity, performs moderately, while DMG-PZ, the most hydrophilic, exhibits the highest size and PDI increases and the lowest transfection, particularly at higher (5%) molar ratios. These findings directly mirror the trends seen in the earlier graphs with PEG-lipids and DMG-pSar, where excessive hydrophilicity - whether from polysarcosine or highly hydrophilic polymer blocks - leads to greater LNP

destabilisation and reduced functional delivery after nebulisation. In both datasets, lower molar percentages (1.5%) of hydrophilic polymer consistently yield better stability and transfection. This pattern is consistent with the literature, which emphasises the need to balance hydrophilicity and surface anchoring for optimal LNP performance under aerosolisation stress. While DMG-PX and DMG-PY offer some advantages over traditional PEG-lipids in terms of initial colloidal stability and potential immunogenicity reduction, neither outperforms DSPE-PEG in maintaining both stability and transfection post-nebulisation, and DMG-PZ's extreme hydrophilicity is detrimental in this context.

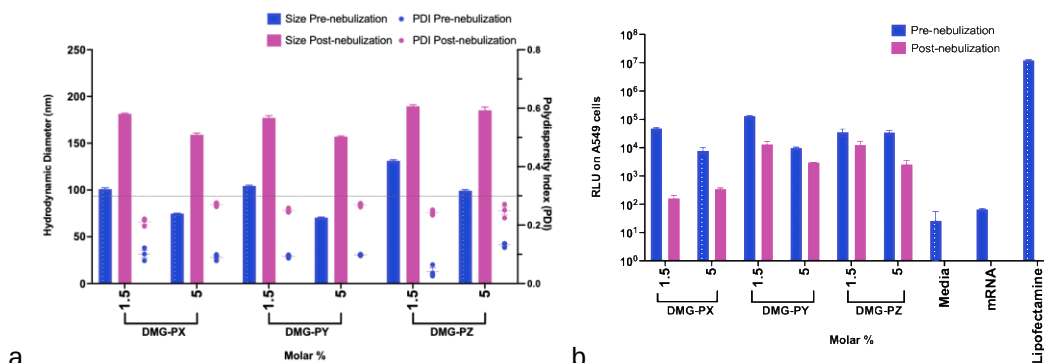


Figure 2. (a) Hydrodynamic diameter and PDI of mRNA-loaded LNPs formulated with DMG-PX, DMG-PY, or DMG-PZ at 1.5% and 5% molar ratios using SM102, measured before (blue) and after (pink) nebulisation. Bars represent mean hydrodynamic diameter (left y-axis), while dots indicate PDI values (right y-axis). (b) Transfection efficiency of mRNA-loaded LNPs containing DMG-PX, DMG-PY, or DMG-PZ at 1.5% and 5% molar ratios, assessed in A549 cells by measuring RLU before (blue) and after (pink) nebulisation. Controls include media only, free mRNA, and Lipofectamine. Data are mean $\pm$ SD ($n = 3$).

Conclusion Although none of the polymer-lipid constructs yet equal DSPE-PEG in stability-efficacy balance, their performance trajectories point toward tuneable chemical space rather than a categorical inferiority. Importantly, the current findings reaffirm DSPE-PEG as the immediate benchmark for nebulised mRNA LNP performance yet also expose its translational limitations. Despite its biophysical advantages, DSPE-PEG's strong anchoring prolongs systemic retention and is linked to anti-PEG antibody formation, complement activation, and hypersensitivity reactions which are effects incompatible with chronic pulmonary dosing where repeated administration is unavoidable. Polymer-lipids such as pSar- or PX/PY-type conjugates, even if less efficient now, offer routes to circumvent PEG-associated immune responses through alternative backbones, biodegradable linkers, or tuneable desorption kinetics. Their tunability enables balancing hydrophilicity and anchor strength to mimic DSPE-PEG-like stability while reducing immunogenic risk. Future efforts integrating biophysical analyses (e.g. SANS and cryo-EM) with iterative polymer design will be key to achieving this next generation of safe, high-performing non-PEG LNP coatings.

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