

The combination of PEG12KL4 peptide and lipid nanoparticles (LNPs) for pulmonary mRNA delivery via nebulisation

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Summary

The messenger RNA (mRNA) therapeutic strategy has garnered significant interest and attention for treating pulmonary diseases, owing to its potential to address various respiratory conditions effectively. PEG12KL4 is a cationic peptide that has demonstrated promising mRNA transfection capabilities in previous studies; however, it still faces notable challenges related to its ability to penetrate and transfect through the pulmonary mucus layer, which acts as a substantial barrier in pulmonary delivery. Lipid nanoparticles (LNPs) are among the most commonly used formulations for achieving efficient mRNA transfection in clinical applications. Nonetheless, it is reported that they are not stable enough to preserve the transfection efficiency of mRNA after nebulisation, which is critical for inhalation-based therapies. To address these issues, this study combined PEG12KL4 and LNP formulations to investigate their stability during nebulisation and their transfection capabilities in the presence of artificial mucus. The results indicated that LNPs containing PEG12KL4 exhibited improved transfection efficiency in artificial mucus compared to the complex formulations without PEG12KL4. Among the tested formulations, the LNPs containing 10% PEG12KL4 (P10NP3) demonstrated the highest stability to nebulisation within the LNPs formulations, revealing their robustness during aerosolisation and their potential for effective pulmonary mRNA delivery. These findings underscore the promising application of PEG12KL4-containing LNP formulations for advancing pulmonary mRNA therapeutics, offering a viable strategy to overcome mucus barrier challenges and improve transfection efficiency in respiratory treatments.

Key Message

This study revealed reliable stability during nebulisation and transfection ability in the mucus of PEG12KL4-containing LNP formulations.

Introduction

mRNA therapy has gained considerable attention for treating pulmonary diseases, such as cystic fibrosis and chronic obstructive pulmonary disease (COPD), due to its ability to produce and replace pathogenesis-related proteins [1]. Achieving effective mRNA delivery and transfection in the lungs requires aerosolization techniques and carriers to ensure the mRNA therapeutics reach the deep lungs and achieve cellular uptake. To achieve deposition in the airways, aerosolisation methods such as nebulisation are essential for generating droplets with size suitable for inhalation. Subsequently, the carrier system is needed to facilitate mRNA penetration through the pulmonary mucus layer and cell membranes to access the cytosol and exert its therapeutic effect. Therefore, an ideal pulmonary mRNA delivery system should be capable of maintaining mRNA stability, penetrating the lung mucus barrier, and achieving efficient transfection. Previously, PEG12KL4, a synthetic cationic peptide with PEG modifications, has demonstrated potential in maintaining mRNA stability and enabling transfection in the lungs [2]; however, mucus penetration remains a significant challenge that needs to be addressed. Lipid nanoparticles (LNPs) have shown potential in mucus penetration [3], but maintaining mRNA integrity during nebulisation remains problematic [4]. This study aimed to develop a combination of PEG12KL4 and LNP formulations to investigate their stability during nebulisation and their transfection efficiency in artificial mucus, providing insights into improving pulmonary mRNA delivery strategies.

Experiment methods

Materials

CleanCap FLuc mRNA was purchased from Trilink Biotechnologies as the mRNA model in this study. The PEG12KL4 peptide (PEG12-KLLLLKLLLLKLLLLK-LNH₂) was synthesized by EZBiolab Inc. Artificial sputum medium (ASM) was purchased from Biochemazone, Inc. Ionizable lipid SM-102 was bought from Cayman Chemical Company. Steric lipid DMG-PEG2000 was bought from Tocris Bioscience. Helper phospholipid DSPC was bought from Avanti Polar Lipids, Inc. Cholesterol was

purchased from Sigma-Aldrich, Co. LLC. Opti-MEM I Reduced Serum Medium (OptiMEM) and DMEM were purchased from Thermo Fisher Scientific Inc.

Preparation of peptide/siRNA complexes and LNPs

The PEG12KL4/mRNA complexes were prepared by mixing PEG12KL4 and mRNA at a mass ratio of 10:1 in ultrapure water with a pipette. To prepare the LNPs formulations, SM-102, DSPC, cholesterol, DMG-PEG2000, and PEG12KL4 were dissolved in ethanol as the organic phase. The mRNA was dissolved in 60 mM citrate buffer (at pH 4.0) as the aqueous phase (Table 1). PEG12KL4 was used to substitute for DMG-PEG2000 in the LNPs. LNP formulation similar to Moderna formulation (without PEG12KL4) was employed as the control for comparison in this study. The two phases were mixed at a volume ratio of 1:3 (organic: aqueous) manually using a pipette, with an N/P ratio of 6:1 for control LNPs and 3:1 (ionizable lipid to mRNA) for PEG12KL4-LNPs. The complexes and LNPs were incubated at room temperature for at least 30 minutes before proceeding to the next experiment.

Table 1. The formulations of LNPs containing PEG12KL4

Formulation ID	N/P	DSPC (mol%)	SM-102 (mol%)	Cholesterol (mol%)	PEG12KL4 (mol%)	DMG-PEG2000 (mol%)
Control LNP	6	10	50	38.5	-	1.5
P1.5NP3	3	10	50	38.5	1.5	-
P5NP3	3	10	50	35	5	-
P10NP3	3	10	50	30	10	-
P20NP3	3	10	50	20	20	-
P40NP3	3	10	50	0	40	-

Nebulisation

A vibrating mesh nebuliser, Aerogen Solo (Aerogen, Ireland), was employed to nebulise the prepared LNPs and complexes. The LNPs and complex solutions were loaded into the Aerogen Solo and nebulised into a 2 mL Eppendorf tube for the following experiments.

Hydrodynamic size and zeta potential

The size, polydispersity index (PDI), and zeta potential of the complexes and LNPs were measured with a dynamic light scattering method with ZetaSizer nano ZS (Malvern Panalytical, UK). To measure the particle size, the complexes and LNPs were diluted in ultrapure water (to a final mRNA concentration of 14 ng/μL). To measure the zeta potential, the complexes and LNPs were diluted in 10% (v/v) PBS (14 ng/μL mRNA).

In vitro mRNA transfection

A549 cells (human lung adenocarcinoma epithelial cells) were seeded in 24-well plates with DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic-antimycotic (100X) at a density of 5×10^4 cells/well 24 h before transfection. The medium was discarded and washed twice with PBS. Cells were incubated in OptiMEM or commercial ASM with LNPs and complexes containing 1 μg/well mRNA in an incubator with 37°C and 5% CO₂ environment. After 5 h of incubation, the medium was removed, and the cells were washed twice with PBS. The cells were cultured in complete culture medium for an additional 19 hours. The expression of luciferase in the cells was evaluated by the luciferase assay system (Promega Corporation, WI, USA). The relative luminescence unit (RLU) was calculated by dividing the luminescence intensity by the total protein mass, and the common logarithm of the RLU was calculated before the statistical analysis to fulfil the Gaussian normality test requirement.

Binding efficiency (BE) and encapsulation efficiency (EE)

To investigate the change in BE of complexes and EE of LNPs during nebulisation, complexes were dissociated in 10 mM SDS, and LNPs were lysed in 2% (v/v) Triton X-100 and 10 mM SDS, both with and without nebulisation. The mRNA content in the solutions was measured using the QuantiFluor RNA assay System (Promega Corporation, WI, USA) according to manufacture protocol. The BE and EE were calculated based on the proportion of free mRNA in the total mRNA content.

Statistical analysis

The statistical analysis was performed using two-way ANOVA followed by a Holm-Sidak post-hoc test to check the differences between groups using GraphPad Prism v10.3.0.

Results and discussion

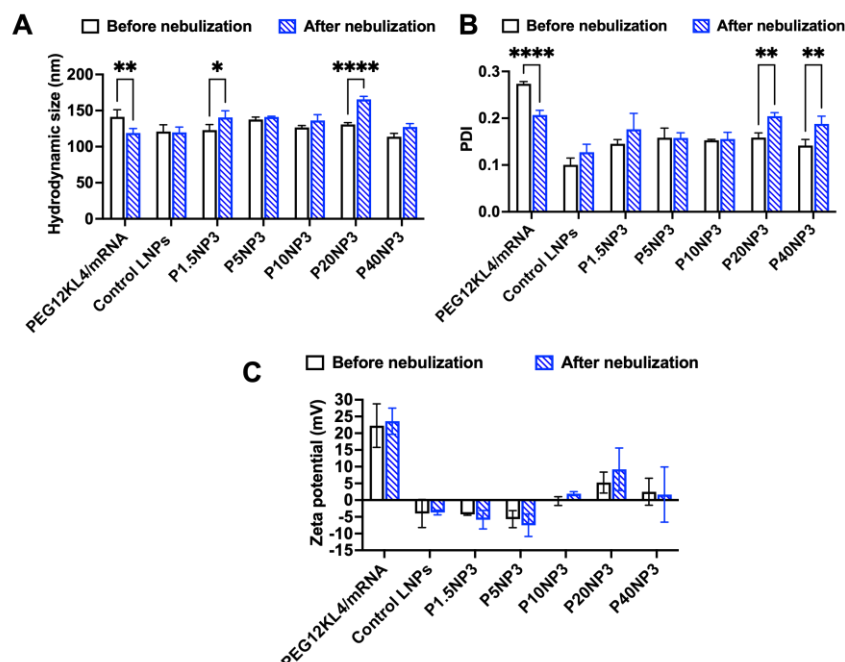


Fig. 1 Hydrodynamic size (A), polydispersity index (PDI) (B), zeta potential (C) of peptide/mRNA complexes and LNP formulations. The statistical analysis was performed using a Two-way ANOVA followed by a Holm-Sidak post-hoc analysis. (mean $\pm$ SD, n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.)

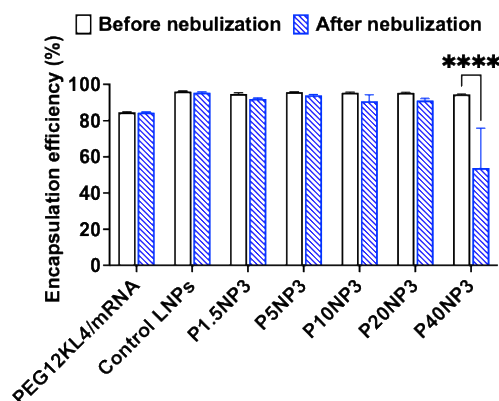


Fig. 2 Binding efficiency (BE) and encapsulation efficiency (EE) of peptide/mRNA complexes and LNP formulations. The statistical analysis was performed using a Two-way ANOVA followed by a Holm-Sidak post-hoc analysis. (mean $\pm$ SD, n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.)

The PEG12KL4/mRNA complexes and LNPs containing varying concentrations of PEG12KL4 were evaluated on how nebulisation affects their properties and transfection capabilities with and without ASM. Both complexes and LNPs initially exhibited a hydrodynamic size of approximately 140 nm (Fig. 1A). Following nebulisation, the complexes showed a significant reduction in particle size, whereas some LNP formulations, specifically P1.5NP3 and P20NP3, displayed a significant increase in particle size. These findings suggest that nebulisation may induce deaggregation of PEG12KL4/mRNA complexes [2] and promote fusion among LNPs [4]. Nebulisation caused an increase in the PDI of P20NP3 and P40NP3, indicating potential instability of these two LNP formulations during the nebulisation process (Fig. 1B). The complexes carried a positive surface charge. In contrast, the LNPs exhibited a neutral surface charge (Fig. 1C). The results suggest the successful encapsulation of PEG12KL4 within the

LNPs and nebulisation did not cause leakage of PEG12KL4. There was a significant decrease in the EE of the P40NP3 LNP formulation, suggesting that a high PEG12KL4 content may contribute to the instability of the LNPs during nebulisation (Fig. 2).

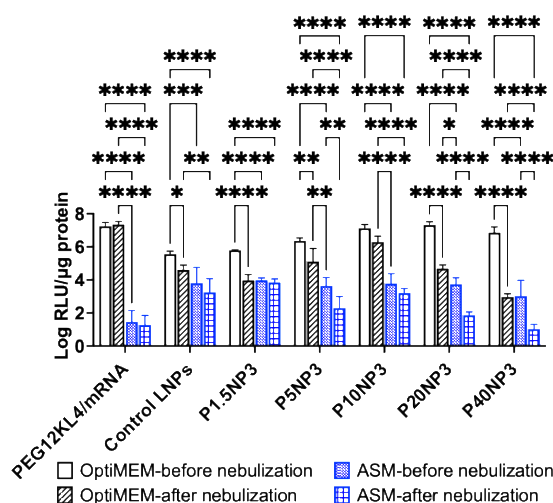


Fig. 3. In vitro transfection of A549 cells with peptide/mRNA complexes and LNP formulations. The statistical analysis was performed using a Two-way ANOVA followed by a Holm-Sidak post-hoc analysis. (mean $\pm$ SD, n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.)

The impact of nebulisation on the transfection efficiency of both complexes and LNPs in the presence or absence of artificial mucus was examined (Fig. 3). The complexes exhibited the highest transfection ability in mucus-free environment but the lowest in the presence of artificial mucus, highlighting the potential of LNP formulations to enhance mucus penetration of PEG12KL4 (Fig. 3). Notably, only the P10NP3 among LNP formulations showed no significant reduction in transfection efficiency in following nebulisation, either in the presence or absence of mucus. Furthermore, this formulation maintained consistent size distribution, zeta potential, and EE after nebulisation, indicating that a PEG12KL4 content of 10% (mol%) may confer the most stable LNP structures within the concentration range examined in this study.

Conclusions

In this study, PEG12KL4-containing complexes and LNP formulations were prepared for the delivery of mRNA in the lungs. The effects of nebulisation on their size distribution, zeta potential, encapsulation efficiency, and transfection ability in the presence of mucus were investigated. Results showed that the LNPs successfully demonstrated higher transfection efficiency in ASM compared to complexes alone. Variations in PEG12KL4 content influenced the properties and transfection capabilities of the LNPs. Notably, the P10NP3 LNP formulation exhibited the most stable characteristics in terms of size distribution, zeta potential, encapsulation efficiency, and transfection ability. These findings suggest that combining PEG12KL4 with LNP formulations holds promise as a pulmonary mRNA delivery system for future applications.

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