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Effective mRNA pulmonary delivery by dry powder formulation of PEGylated synthetic KL4 peptide

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ABSTRACT

Pulmonary delivery of messenger RNA (mRNA) has considerable potential as therapy or vaccine for a range of lung diseases. Inhaled dry powder formulation of mRNA is particularly attractive as it has superior stability and dry powder inhaler is relatively easy to use. A safe and effective mRNA delivery vector as well as a suitable particle engineering method are required to produce a dry powder formulation that is respirable and mediates robust transfection in the lung. Here, we introduce a novel RNA delivery vector, $PEG_{12}KL4$, in which the synthetic cationic KL4 peptide is attached to a monodisperse linear PEG of 12-mers. The $PEG_{12}KL4$ formed nano-sized complexes with mRNA at 10:1 ratio (w/w) and mediated effective transfection on human lung epithelial cells. $PEG_{12}KL4$/mRNA complexes were successfully formulated into dry powder by spray drying (SD) and spray freeze drying (SFD) techniques. Both SD and SFD powder exhibited satisfactory aerosol properties for inhalation. More importantly, the biological activity of the $PEG_{12}KL4$ /mRNA complexes were successfully preserved after drying. Using luciferase mRNA, the intratracheal administration of the liquid or powder aerosol of $PEG_{12}KL4$ /mRNA complexes at a dose of 5 μg mRNA resulted in luciferase expression in the deep lung region of mice 24 h post-transfection. The transfection efficiency was superior to naked mRNA or lipoplexes (Lipofectamine 2000), in which luciferase expression was weaker and restricted to the tracheal region only. There was no sign of inflammatory response or toxicity of the $PEG_{12}KL4$ /mRNA complexes after single intratracheal administration. Overall, $PEG_{12}KL4$ is an excellent mRNA transfection agent for pulmonary delivery. This is also the first study that successfully demonstrates the preparation of inhalable dry powder mRNA formulations with *in vivo* transfection efficiency, showing the great promise of $PEG_{12}KL4$ peptide as a mRNA delivery vector candidate for clinical applications.

1. Introduction

The use of nucleic acids to manipulate gene expression is a powerful therapeutic strategy for the treatment of many diseases. One example is to exploit messenger RNA (mRNA) to produce therapeutic proteins *in vivo* [1]. The successful uses of *in vitro* transcribed mRNA techniques for the production of proteins in animals were first reported in the 1990s [2,3], but these early promising results did not immediately translate into clinic benefits due to the concerns with mRNA instability, risk of innate immunogenicity and inefficient *in vivo* delivery. Thanks to the advancement of biotechnological innovation in the last decade, chemically modified mRNA can now be produced with enhanced stability and reduced immunogenicity, as well as increased protein expression compared to the unmodified mRNA [4,5]. However, safe and efficient *in vivo* delivery remains a major obstacle in mRNA therapeutics development.

mRNA therapeutics have potential for the treatment of various lung diseases such as cystic fibrosis, asthma and lung cancer [6–8]. Local administration of mRNA by inhalation for treatment of lung diseases is desirable due to its non-invasive nature, increased local drug concentration and reduced systemic side effects, hence improving treatment efficacy. In particular, dry powder formulation is highly desirable for pulmonary delivery. While liquid aerosol can be delivered to the lungs of patients through nebulization, dry powder formulation of

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nucleic acids offers several additional advantages such as superior stability, better sterility and longer shelf-life [9]. Dry powder inhalers are also cheaper and easier to operate than nebulizers. However, formulating mRNA into dry powder aerosol is highly challenging. The powder must be highly dispersible and exhibit good aerodynamic properties for effective lung deposition. Generally, particles with aerodynamic diameter between 1–5 μm are optimal for effective lung deposition [10–12]. The integrity and biological activity of the mRNA must also be preserved during the drying process, considering that the long single-stranded mRNA molecule is fragile and labile to thermal and shear stresses [13,14]. Although there are few papers reported the liquid aerosol formulation of mRNA [8,15,16], inhaled dry powder formulations of mRNA to produce therapeutic proteins for pulmonary delivery have not been reported so far.

Our group has previously demonstrated that pulmonary surfactant protein B (SP-B) mimic KL4 peptide is a promising non-viral vector for pulmonary RNA delivery [17]. The synthetic, cationic KL4 peptide can bind with small interfering RNA (siRNA) to form nanosized complexes and mediate effective transfection in lung epithelial cells through endocytosis. However, the clinical application of KL4 peptide as delivery vector is hindered by its poor solubility due to the presence of hydrophobic leucine residues in the sequence. In this study, we aim to further develop the KL4 peptide system for mRNA delivery. To overcome the solubility problem, we adopt the PEGylation strategy [18] in which the hydrophilic polyethylene glycol (PEG) is covalently attached to the KL4 peptide. Furthermore, we investigated two particle engineering techniques, namely spray drying (SD) and spray freeze drying (SFD), to produce inhaled powder formulation of mRNA. The physicochemical properties, aerosol performance, transfection efficiency and the safety profile of the formulations were thoroughly evaluated. The overall goal is to develop a safe, stable and reliable delivery platform for robust mRNA transfection in the airways that could be applied for the treatment of a range of respiratory diseases or mRNA vaccines.

2. Materials and methods

2.1. Materials

KL4 peptide (KLLLLKLLLLKLLLLKLLLLK- NH_2) was purchased from ChinaPeptides (Shanghai, China). PEG_{12}KL4 peptide (with monodisperse dodecaethylene glycol, PEG_{12}) were purchased from EZBiolab (Carmel, NJ, USA) with purity > 90%. The KL4 peptide stock solution was prepared at 1 mg/mL in 1% (v/v) DMSO. The PEG_{12}KL4 stock solution was prepared at 2 mg/mL in distilled water. CleanCap® firefly luciferase mRNA and cyanine-5 EGFP mRNA were purchased from TriLink Bio Technologies (San Diego, CA, USA). The mRNA stock solution was prepared at 1 mg/mL in 1 mM sodium citrate buffer. Dulbecco's modified Eagle's medium (DMEM), Keratinocyte-SFM, Roswell Park Memorial Institute (RPMI) 1640, OptiMEM I reduced serum medium, trypsin-EDTA (0.25%), Fetal Bovine Serum (FBS), Antibiotic - Antimycotic (100×), Lipofectamine 2000, DNA Gel Loading Dye (6×), Hoechst 33,258 were purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA). GelRed nucleic acid stain was purchased from Biotium (Hayward, CA, USA). The luciferase assay system and beetle luciferin potassium salt were purchased from Promega (Madison, WI, USA). Human tumor necrosis factor-alpha (TNF- α), monocyte chemoattractant protein-1 (MCP-1) ELISA kits were purchased from BD Biosciences (New Jersey, NY, USA). Human interleukin-8 (IL-8) ELISA kit, mouse tumor necrosis factor-alpha (TNF- α), monocyte chemoattractant protein-1 (MCP-1), and interleukin-8 (IL-8) ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Mannitol (Pearlitol 160C) was obtained from Roquette (Lestrem, France). Lipopolysaccharide (LPS) from E. coli O111:B4 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were obtained from Sigma-Aldrich (Saint Louis, MO, USA) as analytical grade or better.

2.2. Gel retardation assay

Gel retardation assay was carried out to examine the mRNA binding affinity of KL4 and PEG_{12}KL4 peptides. Both KL4/mRNA and PEG_{12}KL4/ mRNA complexes were prepared from 0.5:1 to 10:1 peptide to mRNA ratios (w/w), with 1 μg of mRNA in 10 μL of TAE buffer. The complexes were incubated at room temperature for 30 min, followed by the addition of 2 μL of gel loading dye. The complexes were loaded into a 2% (w/v) agarose gel stained with GelRed. Electrophoresis was run in TAE buffer at 125 V for 25 min. The gel was visualized under the UV illumination. For mRNA release study, both KL4/mRNA and PEG_{12}KL4/mRNA complexes were prepared at 10:1 ratio (w/w). At 30 min after complexes formation, different concentrations of sodium dodecyl sulfonate (SDS) solution from 1 to 8 mM were added, and the mixtures were incubated at room temperature for 30 min. The samples were loaded into agarose gel and electrophoresis was performed as described above.

2.3. Particle size and zeta potential measurement

For particle size measurement KL4/mRNA and PEG_{12}KL4/mRNA complexes were prepared at 10:1 ratio (w/w) with 4 μg of mRNA in 100 μL of ultrapure water. At 30 min after complexes formation, the hydrodynamic size was measured by dynamic light scattering (Delsa™Nano C, Beckman Coulter, CA, USA). For zeta potential measurement, KL4/mRNA and PEG_{12}KL4/mRNA complexes were prepared at 10:1 ratio (w/w) with 20 μg of mRNA in 500 μL of 2% PBS. At 30 min after complexes formation, the zeta potential was measured in a flow cell using electrophoretic light scattering (Delsa™Nano C, Beckman Coulter, CA, USA). The size and zeta potential of PEG_{12}KL4/mRNA complexes in the SD and SFD powder formulations were also measured after reconstitution.

2.4. Cell culture

A549 cells (human alveolar epithelial adenocarcinoma), BEAS-2B cells (human bronchial epithelial cells) and THP-1 cells (human monocyte) were obtained from ATCC (Manassas, VA, USA). A549 cells were cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic. BEAS-2B cells were cultured in Keratinocyte-SFM supplemented with human recombinant Epidermal Growth Factor (rEGF), Bovine Pituitary Extract (BPE), and 1% (v/v) antibiotic-antimycotic. THP-1 cells were cultured in RPMI-1640 supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic. All the cells were maintained at 5% CO_2, 37 °C, and subcultured according to ATCC instruction.

2.5. mRNA transfection in vitro

A549 cells and BEAS-2B cells were seeded in 24-well plates for overnight at a density of 0.5×10^5 and 1×10^5 cells per well, respectively. KL4/mRNA and PEG_{12}KL4/mRNA complexes formed at ratios from 5:1 to 30:1 (w/w) in OptiMEM I reduced serum medium were added to the cells at 1 μg mRNA per well. Naked mRNA and lipoplexes (Lipofectamine 2000/mRNA complexes) were used as controls. After 5 h of incubation, the transfection medium was replaced with cell culture medium. At 24 h post-transfection, the cells were washed and lysed with reporter cell lysis buffer. The luciferase expression was detected using the luciferase assay system according to the manufacturer's protocol. The luminescence was measured with luminometer (SpectraMax L Microplate Reader, Molecular devices, CA, USA). The protein concentrations of the cell lysates were quantified by Bradford protein assay. The results were expressed as relative light unit (RLU) per mg of total protein. To study the transfection efficiency of PEG_{12}KL4/mRNA as SD and SFD powder formulations, the powders were reconstituted before added to the cells. The luciferase expression

was examined at 24 h post-transfection as described above.

2.6. Cellular uptake study

The cellular uptake of mRNA was studied by flow cytometry and confocal microscopy. For the flow cytometry study, A549 cells were seeded in 24-well plates at a density of 0.5×10^5 cells per well one day before the experiment. The cells were transfected with naked mRNA, KL4/mRNA and PEG_{12}KL4/mRNA complexes at 10:1 ratio (w/w) in OptiMEM I reduced serum medium containing 1 μg of cyanine-5 labelled EGFP mRNA per well. After 4 h of incubation, the cells were washed and trypsinized. Cells from three separate wells of the same treatment were combined and suspended in culture medium. The extracellular florescent signal was quenched with 0.04% (w/v) trypan blue solution. After 2 min, the cells were washed, resuspended in 300 μL of PBS and sieved with a sterile 40 μm cell strainer (BD Biosciences, CA, USA). The fluorescence intensity was analyzed by flow cytometry (BD FACSCantoII Analyzer, BD Biosciences, CA, USA). At least 1×10^4 single cells were analyzed for each sample. For the confocal study, cells were imaged at 4 or 24 h post-transfection. A549 cells were seeded in a 35 mm Mattek glass bottom culture dish (Mattek Corp. Ashland, MA, USA) at a density of 1×10^5 cells per well. Naked mRNA, KL4/mRNA and PEG_{12}KL4/mRNA complexes at 10:1 ratio (w/w) were prepared in Opti-MEM I reduced serum medium and were added to the cells at 2 μg of cyanine-5 labelled mRNA per dish. After 3 to 4 h of incubation, the transfection medium was removed and replaced with culture medium. At 30 min prior to imaging, the nuclei of the cells were labelled with Hoechst stain (5 μg/mL). The cells were visualized by the confocal laser scanning microscope (Zeiss LSM 780 inverted microscope, Jena, Germany).

2.7. In vitro inflammatory study

THP-1 cells were seeded in 24-well plates at 2×10^5 cells per well. Cells were differentiated into macrophage-like cells with 100 nM phorbol 12 myristate 13-acetate (PMA) for 48 h. A549 cells were seeded in 24-well plates at 1×10^5 cells per well. Before the experiment, the cells were starved overnight with fresh medium supplemented with 1% FBS. The cells were then incubated with PEG_{12}KL4/mRNA complexes prepared at 10:1 ratio (w/w) containing 0.25–2 μg mRNA per well in OptiMEM I reduced serum medium. The level of TNF-α, MCP-1, and IL-8 secreted in cell medium were measured by ELISA at 24 h post-transfection. Untreated cells and cells treated with LPS (at 10 and 100 ng/mL for THP-1 cells; at 10 and 100 μg/mL for A549 cells) were used as negative and positive controls, respectively.

2.8. Preparation of dry powder formulations

PEG_{12}KL4/mRNA complexes were prepared at 10:1 ratio (w/w) in ultrapure water. Mannitol (as bulking excipient) was dissolved in water and added to the complexes. The SD and SFD formulations were prepared at 1.5% and 3% (w/v) solute concentrations, respectively, with mRNA concentrations at 0.1% or 0.5% (w/w). Mannitol-only formulations were prepared for comparison. The operation parameters for SD and SFD were adopted from our previous studies [19,20]. For the preparation of SD powder, the solutions were spray dried using a laboratory scale spray dryer with a high performance cyclone in suction mode and closed loop configuration (Mini Spray Dryer B-290 and Dehumidifier B-296; Büchi Labortechnik, Flawil, Switzerland) under the following operating conditions: inlet temperature of 80 °C (outlet temperature of around 50 °C), rate of aspiration at 90% (approximately 35 m^3 /h), liquid feed rate of 1.4 mL/min and compressed air atomization flow rate at 742 L/h. A two-fluid nozzle with an internal diameter of 0.7 mm was used (Büchi stainless steel two-fluid nozzle, Switzerland). For the preparation of SFD powder, the solutions were transferred into a syringe and atomized by the two-fluid nozzle with nitrogen gas flow rate of 601 L/h. The liquid feed rate was 1.5 mL/min. The atomized liquid droplets were frozen and collected in liquid nitrogen. The samples were subjected to freeze drying (FreeZone® 6 L Benchtop Freeze Dry System with Stoppering Tray Dryer, Labconco Corporation, MO, USA) in which the samples were kept under vacuum (chamber pressure below 0.133 mBar) at −25 °C for 40 h, followed by a secondary drying at 20 °C for 20 h. All the dried powders were collected in glass vials and stored in a desiccator with silica gel at ambient temperature until further analysis. One batch of powder was prepared for each formulation. A summary of the drying methods and composition of the dry powder formulations was shown in Table 1. The yield was obtained by dividing the mass of dry powder recovered by the total amount of input material.

Table 1
Summary of spray dried (SD) and spray freeze dried (SFD) formulations of PEG_{12}KL4/mRNA complexes (at 10:1 ratio). Mannitol-only formulations were also prepared as controls.

Formulation	Drying method	% by weight (w/w)			Yield (%)
		mRNA	PEG_{12}KL4/mRNA	Mannitol	
SD-Mannitol	SD	0	0	100	45.8
SD-0.1% mRNA		0.1	1	98.9	75.8
SD-0.5% mRNA		0.5	5	94.5	59.0
SFD-Mannitol	SFD	0	0	100	84.7
SFD-0.1% mRNA		0.1	1	98.9	78.0
SFD-0.5% mRNA		0.5	5	94.5	82.2

2.9. Morphology and aerosol performance of powder formulations

Morphology of SD and SFD powders was visualized using field emission scanning electron microscopy (SEM; Hitachi S-4800 FEG, Hitachi, Tokyo, Japan). Powder samples were sprinkled onto carbon adhesive tape that was mounted on SEM stubs. Excess powders were removed by blowing with clean compressed air. Prior to imaging, the powders were sputter coated with approximately 11 nm gold-palladium alloy in two cycles to avoid overheating. Aerosol performance of the powder formulations was evaluated by the Next Generation Impactor (NGI; Copley, Nottingham, UK) in accordance to the British Pharmacopoeia [21]. For each dispersion, approximately 8.5 ± 0.5 mg and 5.0 ± 0.5 mg of SD and SFD powders, respectively, were loaded in a size 3 hydroxypropyl methylcellulose capsule (Capsugel, West Ryde, NSW, Australia), which was placed in a Breezhaler® (Novartis Pharmaceuticals, Hong Kong). The flow rate and dispersion duration were 90 L/min and 2.7 s, respectively. Prior to each dispersion, a thin layer of silicon grease (LPS Laboratories, Illinois, GA, USA) was coated onto the impactor stages to reduce particle bounce. After dispersion, the powders deposited on the inhaler and NGI stages were collected by rinsing with 4 mL of ultrapure water. Recovered dose was defined as the sum of powder mass assayed on inhaler and all NGI stages in a single run, as calculated with the assayed mannitol obtained from the liquid chromatography (described in the next section). The emitted fraction (EF) referred to the fraction of powder that exited the inhaler with respect to the recovered dose. Fine particle fraction (FPF) was the fraction of powder with aerodynamic diameter < 5.0 μm with respect to the recovered dose. The mass median aerodynamic diameter (MMAD) together with the geometric standard deviation (GSD) were also calculated.

2.10. High performance liquid chromatography (HPLC)

The amount of mannitol (which contributed to at least 94.5% by mass in all formulations) in the dispersed samples of NGI was quantified using HPLC (Agilent 1260 Infinity; Agilent Technologies, Santa Clara,

CA, USA) with a refractive index detector (RID G1362A; Agilent Technologies). Filtered samples with 50 μL in volume were injected and passed through a two ion-exchange ligand-exchange column (Agilent Hi-Plex H column, 7.7 × 50 mm, 8 μm; Agilent Technologies) maintained at 75 °C with ultrapure water running at a flow rate of 0.6 mL/min as the mobile phase. The actual mass of powder deposited in various stages of the NGI was calculated based on the formulation compositions.

2.11. Animals

Female BALB/c mice with average age of 8 to 9 weeks and body weight of 18 to 22 g were used. The mice were housed under a 12 h dark-light cycle at a constant temperature and with *ad libitum* feeding on tap water and standard chow. All mice were obtained from the Laboratory Animal Unit (The University of Hong Kong). All experiments conducted were approved by the Committee on the Use of Live Animals for Teaching and Research (CULATR), The University of Hong Kong.

2.12. Intratracheal administration

Before intratracheal administration, the mice were anaesthetized with intra-peritoneal injection of anaesthetics (80 mg/kg ketamine and 4.5 mg/kg xylazine). A guiding cannula was intubated gently inside the trachea. The liquid or dry powder formulations were administered to the mice intratracheally through the guiding cannula. For liquid aerosol administration, the sample was loaded into a high-pressure syringe (Model FMJ-250; PennCentury Inc., Wyndmoor, PA, USA) and the liquid aerosol was generated by the Microsprayer® Aerosolizers (model IA-1C; PennCentury Inc., Wyndmoor, PA, USA). For powder formulations, the samples were loaded into a 200 μL gel-loading pipette tip which was connected to a 1 mL syringe by a three-way stopcock as previously described [22], and the powder was dispersed with 0.6 mL of air from the syringe.

2.13. mRNA transfection in vivo

In vivo mRNA transfection of $PEG_{12}KL4$/mRNA complexes was carried out with liquid or powder aerosol in mice. For liquid formulations, $PEG_{12}KL4$/mRNA complexes prepared at 10:1 ratio (w/w) containing 5 or 10 μg mRNA in PBS with a final volume of 75 μL were administered as a single dose. Naked mRNA or lipoplexes (Lipofectamine 2000/mRNA complexes at 2:1 ratio w/w), both containing 10 μg mRNA, were used for comparison. For powder formulations, approximately 1 mg of SD-0.5% powder or SFD-0.5% powder (both containing 5 μg mRNA) were administered as a single dose. At 24 h post-administration, luciferin solution was administered intraperitoneally to the mice at a dose of 150 mg/kg body weight under the lethal dose of phenobarbital. The lungs were harvested 10 min after luciferin injection, and bioluminescence imaging of the lungs was performed with an IVIS Spectrum in vivo imaging system (PerkinElmer, USA). The lung tissues were then homogenized and lysed in reporter cell lysis buffer. The samples were centrifuged at 1500 g and 4 °C for 10 min. The luciferase expression in the supernatant was detected using the luciferase assay system as mentioned above. The results were expressed as RLU per mg of total protein.

2.14. Inflammatory and histological study

For inflammatory study, the mice were intratracheally administered with PBS as control, LPS (10 μg), naked mRNA (5 or 10 μg) and $PEG_{12}KL4$/mRNA complexes at ratio 10:1 (w/w) (5 or 10 μg mRNA). All the samples were prepared in 75 μL of PBS and dispersed by Microsprayer® Aerosolizers except LPS which was prepared in 25 μL of PBS and delivered by micropipette. At 24 h post-administration, the mice were injected intraperitoneally with a lethal dose of pentobarbital. The bronchoalveolar lavage fluid (BALF) and the lung tissues were collected. The expression of TNF-α, MCP-1, KC and IL-6 in BALF and lung homogenates were measured by ELISA. For histological study, the mice were intratracheally administered with PBS, LPS (10 μg), naked mRNA (5 μg), $PEG_{12}KL4$/mRNA complexes at ratio 10:1 (w/w) (5 μg mRNA), SD-0.5% mRNA powder (1 mg) and SFD-0.5% mRNA powder (1 mg). Naïve mice without any treatment were also included for comparison. At 24 h post-administration, the mice were injected intraperitoneally with a lethal dose of pentobarbital. The lungs were collected and gently inflated with 4% buffered formalin before fixation in formalin for 24 h. The left lobe of the lung was transferred to 80% of ethanol until they were embedded in a paraffin block. Sections of embedded tissue were mounted on slides and stained with hematoxylin and eosin (H&E). Slides were viewed with an upright microscope (Olympus BX50, Tokyo, Japan) using a UPlanFI 20x/0.5 objective. The images were taken by a digital camera (Sony NEX-6, Tokyo, Japan).

2.15. Statistical analysis

A statistical test was carried out using Prism software version 6 (GraphPad Software Inc., San Diego, CA, USA) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's, Dunnett's or Sidak's post-hoc test unless specified. Differences were considered as statistically significant at $p < 0.05$.

3. Results

3.1. Physicochemical properties of peptide/mRNA complexes

The mRNA binding of peptides was evaluated by gel retardation assay (Fig. 1). The mRNA band intensity decreased as the peptide to mRNA ratio (w/w) increased. For $PEG_{12}KL4$, complete binding was observed at 2.5:1 ratio at which the mRNA band was no longer visible. Compared to KL4 peptide, in which complete binding was achieved at a slightly lower ratio of 2:1, PEGylation did not have a major impact on mRNA binding. The binding affinity was further studied by using SDS to dissociate the complexes and displace the mRNA through competitive binding. The $PEG_{12}KL4$/mRNA complexes were dissociated by 2 mM of SDS as indicated by the presence of mRNA band, whereas the dissociation of the KL4/mRNA complexes required higher concentration of SDS at 4 mM, suggesting a stronger association between mRNA and KL4. The particle size and zeta potential of the KL4/mRNA and $PEG_{12}KL4$/mRNA complexes prepared at 10:1 ratio (w/w) were measured (Table 2). The hydrodynamic diameter of the freshly prepared $PEG_{12}KL4$/mRNA complexes was around 468 nm which was similar to the complexes in SD-0.5% mRNA powder formulations after reconstitution, which was around 432 nm. The particle size of the reconstituted SFD-0.5% mRNA powder formulation was around 375 nm, which was significantly smaller than the freshly prepared complexes. In addition, the freshly prepared $PEG_{12}KL4$/mRNA complexes were significantly larger than the KL4/mRNA complexes which were around 131 nm. The polydispersity index (PDI) of all the samples were similar, from 0.24 to 0.30. The zeta potential of KL4/mRNA and $PEG_{12}KL4$/mRNA complexes were around +26 mV and +27 mV, respectively, which were highly similar to each other. After reconstitution of SD-0.5% mRNA and SFD-0.5% mRNA powder formulations, the zeta potential of the complexes was found to be +28 mV and +31 mV, respectively, which were also similar to the freshly prepared complexes albeit a small increase. The results showed that the physicochemical properties of the $PEG_{12}KL4$/mRNA complexes were not significantly affected by the two drying methods.

3.2. In vitro mRNA transfection, cellular uptake and inflammatory study

mRNA transfection efficiency of the $PEG_{12}KL4$ peptide was studied

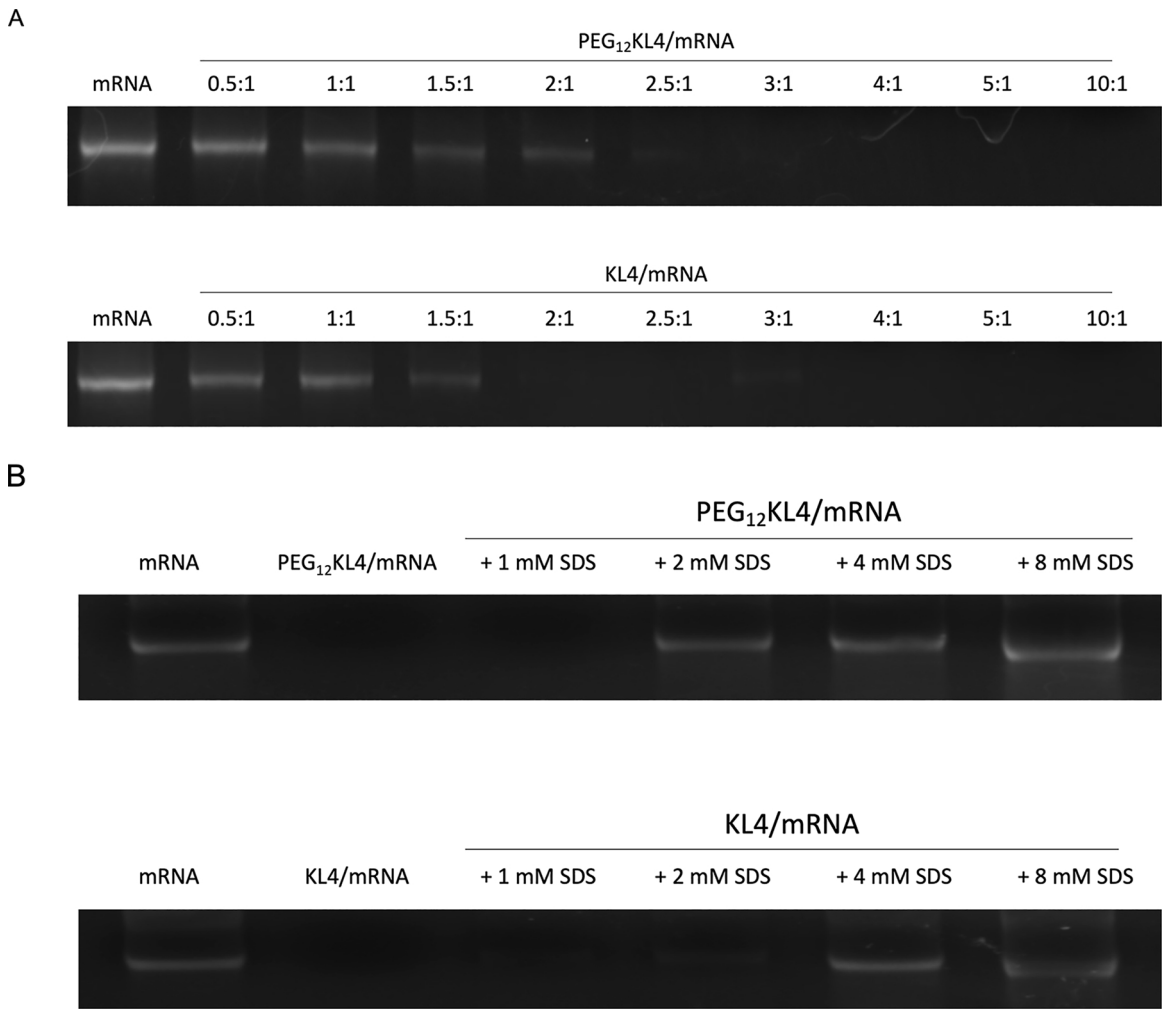

Fig. 1. Gel retardation assay of (A) mRNA binding and (B) mRNA release. For mRNA binding, KL4/mRNA and PEG_{12}KL4/mRNA complexes were prepared at 0.5:1 to 10:1 ratio (w/w). For mRNA release, the complexes were prepared at 10:1 ratio (w/w) and sodium dodecyl sulphate (SDS) solutions at 1–8 mM were added to dissociate the complexes. Unbound mRNA was included as control.

on two human lung epithelial cell lines, A549 and BEAS-2B cells (Fig. 2). The result was compared with KL4 peptide and the commercial transfection agent Lipofectamine 2000. A similar trend was observed on both cell lines. For PEG_{12}KL4 peptide, the transfection efficiency was improved significantly when the peptide to mRNA ratio increased from 5:1 to 10:1, but no further improvement was noticed at higher ratios. In general, PEG_{12}KL4 peptide performed significantly better than KL4 peptide at the corresponding ratios with a 1- to 2-log increase in luciferase expression, suggesting that PEGylation indeed improved mRNA transfection efficiency of KL4 peptide. In addition, the mRNA transfection efficiency of PEG_{12}KL4 peptide was comparable to that of Lipofectamine 2000. To further compare the cellular uptake efficiency of KL4/mRNA and PEG_{12} KL4/mRNA complexes, flow cytometry and confocal imaging studies were carried out on A549 cells (Fig. 3). Over 60% of cells showed uptake of mRNA mediated by PEG_{12}KL4, whereas only 15% of cells showed mRNA uptake by KL4. Both the percentage of cell uptake and the median fluorescence intensity of the PEG_{12}KL4/mRNA were significantly higher than that of naked mRNA and KL4/mRNA complexes. The confocal images showed that the naked mRNA could not enter the cells as expected. Both KL4/mRNA and PEG_{12}KL4/mRNA complexes were taken up by the cells as demonstrated by the intracellular red fluorescent signal after 4 h. Strong red signal of mRNA could still be observed at 24 h post-transfection in cells treated with KL4/mRNA complexes while the signal was less prominent in cells treated PEG_{12}KL4/mRNA complexes. The green fluorescence was present in the PEG_{12}KL4/mRNA treated cells at 4 h and 24 h post-transfection, but not in the KL4/mRNA transfected cells, suggesting that PEG_{12}KL4 was more efficient in mediating EGFP mRNA transfection.

Table 2
Particle size and zeta potential of KL4/mRNA and PEG_{12}KL4/mRNA complexes. The complexes were prepared at 10:1 ratio (w/w). Spray dried (SD) and spray freeze dried (SFD) samples were reconstituted prior to measurement. The data was analysed by one-way ANOVA followed by Dunnett's post-hoc test as compared with the freshly prepared PEG_{12}KL4/mRNA complexes, ***$p < 0.01$, ****$p < 0.0001$. The data was prepared as mean ± standard deviation (n = 3).

	Hydrodynamic diameter (nm)	Polydispersity index	Zeta potential (mV)
KL4/mRNA complexes	131.18 ± 20.96****	0.24 ± 0.05	+25.81 ± 2.26
PEG_{12}KL4/mRNA complexes	467.93 ± 24.93	0.24 ± 0.02	+ 26.50 ± 2.69
Reconstituted SD-0.5 % mRNA	432.03 ± 13.62	0.27 ± 0.01	+ 27.58 ± 0.83
Reconstituted SFD-0.5 % mRNA	375.03 ± 9.90***	0.30 ± 0.01	+ 30.58 ± 2.07

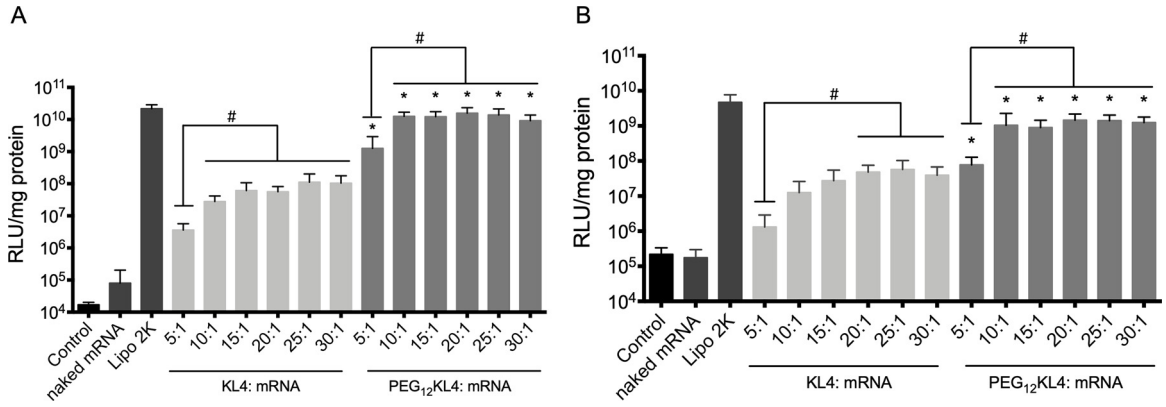

Fig. 2. Luciferase mRNA transfection on (A) A549 cells and (B) BEAS-2B cells. KL4/mRNA or PEG_{12}KL4/mRNA complexes were prepared from 5:1 to 30:1 ratio (w/w). Untreated cells, naked mRNA and Lipofectamine 2000 (Lipo2K)/mRNA complexes (2:1 w/w) were included as controls. Luciferase expression was measured at 24 h post-transfection. The data was expressed as the mean value of relative light unit (RLU) per mg of protein ± standard deviation (n = 3). The data were analyzed by one-way ANOVA followed by Sidak post-hoc test when comparing the KL4/mRNA complexes and the PEG_{12}KL4/mRNA complexes prepared at the same ratio, *$p < 0.05$; and one-way ANOVA followed by Tukey post-hoc test among all the tested ratios of each peptide, #$p < 0.05$.

The *in vitro* inflammatory response of the PEG_{12}KL4 peptide was examined on A549 and THP-1 cells (Fig. 4). The levels of cytokines including MCP-1, TNF-α and IL-8 released from the transfected cells were not significantly different from that of the negative control but were significantly lower than that of the LPS treated samples, suggesting that PEG_{12}KL4 peptide/mRNA complexes did not induce an inflammatory response *in vitro*.

3.3. Morphology, aerosol performance and in vitro mRNA transfection of powder formulations

The morphology of PEG_{12}KL4/mRNA dry powder formulations was examined with SEM (Fig. 5). All the SD formulations appeared to be spherical in shape, and the geometric size of particles was well below 5 μm. There were no striking differences in appearance between the SD-0.1% mRNA and the SD-0.5% mRNA formulations, although the surface of both mRNA containing particles appeared to be rougher than the mannitol only (SD-mannitol) formulation. The particles prepared by SFD were much larger in size of over 10 μm in diameter. These particles were highly porous with small amount of debris noticed in the SEM images. The particles containing mannitol only (SFD-mannitol) were highly aggregating and clumped together. On the other hand, the particles of the SFD-0.5% mRNA formulation appeared to be more discrete and spherical. The presence of peptide/mRNA complexes appeared to increase the physical robustness of the SFD particles.

The aerosol performance of the dry powder formulations was evaluated by the NGI within one month of preparation, and the results were expressed in terms of EF and FPF (Fig. 6). EF which indicates the amount of powder successfully exited the inhaler was satisfactory for all the formulations, with a value of at least 75% or above. FPF represents the respirable fraction of the powder. For the SD formulations, the presence of peptide/mRNA complexes lower the aerosol performance of the powder, with a reduction of FPF compared to the SD-mannitol formulation. The FPF of the SD-mannitol formulation was 60%, and the value decreased to 36% and 41% for the SD-0.1% mRNA and SD-0.5% mRNA formulations, respectively. An opposite trend was observed with the SFD preparations. The FPF of SFD-mannitol was 44%, and the value increased to 62% and 68% for the SFD-0.1% mRNA and SFD-0.5% mRNA formulations, respectively. Overall, the SFD formulations containing peptide/mRNA complexes performed significantly better, both EF and FPF, than their SD counterparts. The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) were calculated based on the NGI data (Table 3). The MMAD of all the formulations were less than 6 μm with GSD less than 5. For both drying methods, the 0.5% mRNA formulation exhibited a smaller MMAD and higher FPF compared to the 0.1% mRNA formulation prepared by the same method, suggesting that formulation containing a higher amount of PEG_{12}KL4/mRNA complexes exhibited better aerosol characteristics for inhalation. The aerosol performance of both SD and SFD powder formulations was also evaluated after six months of storage at room temperature (**Supplementary Fig. S1**). There was no significant difference in terms of EF and FPF between the two time points (i.e. within one month and after six months) of the same formulation, indicating that the aerosol properties of the powder remained stable for at least six months.

To examine the integrity of the mRNA after drying, *in vitro* transfection was carried out with SD and SFD formulations on A549 cells (Fig. 7). The transfection efficiency of all the dry powder formulations was successfully demonstrated. There was no significant difference between the freshly prepared complexes, the samples before and after SD and SFD, indicating that the mRNA remained intact and both of the processes did not compromise their biological activity.

3.4. In vivo mRNA transfection

Initially, the *in vivo* mRNA transfection efficiency of PEG_{12}KL4/mRNA complexes was evaluated with different peptide to mRNA ratios (2.5:1, 5:1 and 10:1 w/w) and at different time-points (4 and 24 h) following intratracheal administration as liquid aerosol in mice. The highest luciferase expression was observed with the complexes formed at 10:1 ratio (**Supplementary Fig. S2**), which was also consistent with the *in vitro* transfection study. Hence, the 10:1 ratio was adopted in the subsequent *in vivo* studies as well as the preparation of dry powder formulations. The luciferase expression in the lung was found to be higher at 24 h post-administration (**Supplementary Fig. S3**), which was used in the subsequent *in vivo* studies. Luciferase expression was shown in the lungs of mice treated with naked mRNA (Fig. 8), but the luminescence was restricted to the trachea region only. On the other hand, the luciferase expression was observed in the lobes of the lungs in mice treated with PEG_{12}KL4/mRNA complexes, and the luciferase expression in the lung tissues was much higher than the naked mRNA group. Similar to the naked mRNA group, the group treated with lipoplexes also demonstrated luciferase expression. However, the expression was limited to the trachea only, and the level of expression was even lower than that of the naked mRNA group. A biodistribution study was carried out at 4 h post-administration using cyanine-5 labelled mRNA to locate the site of aerosol deposition in the lung following intratracheal administration (**Supplementary Fig. S4**). In all three treatment groups, fluorescent signal could be observed in the lobes of the lungs, showing that the aerosol could indeed reach the deep lung

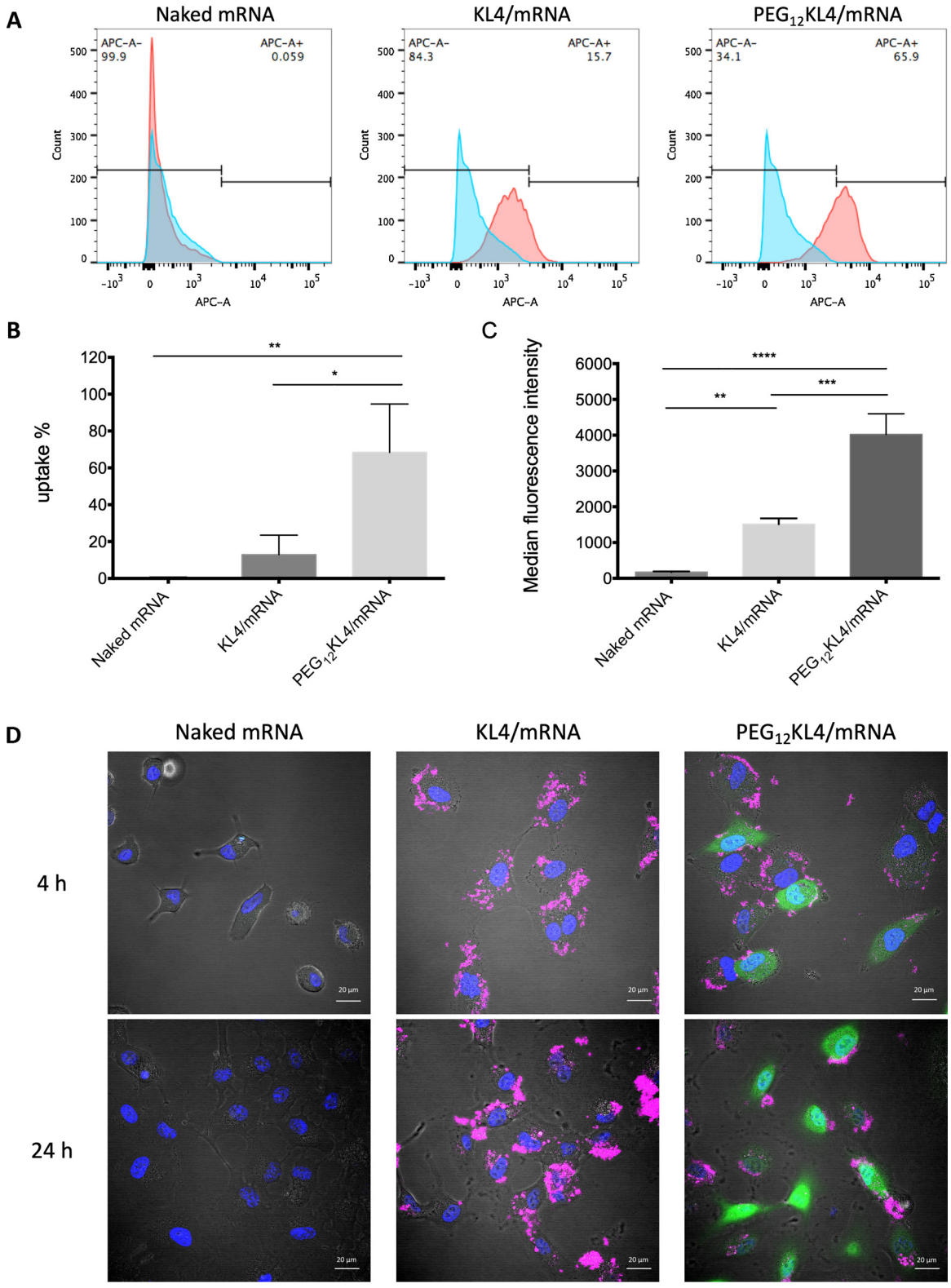

Fig. 3. Cellular uptake study using flow cytometry and confocal microscopy. A549 cells were treated with naked mRNA, KL4/mRNA and PEG_{12}KL4/mRNA prepared at 10:1 ratio (w/w) with cyanine-5 labeled mRNA. Cells were examined at 4 h post-transfection using flow cytometry. (A) representative histograms showing the population of cyanine-5 positive cells (red) compared to the untreated control (blue); (B) percentage of cells with mRNA uptake; and (C) median fluorescence intensity of the cells. Values are the mean ± standard deviation. The data was analyzed by one-way ANOVA followed by Tukey's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ ($n = 3$). (D) Confocal images of cells transfected with cyanine-5 (red) labelled mRNA with EGFP (green) expression; the nuclei (blue) were stained with Hoechst. Images were taken at 4 h and 24 h post-transfection. Scale bar = 20 µm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

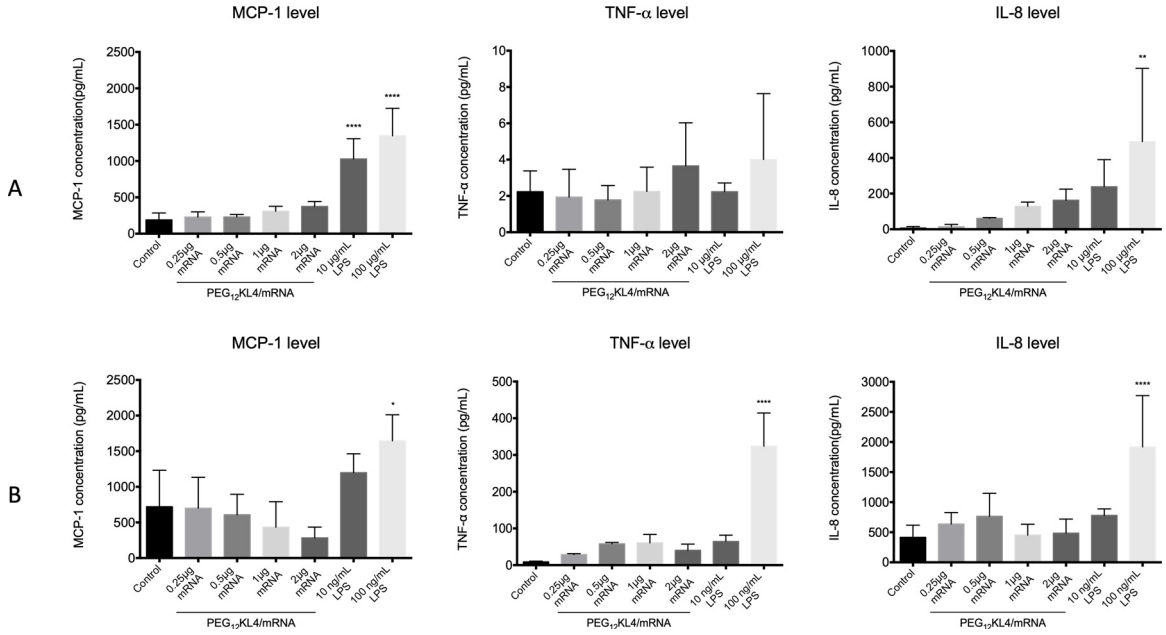

Fig. 4. Release of pro-inflammatory cytokines in (A) A549 cells and (B) THP-1 cells after mRNA transfection. The cells were transfected with $PEG_{12}KL4$/mRNA complexes prepared at 10:1 ratio (w/w) in 0.25 to 2 μg mRNA per well in 24-well plate. Untreated cells and cells treated with LPS were used as negative and positive control, respectively. The level of MCP-1, TNF-α and IL-8 released from the cells were measured at 24 h post-transfection. The data was analyzed by one-way ANOVA followed by Dunnett's post-hoc test as compared with the negative control. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. Data were presented as mean ± standard deviation ($n = 3$).

area, but only the $PEG_{12}KL4$/mRNA complexes could be successfully transfected to express luciferase in the deep lung region, as shown in the bioluminescence images. In addition, it was found that the expression of luciferase mRNA was highly localized in the lung following intratracheal administration of $PEG_{12}KL4$/mRNA complexes, either as liquid aerosol or powder aerosol, but not in other organs at 24 h post-administration (**Supplementary Fig. S5**), suggesting that there was no systemic absorption of mRNA.

The transfection efficiency of the $PEG_{12}KL4$/mRNA complexes at different concentrations in the liquid aerosol were further investigated *in vivo* (Fig. 9). The complexes containing either 5 or 10 μg mRNA (i.e. 50 or 100 μg $PEG_{12}KL4$) were delivered to the mice intratracheally. The luciferase expression in the lung was observed in both treatment groups, with the 5 μg mRNA treatment group displayed a significantly higher luciferase expression. Moreover, the lower dose did not cause any significant change in body weight at 24 h post-administration while the higher dose resulted in about 6% of body weight loss. Therefore, it is concluded that the 5 μg of mRNA dose could achieve high transfection efficiency and low toxicity at the same time. Lastly, the SD-0.5% mRNA and SFD-0.5% mRNA formulations were delivered to the mice as reconstituted liquid aerosol or as powder aerosol (Fig. 10). Both liquid and powder aerosol could express luciferase at 24 h post-

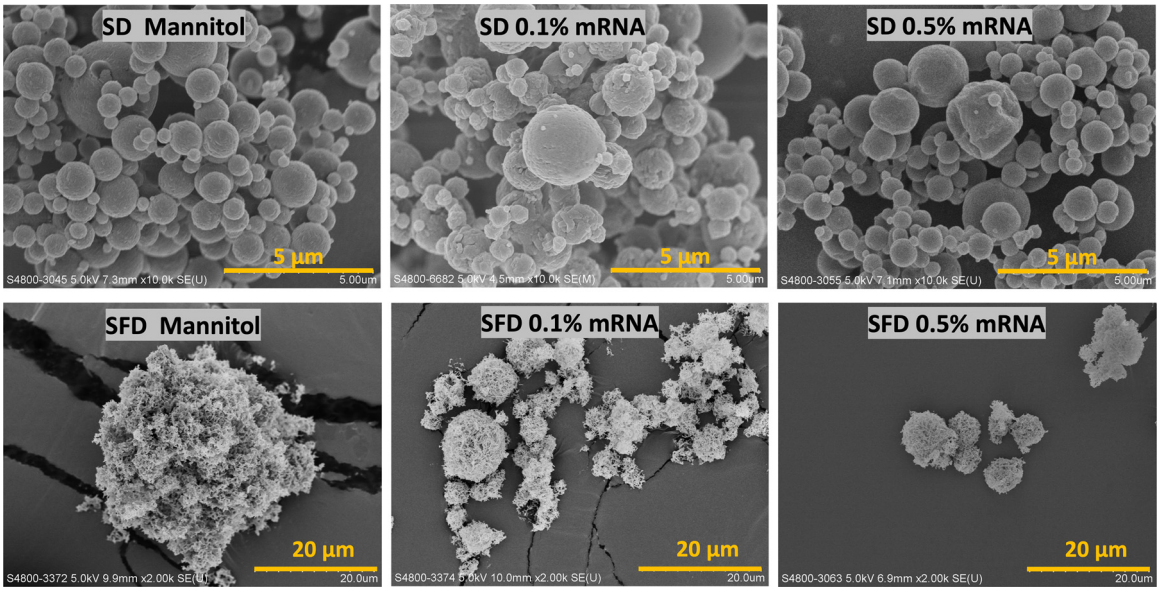

Fig. 5. The scanning electron microscopy (SEM) images of spray dried (SD) and spray freeze dried (SFD) formulations of $PEG_{12}KL4$/mRNA complexes. Formulations containing mannitol only were included for comparison. The images of SD formulations were taken at ×10,000 magnification (scale bar = 5 μm) and the images of SFD formulations were taken at ×2000 magnification (scale bar = 20 μm).

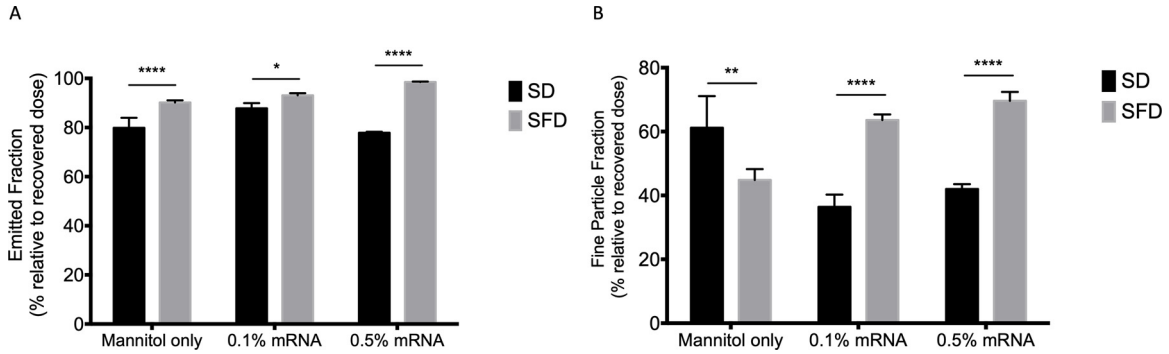

Fig. 6. Aerosolization performance of spray dried (SD) and spray freeze dried (SFD) powder evaluated by the Next Generation Impactor (NGI). (A) Emitted fraction (EF) and (B) fine particle fraction (FPF) were expressed as the percentage by mass of mannitol relative to the recovered mass. Data were presented as mean ± standard deviation (n = 3). The data were analyzed by one-way ANOVA followed by Sidak post-hoc test, *p < 0.05, **p < 0.01, ****p < 0.0001.

Table 3
The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the spray dried (SD) and spray freeze dried (SFD) powder formulations. The values were calculated based on the Next Generation Impactor (NGI) data. The data was presented as mean ± standard deviation (n = 3).

Formulation	MMAD (μm)	GSD
SD-Mannitol	2.43 ± 0.68	4.10 ± 0.94
SD-0.1% mRNA	5.54 ± 0.81	4.71 ± 0.50
SD-0.5% mRNA	4.45 ± 0.36	4.61 ± 0.40
SFD-Mannitol	3.48 ± 0.34	2.79 ± 0.08
SFD-0.1% mRNA	2.13 ± 0.08	2.38 ± 0.02
SFD-0.5% mRNA	1.53 ± 0.15	3.17 ± 0.20

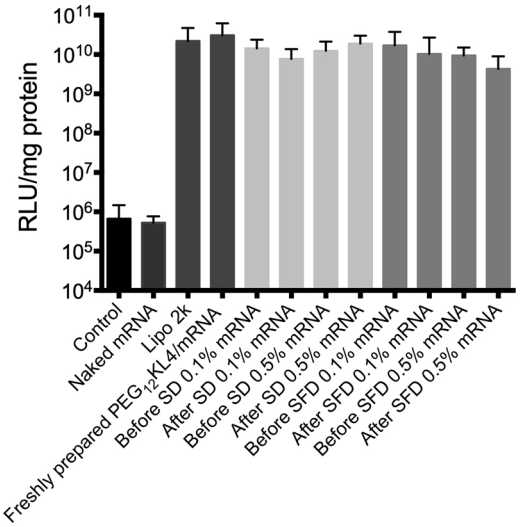

Fig. 7. Luciferase mRNA transfection on A549 cells with dry powder formulations. The powders were reconstituted and added to the cells at 1 μg mRNA per well in a 24-well plate. Naked mRNA, Lipofectamine 2000 (Lipo2k)/mRNA complexes (2:1 w/w ratio) and freshly prepared $PEG_{12}KL4$/mRNA complexes (10:1 w/w ratio) containing 1 μg mRNA were used as controls. Luciferase expression was measured at 24 h post-transfection. The data was expressed as the mean value of relative light unit (RLU) per mg of protein ± standard deviation (n = 3). The data were analyzed by one-way ANOVA followed by Sidak post-hoc test, comparing the same formulation before and after drying, and no significant difference was observed.

administration, but the former displayed significantly higher transfection efficiency. The SD formulation also performed slightly better than the SFD formulation, but not significant difference was observed between the two.

3.5. Safety profile

The inflammatory response of $PEG_{12}KL4$/mRNA complexes were investigated in mice by measuring the level of pro-inflammatory cytokines in BALF and lung homogenates (Fig. 11). The expression of MCP-1, TNF-alpha, KC, and IL-6 was significantly induced by LPS in both BALF and lung homogenates but not affected by naked mRNA and $PEG_{12}KL4$/mRNA, at both 5 and 10 μg mRNA dose, except that the $PEG_{12}KL4$/mRNA at 10 μg mRNA dose significantly induced the MCP-1 expression in BALF. The histological characteristics of the lungs treated with liquid and powder aerosol of $PEG_{12}KL4$/mRNA complexes (5 μg mRNA) were compared with the untreated control, PBS treated and LPS treated groups (Fig. 12). The lungs without any treatment or treated with PBS illustrated a healthy presentation while the lung treated with 10 μg of LPS intratracheally showed irregular distribution of air space and inflammatory cell infiltration into the interstitial and alveolar spaces. The lungs treated with $PEG_{12}KL4$/mRNA complexes, either as liquid or powder aerosol, did not show signs of inflammation. Overall, a single dose of $PEG_{12}KL4$/mRNA at 5 μg mRNA dose per mouse did not show any signs of inflammation and toxicity in the lungs of the animal.

4. Discussion

While we are currently on the verge of the new era of nucleic-acid based medicine, one of the most important barriers to overcome for its clinical translation is delivery [23]. This issue must be addressed before effective *in vivo* nucleic acid therapy can be truly realized. To this end, we previously investigated the use of KL4 peptide for siRNA delivery [17]. As a mimic of SP-B, the synthetic KL4 was initially employed to dissect the role of surfactants on nucleic acid delivery. As observed by many that naked RNA was able to transfect in the lung following pulmonary delivery [24–26], it has been proposed that pulmonary surfactant proteins may act as endogenous transfection agents that facilitate the cellular uptake of RNA in the lung. Although the exact uptake mechanism of naked RNA remained to be elucidated, the promising RNA delivery efficiency of KL4 peptide has prompted us to further develop KL4 peptide as a platform system for RNA delivery. The delivery of mRNA is investigated here because (i) the single-stranded mRNA is a challenging molecule to deliver due to its unstable nature; (ii) there is a lack of studies that explore inhaled dry powder formulation of mRNA (more studies on siRNA) [27,28]; (iii) mRNA has huge therapeutic potential for treatment of many lung diseases as well as prevention of diseases in form of mRNA vaccines [29,30]; and (iv) it is relatively easy to quantify the expression of mRNA with reporter gene without establishing a disease model.

The leucine-rich KL4 peptide has a poor aqueous solubility, limiting its application as non-viral vector. Hence the PEGylation strategy was adopted here to improve its solubility. The monodisperse PEG (instead of the more commonly used polydisperse PEG) was used in this study

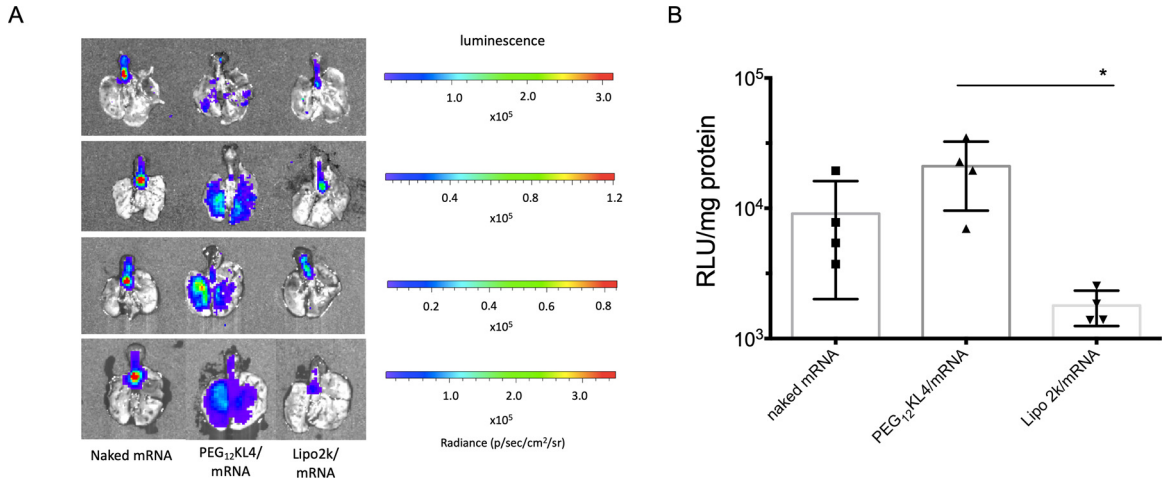

Fig. 8. Pulmonary delivery of mRNA formulations with different transfection agents. BALB/c mice (~20 g) were administered intratracheally with naked mRNA; PEG_{12}KL4/mRNA complexes at 10:1 ratio (w/w); and Lipofectamine 2000 (Lipo2k)/mRNA complexes at 2:1 ratio (w/w). Each mouse received 10 μg of mRNA in a final volume of 75 μL PBS. At 24 h post-administration, (A) the lungs were isolated for bioluminescence imaging; (B) luciferase protein expression of lung tissues were measured, and the data was expressed at the mean value of relative light unit (RLU) per mg of protein ± standard deviation (n = 4). The data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, *$p < 0.05$.

because it gives better homogeneity and reduces batch-to-batch variation. PEGylation is one of the most successful chemical modification strategies in biopharmaceutics, and the safety profile of PEG is well-established. Its use in pharmaceutical formulation is approved by the FDA for different routes of administration including inhalation [31]. In addition to the enhancement in solubility, the PEG chains create steric hindrance which hinder the accessibility of proteases and antibodies, improving peptide stability against degradative enzymes and reducing immunogenicity [32]. Furthermore, it has been reported that PEGylation can improve mucus penetration in the airways by forming a hydrophilic surface, thereby reducing the particle adhesion to mucin fibers through hydrophobic interaction, making it a particularly attractive feature for pulmonary delivery [33]. The effectiveness of the PEG coating for evading mucoadhension is affected by both the PEG grafting density and its molecular weight (MW) [34]. Although the monodisperse linear PEG of 12-mers (MW about 600 Da) used here has a relatively low MW compared to other studies where PEG with average MW > 2000 Da was commonly used, we rationalized that the high

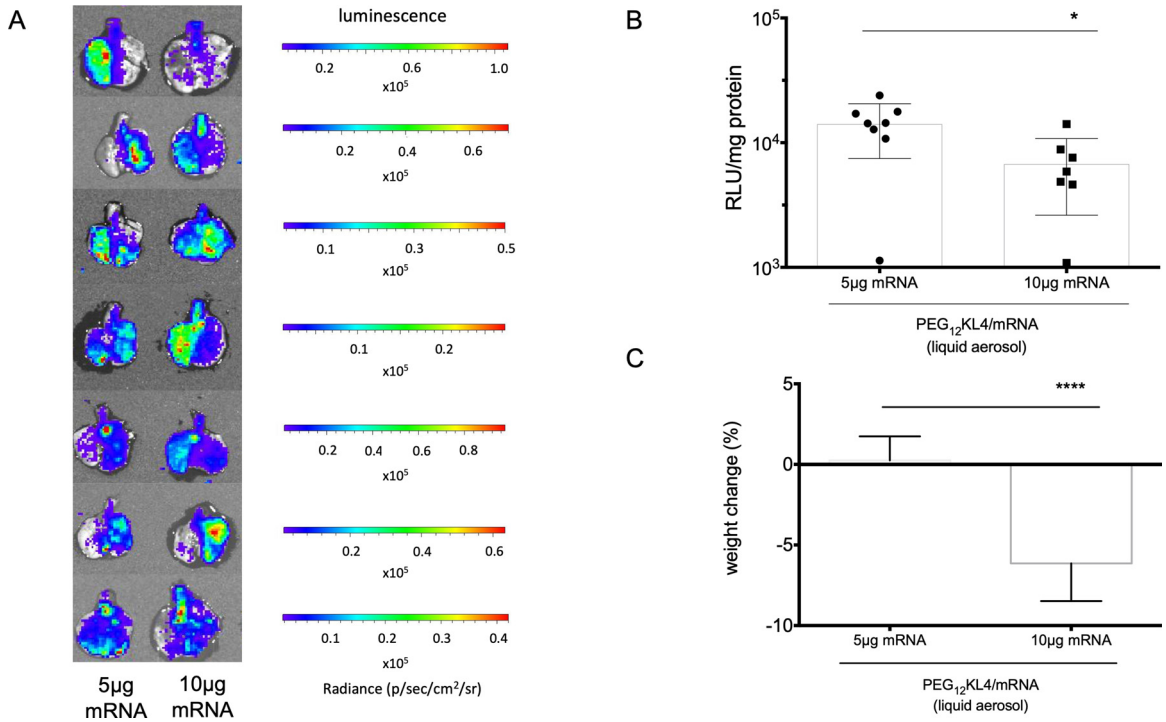

Fig. 9. Pulmonary delivery of mRNA formulations with different mRNA dose. BALB/c mice (~20 g) were administered intratracheally with PEG_{12}-KL4/mRNA complexes at 10:1 ratio (w/w), containing either 5 or 10 μg of mRNA in a final volume of 75 μL PBS. At 24 h post-administration, (A) the lungs were isolated for bioluminescence imaging; (B) luciferase protein expression of lung tissues were measured, and the data was expressed at the mean value of relative light unit (RLU) per mg of protein ± standard deviation. (C) Body weight of the mice was monitored before and at 24 h after administration, and the data was presented as mean value of percentage of weight change ± standard deviation (n = 7–8). The data were analyzed by unpaired two-tailed Student's t-test, *$p < 0.05$, ****$p < 0.0001$.

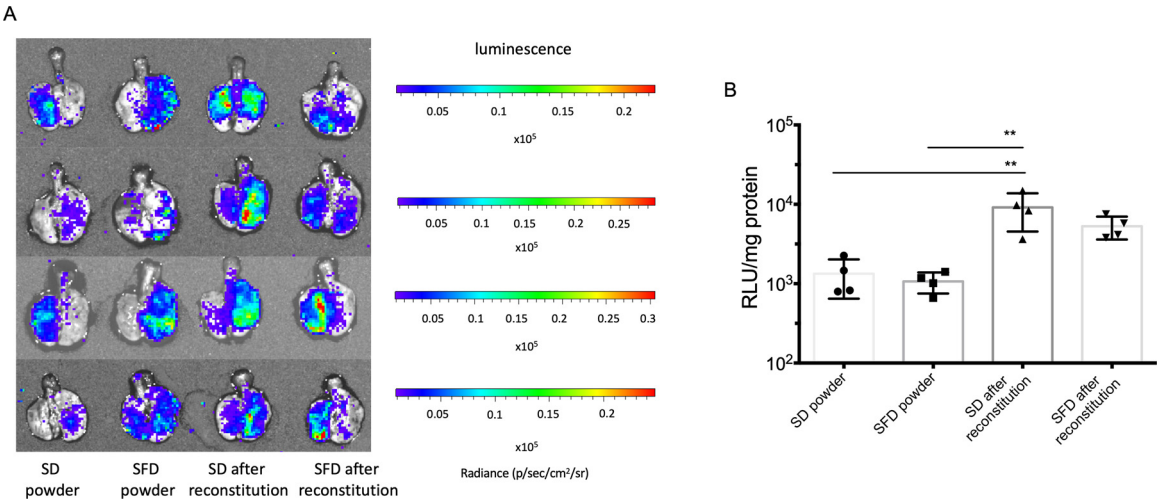

Fig. 10. Pulmonary delivery of mRNA formulations as powder aerosol or reconstituted liquid aerosol. BALB/c mice (~20 g) were administered intratracheally with SD-0.5% mRNA formulation as powder aerosol (1 mg); SFD-0.5% mRNA formulation as powder aerosol (1 mg); SD-0.5% mRNA formulation reconstituted as liquid aerosol (1 mg in 75 μL PBS); and SFD-0.5% mRNA formulation reconstituted as liquid aerosol (1 mg in 75 μL PBS). Each mouse received a dose of 5 μg mRNA. At 24 h post-administration, (A) the lungs were isolated for bioluminescence imaging; (B) luciferase protein expression of lung tissues were measured, and the data was expressed at the mean value of relative light unit (RLU) per mg of protein ± standard deviation (n = 4). The data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, **p < 0.01.

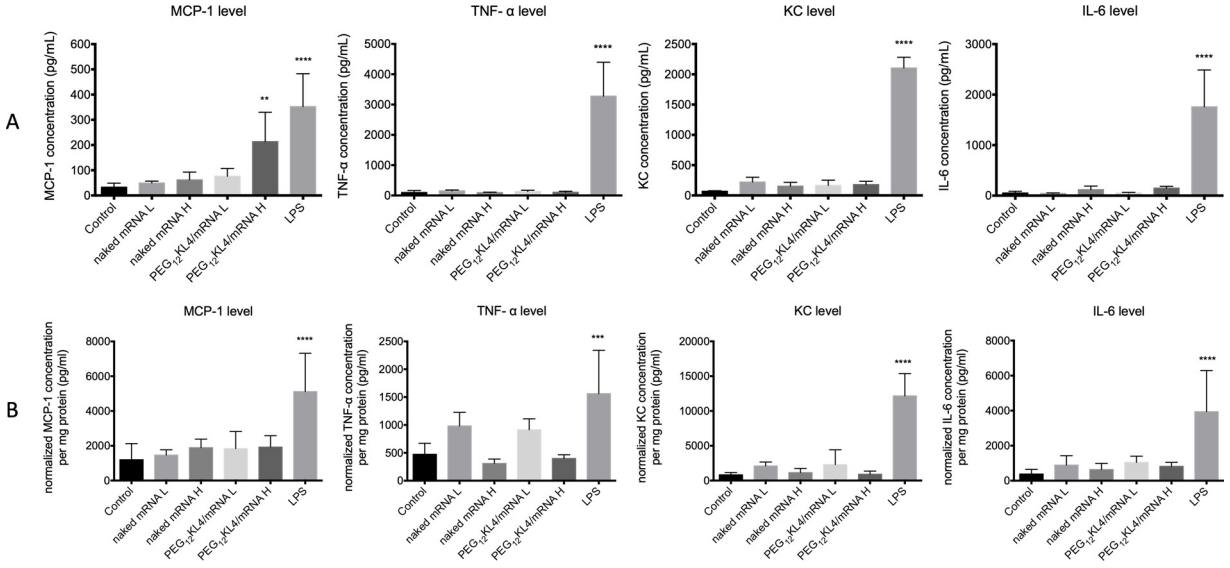

Fig. 11. Level of pro-inflammatory cytokines following pulmonary delivery of mRNA formulations. BALB/c mice were administered intratracheally with PBS as control; naked mRNA L (low dose of 5 μg); naked mRNA H (high dose of 10 μg); PEG_{12}KL4/mRNA L (low dose of 5 μg); PEG_{12}KL4/mRNA H (high dose of 10 μg); and LPS (10 μg), all in a final volume of 75 μL PBS. At 24 h post-administration, cytokines levels in (A) bronchoalveolar lavage fluid (BALF) and (B) lung homogenates were detected by ELISA. Data are expressed as mean ± standard deviation (n = 4–6). Statistical analysis was conducted by one-way ANOVA followed by Dunnett's post-hoc test as compared with control. **p < 0.01, ***p < 0.001, ****p < 0.0001.

density of PEG on the surface of the complexes (high peptide to mRNA ratio of 10:1) could promote muco-inertness. However, further investigation is required to elucidate the interaction between the PEGylated KL4/mRNA complexes and the mucus. After PEGylation, the PEG_{12}KL4 is soluble in water (at least up to 2 mg/ml, whereas KL4 failed to dissolve at 0.5 mg/ml). One of the concerns with PEGylation is that the steric hindrance caused by the hydrophilic PEG may interfere with the binding of mRNA. As showed by the gel retardation assay, the PEG_{12}KL4 required a higher ratio to achieve complete binding compared to KL4. This is because at the same peptide to mRNA ratio, the cationic charge (per weight) of PEG_{12}KL4 was lower than that of the KL4 peptide due the presence of the neutral PEG. The zeta potential of the KL4/mRNA and PEG_{12}KL4/mRNA complexes were similar, indicating that the overall charge of the complexes was not affected significantly. However, the PEG_{12}KL4/mRNA complexes were less compact in size compared to the KL4/mRNA complexes, and these larger and loose complexes were easier to dissociate, which could in fact facilitate mRNA transfection. As demonstrated in the cellular uptake and transfection studies, PEG_{12}KL4 was more efficient than KL4 in delivering mRNA to the cells. The presence of PEG could improve mRNA transfection by promoting cellular entry as well as releasing mRNA in the cells more effectively for protein translation to occur. However, the exact cellular uptake mechanism of the complexes remains to be investigated.

Two particle engineering techniques, SD and SFD, were employed to produce inhaled dry powder formulation of mRNA. It is crucial that the integrity of peptide/mRNA complexes can be preserved after drying while the powders exhibit good aerodynamic properties for efficient

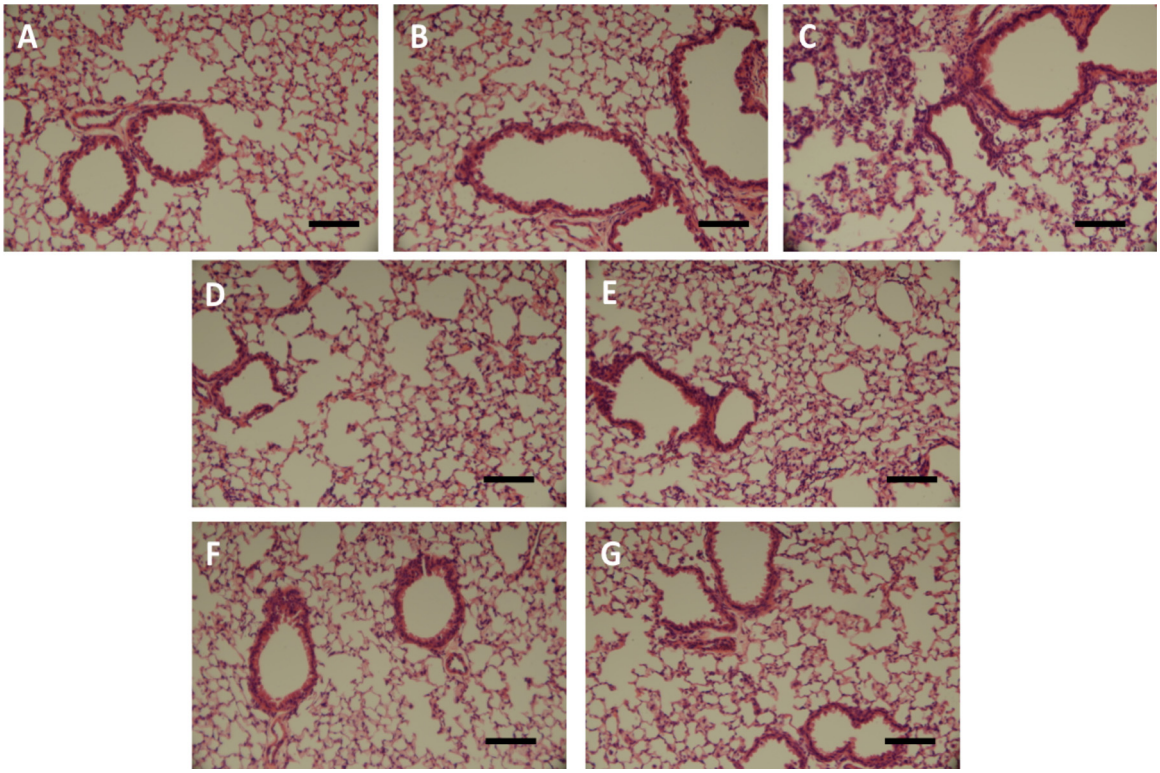

Fig. 12. Histology of the lungs of BALB/c mice following pulmonary delivery of mRNA formulation. (A) untreated control; mice were intratracheally administered with (B) PBS (75 μL); (C) LPS (10 μg in 25 μL PBS); (D) mRNA (5 μg in 75 μL PBS); (E) freshly prepared $PEG_{12}KL4$/mNRA complexes (5 μg mRNA in 75 μL PBS); (F) SFD-0.5% mRNA powder (1 mg); and (G) SD-0.5% mRNA powder (1 mg). Slides were viewed using an upright microscope at 20× magnification (scale bar = 100 μm).

lung deposition. Both of these methods have been previously reported in preparing dry powder formulation of nucleic acids for inhalation [19,20,28,35], and they have their pros and cons. SD is a single step operation that converts feed liquid into dried particles by atomizing the liquid into fine droplets which are immediately brought into contact with a stream of hot drying gas inside a drying chamber. During the process, molecules are exposed to elevated temperature and shear stress, increasing the risk of RNA degradation [36]. However, this drying method is easily scale-up in industry for mass production. SFD is a multi-step process that involves the atomization of a liquid into cryogen (typically liquid nitrogen) in which the particles are instantaneously frozen, followed by the sublimation of the solvent during freeze-drying. The SFD is more suitable for thermo-labile biological molecules and the formation of porous particles usually exhibit good aerosol properties. However, the sudden exposure of extreme low temperature at the spray freeze step or physical impacts during the freeze drying process may jeopardize the integrity of molecules [37]. In addition, the production time is longer and scale-up is more complicated. According to the physicochemical characterization, the $PEG_{12}KL4$/mRNA complexes behaved similarly before and after drying in terms of their particle size, zeta potential and *in vitro* transfection efficiency. In the *in vivo* transfection study, the SD formulation performed better than the SFD formulation although there was no significant difference between the two formulations. The results suggested that the relatively mild SD and SFD conditions employed in this study did not compromise the transfection efficiency of $PEG_{12}KL4$/mRNA complexes.

Both SD and SFD powder formulations exhibited desirable aerosol properties for inhalation. Although the particles of SFD formulations were physically larger, their aerosol performance were indeed better than the SD formulations, reflected by the higher EF and FPF value in all the mRNA containing formulations. This could be attributed to the porous nature of the SFD powder. It is known that SFD can produce porous particles with low density through the sublimation of solvent during the freeze-drying step [38]. The aerodynamic diameter is determined by the physical size and the density. By making the particles porous, the aerodynamic diameter could be reduced, as demonstrated by the MMAD of 4.5 μm in the SD-0.5% mRNA formulation and 1.5 μm in the corresponding SFD formulation. Compared to the mannitol-only powder, the inclusion of $PEG_{12}KL4$/mRNA complexes in the SD formulations had a negative impact on the aerosol performance, resulting in a lower FPF. One possible explanation is that the presence of macromolecules in the non-porous SD particles resulted in larger particle size, as indicated by both SEM images and MMAD values. This phenomenon has also been observed previously when siRNA was included in the SD formulation of mannitol [19]. However, when the amount of mRNA was increased from 0.1% to 0.5%, there was no major difference in FPF between the two. In contrast, the presence of $PEG_{12}KL4$/mRNA complexes improved the aerosol performance of the SFD formulation. The presence of macromolecules in the formulation may enhance the physical robustness of the porous particles, leading to better powder dispersion [20]. In both cases, a further increase in $PEG_{12}KL4$/mRNA complexes loading in the powder formulation can be investigated to explore how this may impact on the aerosol performance. It is desirable to increase the mRNA loading as this could potentially increase the dose of mRNA without excessive powder mass, considering the maximum amount of powder that can be inhaled by human is limited [39]. The aerosol performance of SD powder could also be enhanced by reducing the size or density of the particles or including a dispersion enhancer such as leucine in the formulation. For future industrial development, both drying methods require further optimization while SD has the additional advantage of scale-up feasibility.

The *in vivo* study demonstrated that the $PEG_{12}KL4$ peptide was safe for pulmonary delivery, with low risk of inflammatory response and toxicity at mRNA dose that showed effective transfection efficiency in the lung (5 μg per mouse), although repeated dose is required to

demonstrate its long-term safety. Transfection of naked mRNA and lipoplexes were also observed following intratracheal administration, but the luciferase expression was limited to the trachea and they failed to transfect in the deeper region of the lungs. On the contrary, the $PEG_{12}KL4$ could mediate effective mRNA expression in the deep lung area. It has been reported that PEGylation strategy could enhance the transfection efficiency by navigating and diffusing through the mucus mesh, thereby enhancing the lung distribution [32], which could explain our results. However, as KL4 peptide fails to dissolve in water for *in vivo* study, a direct comparison between KL4 and PEGylated KL4 was not carried out. On the other hand, naked mRNA and lipoplexes are incapable of penetrating the mucus and pulmonary surfactant barriers to reach the epithelial cells in the deeper lung region, resulting in poor transfection [40]. While mRNA transfection in the lung has been shown by others using different types of polymers [15,41,42], none of them reported dry powder formulation for inhalation.

On a closer examination of the *in vivo* transfection study of $PEG_{12}KL4$/mRNA complexes, the liquid aerosol resulted in better mRNA expression in the lung as compared to the powder aerosol despite the same mRNA dose was administered. This could be attributed to the suboptimal powder insufflation during the intratracheal administration which is a challenging procedure [43], resulting in incomplete powder dispersion in the lung of animals. In fact, the cascade impactor study is a more appropriate and relevant method to evaluate powder dispersibility and aerosol performance of the powder formulation. Most importantly, the reconstituted powders were able to mediate good mRNA transfection in the lung of animals.

5. Conclusions

This study is the first to report inhalable dry powder mRNA formulation for pulmonary delivery. The modification of the KL4 peptide by PEGylation resulted in the enhancement of solubility as well as transfection efficiency. Dry powder formulations of $PEG_{12}KL4$/mRNA prepared by SD and SFD techniques were suitable for inhalation and the mRNA integrity could be protected. Effective transfection in the lung was observed when $PEG_{12}KL4$/mRNA complexes were administered intratracheally in mice either as liquid or powder aerosol, with low risk of inflammatory response and toxicity. To conclude, $PEG_{12}KL4$ has considerable potential to be developed as a non-viral vector for mRNA pulmonary delivery, in dry powder form, for therapeutic as well as vaccine applications.

Declaration of Competing Interest

The peptide described in this study is under patent application.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jconrel.2019.10.026.

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