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Dry powder aerosol containing muco-inert particles for excipient enhanced growth pulmonary drug delivery

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Abstract

Tenacious sputum poses a critical diffusion barrier for aerosol antibiotics used to treat cystic fibrosis (CF) lung infection. We conducted a proof-of-concept study using dense poly(ethylene glycol) coated polystyrene nanoparticles (PS-PEG NPs) as model muco-inert particles (MIP) formulated as a powder using an excipient enhanced growth (EEG) strategy, aiming to minimize extrathoracic airway loss, maximize deposition in the airway and further overcome the sputum barrier in the CF lungs. The EEG aerosol formulation containing PS-PEG MIP was prepared by spray drying and produced discrete spherical particles with geometric diameter of approximately 2

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Conflict of Interest

The aerosol technology described in this publication has been filed as a patent application through Virginia Commonwealth University (VCU). Guihong Chai, Qingguo Xu, Michael Hindle and Bruce Rubin are the co-inventors, which are subject to certain rules and restrictions under VCU policy. The terms of this arrangement are being managed by VCU in accordance with its conflict of interest policies.

μm ; and >80% of the powder dose was delivered from a new small-animal dry powder inhaler (DPI). The MIP released from the EEG aerosol had human airway mucus and CF sputum diffusion properties comparable to the suspension formulation. These properties make this formulation a promising pulmonary drug delivery system for CF lung infections.

Graphical abstract:

Dry powder aerosol containing muco-inert particles (MIP) as a promising pulmonary drug delivery platform. Micrometer-sized excipient enhanced growth (EEG) aerosol particles achieve good emptying efficiency from dry powder inhalers and minimize mouth-throat losses. The high humidity in the airway increases aerosol particle size and enhance airway deposition. MIP released from the EEG aerosol particles rapidly diffuse into the sputum to release drug.

Keywords

Dry powder inhaler; spray drying; CF sputum; mucus penetration; multiple particle tracking

INTRODUCTION

Airway mucus obstruction is a hallmark of many respiratory diseases, such as asthma, chronic obstructive pulmonary disease and cystic fibrosis (CF).^{1, 2} CF airway disease includes impaired mucus clearance and chronic airway infection with biofilm producing organisms.^{3–5} Lung disease is the main cause of morbidity in CF patients, and at least 90% of patients die from respiratory failure.⁶ In CF patients, the abnormal ion regulation leads to depletion of the airway surface liquid and tenacious airway secretions, resulting in decreased mucociliary clearance, chronic inflammation and infection.^{7, 8} Aerosolized antibiotics have been used both for eradication of initial infection and for suppression of chronic infection.⁹

Topical administration by aerosol inhalation is more commonly used than oral and intravenous antibiotics for chronic CF infection management.¹⁰ Aerosol inhalation delivers an effective dose to the site of disease rather than administering higher doses systemically to

achieve the same or higher local exposure in the airway. Dry powder inhalers (DPI) are an attractive platform for pulmonary drug delivery as they are propellant-free, intrinsically breath actuated, and have improved formulation stability.¹¹ No external power source is required for a DPI to deliver medication, and delivery is rapid which can reduce the treatment burden and potentially increase adherence.^{12, 13}

The airway deposition of powder aerosols with particle sizes in the range of 2–5 μm mass median aerodynamic diameter (MMAD) from commercially available DPI devices varies from 5% to 40% with 40–70% of the drug depositing at the mouth-throat (MT) region due to poor particle deaggregation or detachment from the carrier during inhalation.^{14, 15} EEG technology delivers a micrometer-sized particle formulation (often ~1–2 μm initially) that has been developed to minimize MT deposition losses and to achieve greater lung retention following hygroscopic growth to 2–5 μm sized particles during inhalation.^{16–18} The emptying efficiency of the EEG powder aerosol formulation from a DPI device is high due to the efficient dispersion of the small particle aerosol, which also allows for minimized extrathoracic loss due to the low inertial of small-particle aerosols.¹¹ After bypassing the MT, the humidity in the airway causes particles containing the hygroscopic excipients to absorb water, increasing the size and weight of these particles enhancing greater and more uniform airway deposition. In an *in vitro* study using a modified HandiHaler, a mouth-throat – tracheobronchial (MT–TB) model showed that about 97% of the emitted particles successfully bypass the MT, resulting a final MMAD of 2.58 μm with a submicrometer fraction of only 3%.¹¹

Although the EEG strategy can improve the delivery efficiency of the dry powder aerosol to the airway, tenacious sputum still poses a barrier for the deposited aerosol particles to deliver the loaded antibiotics into the sputum.^{19, 20} CF sputum is composed of a DNA and mucin fiber network containing a high density of negatively charged glycans sparsely dispersed with periodic hydrophobic regions.²¹ DNA-actin polymers generated from cell necrosis and extracellular traps further increase the adhesivity and meshwork density of the CF sputum.¹⁹ The tenacious sputum barrier has frustrated the effectiveness of conventional particulate formulations that are trapped in the luminal superficial sputum gel layer and are thus readily removed by mucociliary and cough-driven clearance.²⁰ Muco-inert particles (MIP) are a powerful platform to enhance drug delivery by achieving uniform distribution to the mucosal surface and preventing drug particles from being trapped and eliminated by mucociliary clearance.^{22–25} This MIP technology has been applied for various mucosal tissues, including the lung.^{24, 26, 27} Compared to conventional NPs, MIP achieve more uniform distribution throughout the airway and prolonged retention.²²

As a proof-of-concept study, we sought to develop a respirable EEG dry powder aerosol formulation containing MIP as illustrated in Fig. 1. Model MIP, i.e. PS-PEG NPs, were chosen and produced as a dry powder aerosol using spray drying together with the EEG strategy. The particle morphology was characterized and aerosol particle size was measured by laser diffraction after emission from a novel small animal DPI device. The mucus diffusion properties of the MIP both in the form of suspension and from the EEG aerosol were evaluated in healthy human airway mucus and CF sputum.

METHODS

Materials

Red fluorescent, carboxylate-modified polystyrene (PS) beads, 100 nm in size, were purchased from Invitrogen (Life Technologies Corporation, Eugene, OR, USA). N-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimidehydrochloride (EDC), L-leucine and boric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methoxy-terminated PEG (mPEG-NH₂, MW 5k) was purchased from Creative PEGWorks (Winston Salem, NC, USA). Mannitol was donated from Roquette Pharma (Lestrem, France). Poloxamer 188 (Leutrol F68) was donated from BASF Corporation (Florham Park, NJ). All other chemical reagents were analytical grade or better.

PS-PEG NPs preparation and characterization

Red fluorescent COOH-modified PS beads were covalently modified with methoxy-PEG-NH₂ by COOH-amine reaction to obtain a dense PEG coating as previously described.²⁸ This dense PEG coating can minimize adhesive interactions between particles and mucus/sputum by rendering the particle surface muco-inert.^{20, 24} The freshly prepared PS-PEG NPs were resuspended in deionized water to a two-fold dilution of the initial particle volume and stored at 4°C until use. The hydrodynamic diameter, polydispersity index (PDI) and surface charge (ζ potential) of PS and PS-PEG NPs (diluted with 10 mM NaCl, pH 7.4) were measured by NanoZS Zetasizer (Malvern Instruments, Westborough, MA). We confirmed the dense PEG coating on the surface of PS-COOH NPs through the nearly neutral surface charge, whereas uncoated PS-COOH NPs have a highly negative surface charge.^{22, 28, 29}

EEG dry powder aerosol formulation preparation and characterization

To produce a dry powder aerosol formulation, the PS-PEG NPs suspension were formulated into a spray dried micrometer-sized dry powder suitable for the EEG application.¹⁶ In brief, the PS-PEG NPs suspension was suspended in a water: ethanol (80:20, v/v) solution containing mannitol, L-leucine and poloxamer 188 in a ratio of 24:10:1. It was then spray dried using Buchi Nano spray dryer B-90 (Buchi Laboratory Techniques, Flawil, Switzerland) using previously optimized conditions: the drying airflow was 120 L/min, the liquid feed rate was set to 100%, inlet temperature was 70°C, and the spray nozzle was a vibrating mesh (4 μ m).¹¹ As a control formulation, a PS NPs dry powder aerosol was also prepared by spray drying using the same conditions. The spray dried solid particles were collected from the electrostatic precipitator of the spray drier, transferred to sealed capped amber vials and then stored open in a desiccator (RH < 10%) at room temperature for a minimum of 24 hours as a secondary drying process prior to testing. Precious studies have revealed about 2% of water content following spray drying and that an inlet temperature of 70°C was sufficient for particle formation and complete drying.³⁰

The particle size distribution of the dry powder aerosol containing PS-PEG NPs or none muco-inert coated counterpart (PS NPs) was determined using a laser diffraction system. An aerosol generation for these dry powder aerosol formulations was performed using a novel small animal DPI, and then delivered to the measurement zone of the Spraytec® particle size

analyzer (Malvern Instruments, Ltd., Worcestershire, UK). *In vitro* aerosolization performance of each dry powder aerosol formulation was conducted in triplicate.

The morphology of the EEG dry powder aerosol containing MIP was observed using a scanning electron microscopy (SEM). Each dry powder aerosol was mounted onto a SEM stub using double-sided copper tape and then coated with gold by a sputter coater for 2 min under vacuum. The SEM was operated at high vacuum with accelerating voltage 2 kV and specimen working distance 14.5 mm, and images were taken at a magnification of 10k.

Diffusion of MIP formulations in healthy human airway mucus and CF sputum Airway mucus and CF sputum collection

Human airway mucus was collected from endotracheal tubes (ETT) of adult patients admitted for elective surgery at MCV Hospital of Richmond, Virginia. Airway mucus collection was approved by the Institutional Review Board (VCU HM14684_Ame4). These patients required intubation as part of general anesthesia for non-pulmonary surgery and had no known pulmonary disease, respiratory infection, or fever greater than 38.5°C. Salivary contamination on the outside of the tube was first removed to prevent potential dilution effects. The tubes were then spun at $220 \times g$ for 1.5 minutes at room temperature. This process has been validated not affect to keep the structure of the mucus meshwork intact. Airway mucus samples that appeared inhomogeneous or had visible blood contamination were excluded. CF sputum were collected by spontaneously expectorated from CF patients at the CF Centers at Virginia Commonwealth University, which follows is a member of the CF Foundation Therapeutic Development Network. The CF sputum collection was approved by the VCU Institutional Review Board and all sputum donors were anonymous. To minimize any mucolytic effects, sputum was collected from subjects who had dornase alfa treatment 24 hours or more before collection. The collected airway mucus or CF sputa were stored at 4°C and used within 24 hours.

Mucus or sputum diffusion of MIP formulations measured by multiple particle tracking (MPT) MPT methods

The MPT platform was used to evaluate the mucus/sputum diffusion of the MIP particles *ex vivo*.^{31, 32} In brief, 20 μ L of mucus/sputum was added into a customized microscopy chamber, and then 0.6 μ L of diluted MIP suspension (3%, v/v) or <1mg of MIP EEG dry powder aerosol was added to the mucus/sputum samples. After gently stirring, the chamber was sealed to prevent dehydration and incubated for 1.5 hours at room temperature to minimize convection effects. The diffusion of MIP in the mucus/sputum was then viewed by an inverted Olympus IX71 epifluorescence microscope equipped with a 100 $\times$ oil-immersion objective (Olympus Latin America Inc., FL, USA). Trajectory movies were collected for 20 seconds at a temporal resolution of 67 ms using an EMCCD camera (Evolve 512, Photometrics, Tuscon, AZ). For each mucus/sputum sample, six videos of different area of interest were collected by RSIimage software. In each video, more than 100 particles were tracked, at least 50 frames were collected for each particle, and the traced particles in the six videos of each donor's mucus/sputum sample were pooled for analysis.

MPT analysis

The movie analysis was performed using a custom-written code in MATLAB (Mathworks) based on a previously developed algorithm.³³ The x and y positions of particle centroids over time were first extracted and then trajectories were constructed by connecting particle centers between the sequential images. For each trajectory, the time-averaged mean squared displacement (MSD) was calculated as a function of time scale (τ), $\langle r^2(\tau) \rangle = \langle [x(t + \tau) - x(t)]^2 + [y(t + \tau) - y(t)]^2 \rangle$, where $x(t)$ and $y(t)$ represent the particles coordinates at a given time. The ensemble-averaged MSD ($\langle \text{MSD} \rangle$) for all particles in a given sample was calculated by taking the geometric mean of the individual particles' time-averaged MSD.

Statistics

All formulation and characterization experiments were performed in triplicate or quadruplicate, and the results presented as the mean $\pm$ standard deviation and analyzed with ANOVA. To compare the mucus/sputum diffusion of different formulations, at least three donors' mucus/sputum samples were collected, and paired t-test (one-tailed) was used to compare different formulations in each donor's mucus/sputum sample. The statistical analysis was done using IBM SPSS statistics 25, and the statistical significance was assigned at $p < 0.05$. The corresponding symbols in the figures are defined as * $p < 0.05$ and ** $p < 0.01$, respectively.

RESULTS

PS-PEG NPs preparation and characterization

The commercial PS NPs are carboxylate-modified and red fluorescent (580/605 nm) particles, and the surface charge is negative. When covalently modified with methoxy-PEG-NH₂ by COOH-amine reaction, the surface charge approaches neutral. The formation and characterization of PS-PEG NPs is showed in Fig. 2, and the dense PEG coating on the surface of PS NPs was confirmed through the nearly neutral surface charge, whereas uncoated PS NPs have a highly negative surface charge.

EEG dry powder aerosol formulation preparation and characterization

The morphology of the EEG dry powder aerosol formulation containing the MIP showed discrete spherical particles (Fig. 3A). To evaluate the aerosolization performance of the MIP EEG dry powder aerosol formulation, a novel small animal DPI (Fig. 3C) was used to generate the aerosol in the measurement zone of the Spraytec particle size analyzer. The median geometric diameter (D_{v50}) of the EEG dry powder aerosol was approximately 2 μm , and the emitted dose delivered from the novel small animal DPI was more than 80% of the loaded dose (Fig. 3B). This confirmed that this MIP can be formulated into the micrometer sized EEG dry powder aerosol formulation with good emptying from the small animal DPI, and formed into a respirable aerosol suitable for pulmonary delivery. Also shown is the aerosol performance of the EEG formulation without the MIP (Excipients; Fig. 3B) and with PS alone which both showed no significant differences in particle size and emitted dose compared to the MIP formulation.

Diffusion of MIP formulations in human airway mucus and CF sputum

Airway mucus from healthy patients and freshly expectorated CF sputum were used to measure the diffusion of PS-PEG NPs released from the EEG dry powder aerosol, using the MPT technique *ex vivo*. MPT is a robust microscopy technique allowing the simultaneous tracking of thousands of individual particles at high spatiotemporal resolution. This provides transport measurements, including mean square displacement (MSD), which is a measure of the distance traveled by particle over a given time interval.³⁴

Diffusion of MIP formulations in human airway mucus

The diffusion properties of the MIP formulations were first evaluated in healthy human airway mucus. The MSD results showed that the PS-PEG NPs from the suspension formulation diffused rapidly due to the dense muco-inert PEG coating on the surface, whereas the PS NPs were restrained in the airway mucus (Suspension; Fig. 4A). After formulating as an EEG dry powder aerosol, the PS-PEG NPs released from this aerosol maintained rapid mucus diffusion that was 22-fold faster than that of PS NPs from the corresponding EEG powder aerosol (Aerosol; Fig. 4A). A representative single particle trajectory demonstrates a long-distance movement of PS-PEG NPs in the airway mucus both from the suspension formulation and released from the EEG dry powder aerosol, whereas the PS NPs were restrained in the airway mucus (Fig. 4B). The diffusion rate distribution of the PS-PEG NPs in the mucus also showed a significant fraction of quickly diffused particles from the formulations of suspension and EEG dry powder aerosol while most of the PS NPs were restrained (Fig. 4C). The trajectory movies of the conventional NPs or the MIP from different formulations in the mucus are shown in the Supplementary Materials (Movie S1–S4).

Diffusion of MIP formulations in CF sputum

The diffusion of the PS-PEG NPs released from the EEG dry powder aerosol formulation in freshly expectorated CF sputum was measured and compared with the EEG dry powder aerosol prepared with conventional PS NPs and the PS-PEG NPs suspension formulation. Particle diffusion in the CF sputum reflected by the $\langle \text{MSD} \rangle$ (Fig. 5A) showed that the conventional PS NPs, either in the form of suspension or EEG dry powder aerosol formulation, were greatly restrained in the CF sputum, whereas the PS-PEG NPs released from the EEG dry powder aerosol has 11-fold greater diffusion in the CF sputum. A representative single particle trajectory shows greater movement of the PS-PEG NPs released from the EEG dry powder aerosol, while the PS NPs were stuck in the CF sputum (Fig 5B). The diffusion rate distribution also demonstrated that PS-PEG NPs released from the EEG dry powder aerosol moved similarly to those from the suspension (Fig. 5C). Trajectory movies in CF sputum of the conventional NPs or the MIP from different formulations are shown in the Supplementary Materials (Movie S5–S8). This demonstrates that dense PEG coated PS-PEG NPs, can be formulated into micrometer-sized dry powder for aerosol by the EEG technique, and that the MIP released from this dry powder aerosol maintain their ability to rapidly diffuse in both airway mucus and CF sputum.

DISCUSSION

The airway mucus is a viscoelastic and adhesive gel that protects the lung from pathogens and hydrates the epithelium.²¹ In CF, the sputum is more viscoelastic than normal mucus.^{3, 19, 35, 36} Most inhaled particles, including conventional particulate drug aerosols, are trapped in the superficial gel layer by steric obstruction and/or adhesion. Trapped particles are cleared from the airway by the mucociliary or cough-driven clearance. This limits the retention time of therapeutic aerosols in the lung.^{20, 29, 37} Muco-inert particles, e.g. PS-PEG NPs, PLGA-PEG NPs and PLGA F127 NPs, have been shown to limit mucus clearance and can sustain drug delivery for local airway therapy.^{22, 23, 27, 29} The dense PEG coating on the surface of PS NPs allow PS-PEG NPs to rapidly diffuse through highly viscoelastic CF sputum by minimizing adhesive interactions between particles and the sputum constituents. In addition, the particle size of PS-PEG NPs (100 nm) is smaller than mucus/sputum meshwork,^{24, 35, 36, 38} which was estimated 500 nm for airway mucus and 150 nm for CF sputum,¹ allowing these muco-inert particles to efficiently diffuse through airway mucus and CF sputum. MIP NPs with 300 nm in size exhibited uniform distribution and significantly enhanced retention in mice airway compared to size-matched mucoadhesive NPs,²² and as large as 200 nm have been proven to diffuse through undiluted CF sputum 90-fold faster than similar-size conventional particles.²⁴ This MIP technique is being evaluated for drug and gene delivery to treat CF lung diseases.²³

Dry powder aerosols can potentially decrease treatment time, and improve formulation stability and sterility compared to liquid formulations.¹² The MT deposition of EEG powder aerosol was less than 3% of the drug dose, which is at least one order of magnitude lower than with conventional or state-of-the-art inhalers.^{11, 39} In addition, the EEG aerosol achieved increased deposition in the lower TB region by a factor of 20–30 times higher compared with conventional DPI.³⁹ We showed that MIP can be formulated into micrometer-sized EEG powder aerosol with good emptying from a novel small animal DPI. This is dispersed into a respirable aerosol powder suitable for pulmonary delivery. Future work is warranted to focus on assessing maximum MIP loading capacity in the EEG powder aerosol formulation.

The MIP in the EEG dry powder aerosol maintained the rapid mucus/sputum diffusion properties without losing their unique feature of deep mucus/sputum penetration. Compared to the suspension, the diffusion of MIP from the dry powder aerosol was a bit lower, which may be due to the mannitol excipient used in the EEG dry powder aerosol. This hygroscopic excipient can absorb a small amount of water and can potentially increase the interstitial fluid viscosity. Regarding the suspension formulation, a small amount of water (3%, v/v) was added to the mucus. This difference in interstitial water content may result in small differences in MIP diffusion. This could be further optimized by changing the excipients and the ratio of excipients to MIP combined. Unlike *ex vivo* study, the EEG aerosol will absorb water during the inhalation process, and most absorption of water will be happened prior to deposition on the superficial mucus/sputum gel, and this will not significantly affect the mucus/sputum properties. Antibiotic particles developed in this manner could be more effectively delivered to the airway with less drug loss in the mouth and throat, deeper airway penetration, longer airway retention, and rapid diffusion through sputum. Various MIP drug

delivery systems have been developed for mucosal surface delivery, such as PLGA MIP,^{22, 31, 32} liposomal MIP,⁴⁰ non-viral vector MIP,⁴¹ etc., providing diverse drug carriers that can be selected for combination with the EEG technique. Muco-inert non-viral gene carriers, such as PEI-PEG and PBAE-PEG nanoparticles, have excellent transfection efficacy in animal lungs because of the rapid penetration through sputum and mucus barriers.⁴² If these gene delivery systems can be combined in the EEG aerosol to maintain the mucus/sputum penetration properties, it could be an effective strategy to improve non-viral gene delivery to the airway. This promising EEG dry powder aerosol may also benefit other lung diseases of patients with airway mucus dysfunction by enhancing mucus/sputum drug delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS:

CF	cystic fibrosis
PS	polystyrene
PEG	poly(ethylene glycol)
NPs	nanoparticles
MIP	muco-inert particles
EEG	excipient enhanced growth
DPI	dry powder inhalers
MMAD	mass median aerodynamic diameter
MT	mouth-throat
TB	tracheobronchial
SEM	scanning electron microscopy
EET	endotracheal tubes
MPT	multiple particle tracking
MSD	mean squared displacement

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Highlights:

- Excipient enhanced growth (EEG) powder aerosol minimize extrathoracic airway losses and enhance airway deposition
- Muco-inert particles (MIP) with superior airway mucus penetration can be formulated as an EEG dry powder aerosol
- MIP released from the EEG aerosol had human airway mucus and CF sputum diffusion properties comparable to the suspension formulation
- EEG aerosol containing MIP could be a promising pulmonary drug delivery system for CF lung infections.

Figure 1. Schematic of EEG dry powder aerosol containing MIP as a promising pulmonary drug delivery platform. Micrometer-sized EEG aerosol particles minimize MT loss. The high humidity in the airway increases aerosol particle size and facilitates airway deposition. MIP released from the EEG aerosol particles rapidly diffuse into the sputum to release drug.

Figure 2. PS-PEG NPs preparation and characterization. (A) Schematic of PS-PEG NPs formation; (B) Particle size, size distribution and surface charge (ζ potential) of PS-PEG NPs.

Figure 3.

Characterization of EEG dry powder aerosol formulation containing the MIP. (A) SEM image of the EEG dry powder aerosol formulation; (B) *In vitro* aerosolization performance measured by Spraytec instrument; (C) Novel small animal DPI used to generate aerosol in the measurement zone of the Spraytec instrument for particle size measurement. Mean $\pm$ SD, n=3 replicates. One-way ANOVA followed by Bonferroni *post hoc* test for multiplicity. No statistically significant difference was found among Excipients, PS and PS-PEG aerosols in either Geometric size or Emitted dose.

Figure 4.

Diffusion of the MIP in human airway mucus from suspension formulation or released from the EEG dry powder aerosol. (A) Ensemble averaged MSD ($\langle \text{MSD} \rangle$); (B) Representative single particle trajectory; (C) Particle diffusion rate distribution. Red dash lines denote median values. Mean $\pm$ SEM, $n=3$ mucus donors. T-test (one-tailed) $*p<0.05$. No statistically significant difference was found for PS suspension vs PS aerosol, and PS-PEG suspension vs PS-PEG aerosol.

Figure 5. Diffusion of the MIP in CF sputum from suspension formulation or released from the EEG dry powder aerosol. (A) Ensemble averaged MSD ($\langle \text{MSD} \rangle$); (B) Representative single particle trajectory; (C) Particle diffusion rate distribution. Red dash lines denote median values. Mean $\pm$ SEM, $n=3$ CF sputum donors. T-test (one-tailed) $*p<0.05$, No statistically significant difference was found for PS suspension vs PS aerosol, and PS-PEG suspension vs PS-PEG aerosol.