Insights into the mechanisms of interaction between inhalable lipid-polymer hybrid nanoparticles and pulmonary surfactant

Xu, You; Parra-Ortiz, Elisa; Wan, Feng; Cañadas, Olga; Garcia-Alvarez, Begoña; Thakur, Aneesh; Franzyk, Henrik; Pérez-Gil, Jesús; Malmsten, Martin; Foged, Camilla

Published in:
Journal of Colloid and Interface Science

DOI:
10.1016/j.jcis.2022.11.059

Publication date:
2023

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY-NC-ND

Citation for published version (APA):
Insights into the mechanisms of interaction between inhalable lipid-polymer hybrid nanoparticles and pulmonary surfactant

You Xu, Elisa Parra-Ortiz, Feng Wan, Olga Cañadas, Begoña García-Alvarez, Aneesh Thakur, Henrik Franzyk, Jesús Pérez-Gil, Martin Malmsten, Camilla Foged

Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark

Department of Biochemistry and Molecular Biology, Faculty of Biology, Complutense University, 28040 Madrid, Spain

Research Institute "Hospital 12 de Octubre (imas12)", Madrid, Spain

Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Jagtvej 162, 2100 Copenhagen Ø, Denmark

Department of Physical Chemistry 1, University of Lund, SE-22100 Lund, Sweden

Department of Biochemistry and Molecular Biology, Faculty of Chemistry, Complutense University, 28040 Madrid, Spain

Abstract

Pulmonary delivery of small interfering RNA (siRNA) using nanoparticle-based delivery systems is promising for local treatment of respiratory diseases. We designed dry powder inhaler formulations of siRNA-loaded lipid-polymer hybrid nanoparticles (LPNs) with aerosolization properties optimized for inhalation therapy. Interactions between LPNs and pulmonary surfactant (PS) determine the fate of inhaled LPNs, but interaction mechanisms are unknown. Here we used surface-sensitive techniques to study how physicochemical properties and pathological microenvironments influence interactions between siRNA-loaded LPNs and supported PS layers. PS was deposited on SiO₂ surfaces as single bilayer or multilayers and characterized using quartz crystal microbalance with dissipation monitoring and Fourier-transform infrared spectroscopy with attenuated total reflection. Immobilization of PS as multilayers, resembling the structural PS organization in the alveolar subphase, effectively reduced the relative importance of interactions between PS and the underlying surface. However, the binding affinity between PS and LPNs was identical in the two models. The physicochemical LPN properties influenced the translocation pathways and retention time of LPNs. Membrane fluidity and electrostatic interactions were decisive for the interaction strength between LPNs and PS. Experimental conditions reflecting pathological...
microenvironments promoted LPN deposition. Hence, these results shed new light on design criteria for LPN transport through the air–blood barrier.

© 2022 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Nanoparticle-based pulmonary drug delivery is challenging, because inhaled nanoparticles must overcome complicated transport issues related to airway geometry, humidity, and clearance mechanisms, as well as the challenge of pathological conditions [1]. Upon deposition in the respiratory tract, nanoparticles come into contact with the pulmonary surfactant (PS) layer before phagocytosis by alveolar macrophages [2]. Hence, PS represents a main biological barrier after particle deposition in the lower airways, and it influences the retention time of inhaled particles and determines pulmonary drug bioavailability [2].

PS is produced by type II alveolar cells, and it is mainly composed of lipids (approx. 90 % by surfactant weight), i.e., phosphatidylcholine (PC), phosphatidylglycerol (PG), and cholesterol. The additional 8–10 wt% consists of a mixture of surfactant proteins (SPs), i.e., the hydrophilic proteins SP-A and SP-D, and the hydrophobic proteins SP-B and SP-C [3]. Curosurf® is a clinical PS formulation used for lung surfactant replacement therapy. It is extracted from porcine lungs, and it consists mainly of the phospholipids PC, PG, and sphingomyelin, and the hydrophobic proteins SP-B and SP-C. Curosurf® and natural PS display comparable biophysical activity, and Curosurf® has been widely used as a model system for PS [4,5]. In addition, the hydrophobic fraction (organic extract) of native PS from porcine lungs has also been widely used as PS model, because it contains the main surface-activity components of native PS, including SP-B and SP-C, as well as the lipid fraction [6–8]. These two PS model systems display comparable surface activity under conditions mimicking the breathing dynamics (unpublished data).

Interactions between inhaled particles and the PS system are of increasing interest. Recent work has addressed the biophysical function of PS in the presence of drug substance or nanoparticles [9]. The surface of nanoparticles can also be modified with PS components, which is referred to as a biomolecular corona, which can modulate the interactions of nanoparticles with target cells, and it thus has an impact on their biological activity [10–12]. The physicochemical properties of nanoparticles, including particle size, surface charge, and hydrophobicity, may influence the interactions between nanoparticles and PS, and they represent critical quality attributes for the pulmonary delivery efficiency of nanoparticles [2]. A better understanding of the effect of the physicochemical properties of nanoparticles on nanoparticle-PS interactions is therefore needed to extend the application of nanoparticles for inhalation therapy. However, little is known about how the physicochemical properties of nanoparticles influence the interactions with PS.

The interactions between nanoparticles and PS may also be affected by the biological microenvironment. The pH of the lung-lining fluid is almost neutral (pH = 6.6 to 7.1) under normal physiological conditions [13]. However, chronic bacterial infections in the respiratory tract alter airway homeostasis, and may cause intrinsic acidification during infectious and inflammatory diseases [14]. It has also been suggested that the composition of the lining fluid in diseased airways can be abnormal, as compared with healthy pulmonary conditions, notably displaying a higher content of Na+ and Cl- [15]. Studying interactions between PS and nanoparticles may therefore help to elucidate design criteria for inhaled therapeutic nanoparticles intended to treat chronic respiratory diseases, e.g., asthma and chronic obstructive pulmonary disease, as well as to predict the fate of nanoparticles after deposition in the airways.

The interaction between nanoparticles and pulmonary barriers has previously been studied using a number of experimental techniques [16], e.g., the quartz crystal microbalance with dissipation monitoring (QCM-D), and Fourier-transform infrared-attenuated total reflectance (FTIR-ATR) spectroscopy. QCM-D is a highly sensitive and versatile tool for studying the properties of both model and complex layers adsorbed on the surface of a quartz crystal. Information about both changes in mass and viscoelastic properties of the adsorbed layers [17] is provided by measuring changes in the resonance frequency (Δf) and dissipation (ΔD) [18]. FTIR-ATR, in turn, is a versatile tool to study interactions between biomaterials and surfaces, and it can be applied in either a qualitative or a quantitative manner with limited interference from the solution beyond the interfacial region [19]. In contrast to QCM-D, contributions from adsorbates and solvent can be investigated separately using FTIR-ATR [18]. Hence, we used these two techniques to provide complementary information about the interactions between nanoparticles and PS. For both techniques, supported phospholipid bilayers are formed on a solid substrate to investigate the mechanisms involved in the interaction between nanoparticles and PS [18]. However, it is not known, if there is a difference between supported phospholipid bilayers on a solid substrate and the free-floating PS (multilayers) found in the airways.

Lipid-polymer hybrid nanoparticles (LPNs) have been reported to display an unprecedented ability to deliver nucleic acid cargoes to cells, both in vitro and in vivo [20,21]. The LPNs used in this study consist of the ionizable cationic lipid-like material referred to as lipidoid L5 [22] (Fig. S1), which displays a tetraamine backbone linked to five aliphatic chains (Table S1), thereby providing efficient interaction with polyanionic nucleic acids via attractive electrostatic interactions, and mediating cellular uptake, endosomal escape, and cytotoxic delivery. The LPNs also contain the biodegradable polymer poly(εcaprolactone-co-glycolic acid) (PLGA), which we hypothesize forms the core of the LPNs, surrounded by a shell of L5 monolayer and bilayer(s), and enabling sustained release of siRNA [20,23]. Recently, we designed dry powder inhaler formulations containing tumor necrosis factor (TNF)-α siRNA-loaded LPNs with aerosolization properties optimized for inhalation therapy targeting lung inflammation [24,25]. We previously designed ternary excipient mixtures composed of leucine, trehalose and dextran that protect TNF-α siRNA-loaded LPNs during spray drying, and result in dry powder inhaler formulations with preserved chemical siRNA integrity [24]. In addition, these dry powder inhaler formulations deposited homogeneously in the lower airways upon pulmonary administration. In the present study, the aim was to optimize the composition of the LPNs for pulmonary delivery. To that end, we evaluated the effect of L5 chain length and L5 content, which affect the physicochemical properties of the nanoparticles, on the interaction between LPNs and surfactant bilayers. PS was modelled using both planar supported bilayers and multilamellar vesicles (MLVs) in dispersion to model the interaction of the nanoparticles with PS in the alveolar fluid.
2. Materials and methods

2.1. Materials

2′-O-Methyl-modified dicer substrate asymmetric siRNA duplex directed against TNF-α was provided by GlaxoSmithKline (Stevenage, UK) as a dried, purified, and desalted duplex. The siRNA had the following sequence and modification pattern: TNF-α sense 5′-pGUCUCAGCCCUCCUCAUUCCUCGct-3′ and antisense 5′-ACGACAAUGACAGAGGCGUAGACAU-3′, where lower case letters represent deoxyribonucleotides, underlined capital letters represent 2′-O-methylribonucleotides, and p represents a phosphate residue. PLGA (lactide:glycolide molar ratio 75:25, M_W: 20 kDa) was rinsed twice with 99 % (v/v) ethanol to remove residual chloroform/methanol, eventually resulting in a thin dry lipid film. The lipid film was rehydrated in Tris buffer (10 mM, pH 7.4). The mixture was bath-sonicated for 5 min using an ultrasonic cleaner (Branson Ultrasonic Cleaner, Danbury, CT, USA), and subsequently heated at 60 °C for 1 h in a water bath with vortexing every 10th min. After 20 min of rehydration, the liposomes were tip-sonicated for 20 s with a MISONIX S-4000 probe sonicator (LLC, Newtown, CT, USA) (amplitude 70, power 16 W) to reduce their size, and they were subsequently vortexed for 1 min and annealed for 1 h at room temperature.

2.2. Preparation of TNF-α siRNA-loaded LPNs and physicochemical characterization

L_5N_{12} modified LPNs loaded with TNF-α siRNA were prepared using the double emulsion solvent evaporation method, essentially as reported previously [23]. The L_5N_{12} content, relative to the total solid content (L_5N_{12} and PLGA), was 15 % (w/w), and the L_5N_{12} TNF-α siRNA ratio was 15:1 (w/w), based on previous optimization [27]. LPNs were prepared with: (i) L_5 analogues of different chain lengths (L_5N_8, L_5N_{12}, L_5N_{14}, and L_5N_{16}, Table S1) were synthesized, purified, and characterized as previously reported [20,26]. Curosurf® (Poractant Alfa) was purchased from Chiesi Pharmaceuticals (Parma, Italy). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). The fluorescent dye 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Poly-L-lysine with a molecular weight of 70–150 kDa was acquired from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water of Milli-Q quality was used for all buffers and solutions.

2.3. Preparation of lipid vesicles

Curosurf® small unilamellar vesicles (SUVs) were prepared by sonication of Curosurf® suspensions (0.5 mg/ml) in an ice-water bath using four cycles of sonication at an amplitude of 80 % (50 W, UP50H, Hielscher Ultrasونics, Teltow, Germany) with 2 min wait time in between the cycles to avoid heating the suspensions.

DiO-labeled DPPC vesicles were prepared using the thin film method as previously described [28]. Briefly, DPPC and 2 % DiO (w/w) were dissolved in chloroform/methanol (9:1, v/v) in a round-bottomed flask. The organic solvents were removed using rotary evaporation (Laborota 4011 digital, Heidolph Instruments, Schwabach, Germany) under vacuum. Subsequently, the lipid film was rinsed twice with 99 % (v/v) ethanol to remove residual chloroform/methanol, eventually resulting in a thin dry lipid film. The lipid film was rehydrated in Tris buffer (10 mM, pH 7.4). The mixture was bath-sonicated for 5 min using an ultrasonic cleaner (Branson Ultrasonic Cleaner, Danbury, CT, USA), and subsequently heated at 60 °C for 1 h in a water bath with vortexing every 10th min. After 20 min of rehydration, the liposomes were tip-sonicated for 20 s with a MISONIX S-4000 probe sonicator (LLC, Newtown, CT, USA) (amplitude 70, power 16 W) to reduce their size, and they were subsequently vortexed for 1 min and annealed for 1 h at room temperature.

2.4. Dynamic light scattering (DLS) measurements

DLS was used to characterize the effect of surfactant components on the nanoparticle size distribution [9]. The colloidal stability of LPNs and Curosurf® SUVs was also investigated at 37 °C for 10 min. The z-average (nm) and zeta potential of LPNs and Curosurf® SUVs were measured at 25 °C using a Zetasizer Nano ZS equipped with a 633 nm laser and 173° detection optics (Malvern Instruments, Worcestershire, UK). The LPNs (0.1 mg/mL) were incubated at 37 °C for 10 min with Curosurf® SUVs (0.1 mg/mL) in 10 mM acetate buffer (pH 5.4) supplemented with 150 mM NaCl. The size distribution of the LPN and Curosurf® SUV mixture was also measured at 37 °C using a Zetasizer Nano ZS.

2.5. Quartz crystal microbalance with dissipation

QCM-D measurements were performed using a QSense Analyzer instrument equipped with standard modules (Biolink Scientific, Västra Frölunda, Sweden). Supported PS single bilayer was prepared by deposition of Curosurf® on a SiO_2 surface (QSense QXS 303re, 4.95 ± 0.05 MHz, 14 mm diameter, 0.3 mm thickness, 17.7 ng/cm² mass sensitivity), as described previously [29]. Cells and tubes were thoroughly cleaned with 2 % Hellmanex solution and rinsed multiple times with MQ, followed by rinsing in absolute ethanol, and dried using a N_2 flow. Prior to the experiments, the SiO_2 surfaces were first rinsed using 2 % Hellmanex solution, then washed with MQ water and ethanol, dried with N_2, and plasma cleaned (Model PDC-32G, Harrick Plasma, Ithaca, New York, NY, USA) for 2 min before cell assembly. SiO_2 surfaces were treated with divalent calcium cations (CaCl_2) at a concentration of 2 mM for 5 min before forming PS bilayers. Supported PS bilayers were formed by deposition and fusion of Curosurf® SUVs (z-average of approx. 64.0 nm) in MQ (0.1 mg/ml in MQ) at a flow rate of 100 µl/min at 50.0 °C using an external peristaltic pump in the presence of 2 mM Ca²⁺ ions. As indicated by the maximum Δf and ΔD, the critical vesicle coverage (red arrow in Fig. S2) was reached after approx. 4 min of injection. SUV deposition, rupture, and full formation of a PS layer was confirmed by a Δf value of –23 ± 1 Hz and a ΔD value of (0.010 ± 0.007) × 10⁻⁶ with respect to MQ water at 37.0 °C, as previously reported [29]. When stable readings from the instrument were observed, buffer (10 mM acetate buffer at pH 5.4 supplemented with 150 mM NaCl, or 10 mM Tris buffer at pH 7.4, supplemented with 30 mM NaCl or 150 mM NaCl) was injected. When stable readings were observed, the PS layer was exposed to 0.1 mg/ml nanoparticles for 20 min, followed by incubating the nanoparticles with PS for additional 20 min, and rinsing with the corresponding buffer for 20 min.

To form PS multilayers on the SiO_2 surface, the first PS layer was rinsed with 10 mM Tris buffer (pH 7.4) for 20 min. Subsequently, poly-L-lysine, used as an electrostatic polymer linker, was added to the first PS layer at 10 µg/mL for 20 min, incubated for 20 min, and then rinsed for 20 min using the same buffer. To form the second PS layer, Curosurf® SUVs in buffer (without CaCl_2) were added at 0.1 mg/ mL until the critical coverage of adsorbed vesicles was reached, followed by a rinsing step for 20 min using the same buffer.
buffer. To form the third PS layer, the steps to form the second layers were repeated, i.e., deposition of a poly-L-lysine layer and injection of Curosurf® SUVs. Both Δf and AD at the 3rd, 5th, 7th, 9th and 11th overtones were recorded simultaneously. For quantification, the 7th harmonic was chosen for maximum data robustness. All measurements were performed at 37.0 °C in triplicate.

2.6. Fluorescence spectroscopy

Deposition of one, two, and three bilayers, respectively, on the SiO₂ surfaces was quantified using fluorescence spectroscopy. DIO-labeled DPPC vesicles were mixed with Curosurf® SUVs at a weight ratio of 5:95 to label the PS bilayers. Subsequently, the first bilayer was deposited on the SiO₂ surface, the surface was washed with buffer for 20 min, followed by (i) pumping air at 0.2 ml/min for 2 min to remove any remaining liquid, and (ii) rinsing the surface with absolute ethanol for 20 min to remove PS from the surface and quantify the PS content in the collected PS samples. After ethanol evaporation, the samples were freeze-dried and reconstituted with 200 μl MQ to measure the fluorescence intensity. The same procedure was used to collect the PS materials after formation of two and three bilayers, respectively. The fluorescence in the samples was quantified using a fluorescence plate reader (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany) at excitation and emission wavelengths of 484 nm and 501 nm, respectively. The fluorescence intensities of one, two, and three bilayers, respectively, were compared to determine the percentage of coverage for each layer. All measurements were performed at 37.0 °C in triplicate.

2.7. Fourier transform infrared-attenuated total reflectance (FTIR-ATR)

Infrared spectra were recorded using a Nicolet i50 FTIR Spectrometer (Thermo Fisher Scientific) equipped with a flow-through gateway ATR flow-cell accessory (Specac Ltd., Kent, UK), thermostated at 37 °C using a water recirculation bath, and purged using dry compressed air. A peristaltic pump (IPC, Ismatec, Wertheim, Germany) was used to inject the samples and run the liquid through the flow cell at 0.2 ml/min. Trapezoid Silicon crystals (70 x 10 x 6 mm, 45° angle, Sil'tronix, Archamps, France) were cleaned with 1 % sodium dodecyl sulfate (SDS), rinsed thoroughly in MQ and absolute ethanol, and dried under a N₂ flow. The O-ring, the HPLC tubing, and the connectors were cleaned with 1 % (v/v) SDS and rinsed 15 times with MQ. Background spectra were obtained from 128 averaged interferogram scans for full characterization, acquired at 4 cm⁻¹ spectral resolution for wavenumbers between 4000 and 1500 cm⁻¹ in silicon crystals with MQ. Silicon crystals were pre-treated with CaCl₂ and Curosurf® SUVs prepared as described above. The method for forming PS bilayers was similar to that employed for the QCM-D measurements described above. After PS characterization, 2 ml 0.1 mg/ml nanoparticles was injected into the cell at a rate of 0.2 ml/min, while recording 32 scans per second (4 cm⁻¹ spectral resolution). This was followed by incubation for 60 min while recording 64 scans every 60 s. The PS was then rinsed (20 min, 0.2 ml/min) and characterized in acetate buffer (10 mM, pH 5.4) supplemented with 150 mM NaCl. Acquisition, background subtraction, and spectral analysis were performed using the OMNIC 9 software (Thermo Fischer Scientific). All experiments were done at 37.0 °C in duplicate.

2.8. Contact angle measurements

Contact angles were measured by sessile-drop goniometry using a theta-flex optical tensiometer (Biolin Scientific, Västra Frölunda, Sweden). A video of a drop of buffer (20 μl, 10 mM acetate buffer, pH 5.4, supplemented with 150 mM NaCl) on a solid lipid surface was recorded, and the contact angle between the buffer drop and the surrounding gas was determined from the video images using a fitting procedure.

2.9. Cryogenic transmission electron microscopy

Native PS was purified from porcine lungs obtained from a slaughterhouse, as previously reported [30]. The hydrophobic fraction of native PS was prepared by chloroform/methanol extraction, as described previously [31], and it contains all surfactant lipids and the hydrophobic surfactant proteins SP-B and SP-C. The total phospholipid concentration in PS was determined by phosphorus analysis, as previously described [32]. The hydrophobic fraction of native PS was reconstituted into MLVs in Tris buffer (5 mM, pH 7.4, supplemented with 150 mM NaCl), as described previously [33]. Briefly, MLVs were prepared by hydrating the dry proteolipid film in Tris buffer, and allowing it to swell for 1 h at 45 °C under interval mixing by vortexing (10 min mixing and 10 min wait time).

To study the influence of LPNs on the structure of PS, MLVs and LPNs were incubated at 37.0 °C for 10 min before the experiment. MLVs, LPNs, and the mixture of MLVs and LPNs, respectively, were applied to holey carbon copper grids (R2/2; Quantifoil, Großlőßnitz, Germany) and vitrified using a Cryoplunge (Gatan, Pleasanton, CA, USA). Sample imaging was carried out using a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan), operated at 100 kV, and recording at a final magnification of 2.84 Å/px using an F416 CMOS camera from TVIPS (Gauting, Germany) at 40,000 x nominal magnification.

2.10. Statistics

Data was analyzed using Origin software (OriginLab Corporation, MA, USA) and GraphPad Prism (version 8.4.2, La Jolla, CA, USA). Data were fitted with a 2FI model using Design-Expert (Stat-Ease, Minneapolis, MN, USA). Differences in means between groups were compared by one-way analysis of variance (ANOVA), and pair-wise comparison was performed using Tukey's post-test. A p-value ≤ 0.05 was considered statistically significant, p < 0.05 (**), p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****).

3. Results

3.1. Preparation and characterization of TNF-α siRNA-loaded LPNs

Formulations of TNF-α siRNA-loaded LPNs, modified with L₅ analogues of different chain lengths, and with different L₅ content, displayed z-averages of approx. 200 nm (Fig. S3A, C), and PDIs below 0.25 (Fig. S3B, D), as reported previously [26]. The encapsulation efficiencies of the siRNA-loaded LPNs were in the range of 44 % to 74 % (Table S2), also as reported previously [26].

3.2. Formation and evaluation of supported PS membrane bilayers and multilayers as models for studying interactions with nanoparticles

QCM-D was employed to characterize the interactions between LPNs and PS, with respect to Δf and ΔD using either: (i) single PS membrane bilayers, or (ii) multilayer PS membranes. The term Δf represents the mass change in the system, i.e., adsorption (a negative shift) or desorption (a positive shift), whereas ΔD is qualitatively related to the viscoelastic properties of the adsorbed layer. CaCl₂ was used to stabilize the vesicle-substrate interaction, because CaCl₂ has previously been shown to enhance spontaneous formation of negatively charged flat lipid bilayers [35]. The process of full PS membrane bilayer formation by Curosurf® SUV deposition and fusion is illustrated in Fig. S2. The Δf was 23 ± 1 Hz, indi-
cating a high PS membrane bilayer coverage [29]. In line with this, the \( \Delta D \) (0.010 ± 0.007) \( \times 10^{-6} \) was close to zero, supporting the notion of a smooth and rigid bilayer, confirming the formation of a single membrane bilayer.

Im mobilization of multilayer PS membranes on SiO\(_2\) surfaces was employed to minimize the impact of PS-substrate interactions. For PS multilayers, anionic SUVs adhered first to cationic poly-\( \epsilon \)-lysine (in 10 mM Tris buffer) via strong electrostatic attraction, followed by vesicle rupture and bilayer formation [36]. Poly-\( \epsilon \)-lysine is positively charged at pH 7.4 (\( p_\mathrm{Ka} \sim 10 \)) [37]. Poly-\( \epsilon \)-lysine was used not only to link the bilayers, but also as a fusogen to stimulate the formation of planar bilayers from negatively charged vesicles [36]. PS was labeled with DiO, and fluorescence spectroscopy was used to quantify the mass of each deposited layer, which is represented as the percentage of coverage. Addition of poly-\( \epsilon \)-lysine to the first PS bilayer lead to a \( \Delta f \) of 9.4 ± 1 Hz with \( < 1.0 \times 10^{-6} \) shift in \( \Delta D \) [at the 7th overtone, Fig. 1A (*)]. The low value for \( \Delta D \) indicates that poly-\( \epsilon \)-lysine is tightly associated to the bilayers. Addition of a second PS membrane bilayer resulted in a high coverage with a \( \Delta f \) of −23 ± 2 Hz and a \( \Delta D \) of (4.0 ± 0.6) \( \times 10^{-6} \) (Fig. 1A (II) and B). Subsequently, poly-\( \epsilon \)-lysine was added to the two PS membrane bilayers, resulting in a \( \Delta f \) shift of 2 ± 1 Hz, and a \( \Delta D \) shift of (2.0 ± 0.4) \( \times 10^{-6} \), indicating that poly-\( \epsilon \)-lysine binding causes dehydration of the gap between the membranes [36]. Addition of a third PS bilayer with high coverage resulted in a \( \Delta f \) of −28 ± 3 Hz and a \( \Delta D \) of (5.0 ± 0.5) \( \times 10^{-6} \) after buffer washing (Fig. 1A(III) and B). The \( \Delta D \) for the third PS bilayer was higher than the \( \Delta D \) associated with the second PS bilayer, although this difference was not statistically significant. The \( \Delta f \) upon PS binding was rather similar to that for the first PS bilayer, which suggests a largely intact second PS bilayer [36], although osmotic de-swelling and related water loss upon PS binding to the second poly-\( \epsilon \)-lysine layer precludes more detailed analysis of this phenomenon. The mass change of each bilayer was also confirmed by measuring the fluorescence intensity of each bilayer, which is consistent with the results of the \( \Delta f \) change (Fig. 1C). Thus, the finding that the intensity increase of the second and third bilayers was nearly equal to that of the first bilayer suggests that the SUVs fuse into consecutive bilayers, on top of the initial substrate-bound bilayer. The high dissipation of the third bilayer is also characteristic of an acoustically free-floating membrane bilayer [38]. Therefore, the consecutive and high viscoelastic properties of multilayers suggest weak substrate-PS interactions and PS multilayers displaying a high coverage.

3.3. Interactions between PS layers and LPNs using PS bilayers and multilayers

Having characterized the PS membrane bilayers and multilayers, LPNs were next added. First, we confirmed that no measurable aggregation occurred during the QCM-D study (up to 120 min) by incubating the nanoparticles in the desired buffer and temperature and measuring their size (Fig. S4).

The nanoparticle-PS interaction was determined by QCM-D measurements during nanoparticle injection (I), incubation (II), and buffer rising (III) using both PS membrane bilayers and multilayers (Fig. 2). For this, 25 % (w/w) \( \mathrm{L}_5\mathrm{N}_{12} \)-modified LPNs were employed and compared to non-modified PLGA nanoparticles (0 % \( \mathrm{L}_5\mathrm{N}_{12} \)) and siRNA-\( \mathrm{L}_5\mathrm{N}_{12} \) lipoplexes (100 % \( \mathrm{L}_5\mathrm{N}_{12} \)). After exposure to non-modified PLGA nanoparticles, both the \( \Delta f \) and \( \Delta D \) of PS multilayers were very close to the \( \Delta f \) and \( \Delta D \) values of PS bilayers, indicating no possibility for deformation and material exchange with the PS layers (Fig. 2A.1 and A.2). In contrast, \( \mathrm{L}_5\mathrm{N}_{12} \) modification of PLGA nanoparticles affected their interaction with PS. For 25 % (w/w) \( \mathrm{L}_5\mathrm{N}_{12} \)-modified LPNs, the value for \( \Delta f \) and \( \Delta D \) of PS bilayers returned to the initial values after buffer rinsing (Fig. 2B.1). However, \( \Delta f \) increased to 30 Hz after buffer rising, suggesting that material, e.g., adsorbed LPNs or LPNs modified with PS components, are removed from the PS multilayers (Fig. 2B.2). As compared to the interaction of \( \mathrm{L}_5\mathrm{N}_{12} \)-siRNA lipoplexes with PS bilayers (Fig. 2C.1), \( \mathrm{L}_5\mathrm{N}_{12} \)-siRNA lipoplex interaction with PS multilayers exhibited a significant increase in dissipation (Fig. 2C.2). In plots of \( \Delta f \) versus \( \Delta D \), the slope (i.e., the binding affinity [39]) of adsorption of nanoparticles to the two PS layer models was identical, indicating that the number of bilayers has no effect on the LPN binding affinity. For non-modified PLGA nanoparticles (Fig. 2A.3), the plot of \( \Delta f \) versus \( \Delta D \) showed very weak interactions between PS and nanoparticles. There was a progressive increase in dissipation accompanying the decay in resonance frequency upon LPN deposition (Fig. 2B.3), which suggests the formation of a diffuse layer. Upon reaching a turning point at which membrane saturation has been achieved, \( \Delta D/\Delta f \) shows a reverse trace for the membrane multilayer model, illustrating a decrease in mass and viscoelasticity, caused by detachment of deposited LPNs. When LPNs were applied to a single membrane bilayer, the dynamic response was slower than the response for binding of LPNs to multilayer membranes. In addition, siRNA-\( \mathrm{L}_5\mathrm{N}_{12} \) lipoplexes (Fig. 2C.3) changed the \( \Delta f/\Delta D \) trace direction. This indicates that lipid

---

**Fig. 1.** Representative QCM-D measurements of \( \Delta f \) (red) and \( \Delta D \) (blue) at the 3rd, 5th, 7th, 9th and 11th overtones of the PS membrane multilayers at pH 7.4 starting from I (first bilayer) the formation of PS from SUVs onto the SiO\(_2\) surface in the presence of 2 mM CaCl\(_2\), (*) poly-\( \epsilon \)-lysine (10 \( \mu \)g/ml) with a molecular weight range of 70–150 kDa was added, and SUVs were injected without CaCl\(_2\) to form the second (II) and the third (III) bilayers (A). \( \Delta f \) and \( \Delta D \) change in different PS bilayers (B). Fluorescence intensity ratio of the second and third bilayer, relative to the fluorescence intensity of the first bilayer (C). For B and C, bars represent mean values ± SD, \( n = 3 \). Significantly different results are indicated: *** \( p < 0.001 \), and **** \( p < 0.0001 \). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
exchange with PS resulted in an increased viscoelasticity of the layer without affecting the deposited mass, which suggests either an irreversible change of membrane structure, or that LPNs are trapped at the membrane interface [40]. The different changes observed for the different overtones indicate that lipid exchange/removal takes place, especially in the outer membrane bilayer [40] (Fig. S5).

Experiments performed using single and multiple PS membrane bilayers revealed that the binding affinity was almost identical at the onset of nanoparticle deposition for both models. Furthermore, single PS membrane bilayers and multilayers exhibited similar behavior with respect to frequency and dissipation during LPN deposition. However, PS multilayers were more susceptible to interaction between PS and LPNs and subsequent removal of material from the multilayers. Nevertheless, when comparing LPN binding affinity, PS membrane bilayers reflect the behavior also of more complex multilayer systems, while at the same time being more robust. Therefore, PS membrane bilayers were subsequently used to investigate the effect of formulation variables and microenvironment on the interactions between LPNs and PS.

3.4. Effect of formulation variables on the interaction between LPNs and PS

In order to demonstrate the effects of the physicochemical properties of LPNs, Lα analogues with 8, 12, 14 and 16 carbon atoms (Table S1, Fig. S1) were studied to examine the effect of aliphatic chain length, which confers variation in LPN surface hydrophilicity and fluidity. Furthermore, the effect of LPN surface charge was studied by varying the Lα/N2 content. The interaction between LPNs displaying different physicochemical properties and PS was studied under different experimental conditions, e.g., (i) pH 7.4/ionic strength 30 mM, (ii) pH 7.4/ionic strength 150 mM, and (iii) pH 5.4/ionic strength 150 mM. The interaction strength between LPNs and PS at pH 7.4 (ionic strengths of 30 and 150 mM, respectively) was weaker than at pH 5.4 and an ionic strength of 150 mM, as demonstrated below. Therefore, the latter condition was chosen to study the effect of formulation variables on the interaction between nanoparticles and PS.

3.4.1. Physicochemical properties of LPNs

As outlined above, surface hydrophobicity affects the interaction between LPNs and PS, given that both systems contain hydrophobic components. Biophysical studies have shown that the inhibitory effect of PS increases with increasing nanoparticle surface hydrophobicity due to adsorption of hydrophobic SP-B and SP-C, which are key to inhibiting the increase in surface tension [41]. The same is expected for membrane fluidity, because fluidity has been found to influence the stability and permeability of nanoparticles in mucus, as well as their interaction with cells [42,43]. In addition, surface charge is a key factor to define the fate of nanoparticles after pulmonary delivery, i.e., translocation of nanoparticles into the blood circulation or the lymphatic system.
and mucociliary or alveolar macrophage clearance [2]. Hence, we first determined: (i) the hydrophobicity of the L5 analogues by contact angle measurements, and (ii) the zeta potential of the LPNs. As expected, the longer the chain length of the L5 analogues, the higher the hydrophobicity (higher contact angle values) (Fig. 3A) and the lower the membrane fluidity [26]. However, all LPNs displayed almost identical zeta potentials, independently of the L5 chain length (Fig. 3B). In contrast, LPNs with a higher L5 content (and correspondingly lower PLGA content to keep the solid content constant) displayed a higher positive surface charge (Fig. 3C), ranging from −1.6 mV to 25.1 mV in an L5N12 content-dependent manner (0% to 100%).

3.4.2. Effect of L5 analogue chain length on the interaction between nanoparticles and PS

We then studied the influence of surface hydrophobicity and membrane fluidity of LPNs on their interactions with PS. LPNs modified with L5 analogue of different chain lengths were found to interact differently with PS. First, we used DLS to evaluate the interaction of LPNs with PS. The particle size distribution of LPNs/PS mixtures was compared at different L5 analogues chain lengths, i.e., ranging from L5N8 to L5N16. Curosurf® SUVs displayed an average hydrodynamic diameter of 60 ± 21 nm (Fig. S6). LPNs modified with L5 analogues of different chain lengths displayed comparable hydrodynamic diameters of approx. 190 nm and narrow size distributions (Fig. S6). DLS analysis of LPNs modified with the L5 analogues of shorter chain length displayed a stronger interaction with PS than LPNs modified with L5 analogues of longer chain length. Curosurf® SUVs mixed with L5N8 or L5N12-modified LPNs displayed one peak with increased hydrodynamic diameter (Fig. S7), as compared to LPNs alone, indicating fusion with PS and formation of a corona. Additionally, Curosurf® SUVs mixed with L5N14- or L5N16-modified LPNs displayed a small-sized peak of approx. 60 nm (Fig. 4A). Curosurf® SUVs mixed with L5N8- or L5N12-modified LPNs displayed one peak with increased hydrodynamic diameter compared with LPNs (Fig. 4A), indicating fusion with PS and formation of a corona.

The size distribution of Curosurf® SUVs incubated with LPNs in acetate buffer (pH 5.2) without NaCl was also measured to investigate the influence of electrostatic interaction. Under these conditions, the small-sized peak observed for L5N14- and L5N16-modified LPNs in the presence of NaCl (Fig. 4A) also appeared for L5N12-modified LPNs (Fig. S7). This indicates that formation of these small-sized aggregates is not due to electrostatic interaction. Taking into account the chemical structure of L5 molecules with a small headgroup and five chains of varying length, one would expect that these molecules would show a tendency to form non-lamellar phases, which, in turn, would lead to the formation of uneven coatings on the nanoparticle surface, as observed by cryo-TEM (Fig. 6). Since increasing the length of the L5 analogue chain would favor the formation of such non-lamellar phases, it is conceivable that for LPNs with L5 analogues with chains of 14 and 16 carbon atoms, some L5 molecules could detach from the nanoparticle surface forming micelles that may incorporate surfactant lipids.

Compared to L5N8-modified LPNs (Fig. 4B), L5N12-modified LPNs displayed the same trend for the changes in Δf ~ -9.8 Hz and ΔD ~ 9.0 × 10⁻⁶, but quantitatively, these effects were smaller than those observed for L5N8-modified LPNs (Fig. 4C). Thus, binding of L5N14-modified LPNs to PS resulted in a Δf value of ~ -3.7 Hz and a ΔD value of 2.6 × 10⁻⁶ after 1 h incubation, both returning to the initial values after rinsing with buffer (Fig. 4D). In contrast, L5N16-modified LPNs displayed a dissipation of < 1.3 × 10⁻⁶, while Δf decreased steadily (Fig. 4E), indicating LPN deposition on dense surface layers, or LPN incorporation into the PS bilayers. Taken together, the DLS and QCM-D results demonstrate that LPNs containing L5 analogues with shorter aliphatic chains interact stronger with PS. Next, we modelled the QCM-D results for the Δf of LPNs with different fluidity (estimated based on the mean molecular area of the L5 analogue [26]) and hydrophobicity (contact angle of a buffer droplet onto different lipid surfaces using 2FI). The predicted R² of 0.9791 is well in agreement with the adjusted R² of 0.9872. The p-value is below 0.0001, which indicates that the model terms are significant. Response surface plots were constructed for Δf as a function of fluidity and hydrophobicity (Fig. 4F). The model was used to predict that LPN deposition onto PS increases with increased LPN fluidity. A decrease in frequency from ~1 Hz to ~8 Hz was observed when the mean molecular area was increased from 40 Å² to 150 Å². However, the frequency was negatively influenced by the LPN hydrophobicity, i.e., the lower the hydrophobicity, the larger the deposition, as indicated by the decreased Δf from ~1 Hz to ~8 Hz when the contact angle was increased from 25° to 95°. The increased adsorption of LPNs modified with the L5 analogues of shorter chain length (i.e., L5N8 and L5N12) on PS could be the result of a more flexible structure of the L5 analogues of shorter chain length, while L5N14- and L5N16-

Fig. 3. Contact angle (hydrophobicity) of L5 analogues with different chain lengths (A), zeta potential of siRNA-loaded LPNs modified with 15% (w/w) L5 analogues of different aliphatic chain lengths dispersed at pH 5.4/ionic strength 150 mM (B), and zeta potential of siRNA-loaded LPNs modified with L5N12 at different weight ratios, dispersed in acetate buffer (pH 5.4, 150 mM) (C). Data points represent mean values ± SD (n = 1, three technical replicates).
modified LPNs displayed a more compact structure due to stronger hydrophobic interactions between the aliphatic chains. In contrast to the higher fluidity of the L₅ analogues of shorter chain length (L₅N₈ and L₅N₁₂), L₅N₁₄- and L₅N₁₆-modified LPNs displayed smaller average molecular areas due to stronger hydrophobic interactions. The finding that LPNs modified with the L₅ analogues of shorter chain length interact stronger with PS than LPNs modified with the L₅ analogues of longer chain length, indicates that the fluidity of LPNs influences their interaction strength with PS.

### 3.4.3. Effect of L₅ content on interactions between LPNs and PS

Next, we investigated the effect of L₅ content on the interactions between LPNs and PS using an L₅ analogue of medium aliphatic chain length, i.e., L₅N₁₂, at pH 5.4/ionic strength 150 mM. The interactions between LPNs and PS were found to be dependent on the L₅N₁₂ content. The particle size distribution of LPN/PS mixtures was compared at different L₅N₁₂ contents, i.e. ranging from 0:100 to 100:0 (Fig. 5A). For 15 % L₅N₁₂-modified LPNs, negatively charged Curosurf® SUVs were observed to fuse with the LPNs, resulting in the appearance of a peak in the DLS measurement. The size of LPNs incubated with Curosurf® SUVs shifted towards higher sizes with increased L₅N₁₂ content, indicating stronger interaction. At 50 % (w/w) L₅N₁₂, it is likely that aggregates are formed. However, L₅N₁₂-siRNA lipoplexes incubated with Curosurf® SUVs displayed similar size distributions as lipoplexes, which might be related to the high miscibility of L₅N₁₂ with PS.

Continuous LPN deposition was also detected using QCM-D without shedding during buffer rinse. For all formulations containing more than 15 % L₅N₁₂, extensive deposition was observed after injection (Fig. 5B-F). In parallel to the expected large shift in frequency, a large Δf was also detected (*) during injection of the formulation. However, both the frequency and the dissipation decreased after reaching a maximum (Fig. 5D-G), possibly due to disruption of PS bilayers and removal of both PS molecules and LPNs. This is supported by the formation of LPNs/SUVs aggregate or large vesicles at L₅N₁₂ contents above 50 %. With increasing L₅N₁₂ content, the affinity of LPNs to PS was increased, the time required to reach maximum frequency and dissipation change point (*) was shorter, and the slope of the Δf - AD plots was reduced (Fig. 5H), the latter related to faster kinetics of LPN binding to PS.

In the lungs, surfactant lipids form a lipid monolayer at the alveolar air–liquid interface, which is associated to membrane reservoirs, including MLVs. The structural change of MLVs in the presence of LPNs was studied using cryo-TEM (Fig. 6). The MLVs were polydisperse, containing a mixture of multilayer stacks, unilamellar vesicles, and multi-vesicular bodies (Fig. S8), which define the typical structure of PS [26]. As expected, non-modified PLGA nanoparticles displayed a spherical shape (Fig. 6A), and showed limited interaction with PS vesicles (Fig. 6B, red arrow), which is consistent with the QCM-D results. In addition, 15 % and 50 % (w/w) L₅N₁₂-modified LPNs displayed spherical nanoparticles with a size consistent with the particle size measurements and an electron-dense core (Fig. 6C and D), as reported previously [26]. The siRNA-loaded LPNs modified with 15 % (w/w) L₅N₁₂ exhibited a strong interaction with PS MLVs, generating negatively curved structures in the MLVs (red arrow, Fig. 6E). The resultant structures might facilitate interaction between the surfactant film and the multilayered structures that are attached in the alveolar fluid (the surfactant reservoir) [3]. Further increase in the lipidoid content to 50 % altered the interactions between surfactant layers that maintain PS structure, e.g., membrane-opened vesicles and smaller unilamellar vesicles (red arrow, Fig. 6F), which might be related to the possible nanomaterial-induced withdrawal of SPs. Taken together, the increased surface charge of LPNs not only leads to faster LPN binding kinetics to PS, but also enhances the interaction strength with PS.

### 3.5. Effect of pathological microenvironments on the interaction between LPNs and PS

To get insights into the effect of the local environment on LPN-PS interactions, siRNA-loaded LPNs modified with L₅ analogues of different chain lengths were used. Since siRNA-loaded LPNs modi-
fied with \( L_5 \) analogues of different chain lengths displayed comparable results at (i) pH 7.4/ionic strength 30 mM, (ii) pH 7.4/ionic strength 150 mM, and (iii) pH 5.4/ionic strength 150 mM (Fig. S9). \( L_5 \)-modified LPNs were selected for the subsequent studies. The condition of pH 5.4/ionic strength 150 mM was used to reflect the pathological microenvironment in human lungs [39]. In addition, the condition of pH 7.4/ionic strength 150 mM was employed to investigate the effect of ionic strength.

The zeta potential of Curosurf \( /C_210 \) SUVs was significantly less negative at an ionic strength of 150 mM than at lower ionic strength (30 mM) (Fig. 7A), which might be related to a structural change of DPPC at higher ionic strength [44] leading to a less negatively charged surface. Hence, NaCl shields the charge, resulting in a less negatively charged surface at higher ionic strength. In contrast, the ionic strength had a non-significant impact on the surface charge of the LPNs, and decreasing the pH from 7.4 to 5.4 led to a slightly reduced negative zeta potential in the presence of NaCl, possibly due to the high degree of protonation of \( L_5 \) at lower pH. Mirroring this, \( L_5 \)-modified LPNs exhibited limited deposition at pH 7.4/ionic strength 30 mM, where both LPNs and Curosurf \( /C_210 \) SUVs displayed negative zeta potentials (Fig. 7B). Increasing the buffer salinity to 150 mM led to slightly higher LPN adsorption (Fig. 7C), because the surface charge of the LPNs/Curosurf \( /C_210 \) SUVs is shielded by Na\(^+\) and Cl\(^-\) ions. In contrast, LPN binding to PS at pH 5.4 was much more pronounced, evident from a large change in frequency, reaching \(-5.6 \) Hz after 20 min injection at pH 5.4/ionic strength 150 mM (Fig. 7D), since both \( L_5 \) Na\(^+\) and Curosurf \( /C_210 \) SUVs are fully protonated. In parallel to the decrease in \( \Delta f \), \( \Delta D \) increased (reaching \( \Delta D \approx 3.9 \times 10^{-6} \) over the same time period). This relatively slight increase in \( \Delta D \) might reflect attachment to or incorporation of adsorbed LPNs into PS. However, continuous water adsorption may also lead to a continuous increase in frequency and dissipation, which complicates more in-depth analyses. The values for \( \Delta f \) and \( \Delta D \) changed to \(-15.1 \) Hz and \(-10.9 \times 10^{-6} \), respectively, due to partial desorption accompanied by layer disorganization during buffer rinsing. Since this effective hydration is expected to depend on both the structure and composition of the adsorbed layer, but also on the environmental conditions, we next performed FTIR-ATR studies to dissect these effects at the molecular level.

### 3.5.1. FTIR-ATR studies of the interactions between LPNs and PS

Having probed the overall formation of single PS membrane bilayers and multilayers, as well as the interactions of LNPs with these systems, we then used FTIR-ATR to compare the interaction between LPNs and PS in a full compositional and kinetic study. Curosurf \( /C_210 \) SUVs were deposited onto SiO\(_2\) crystals, and their IR absorbance was monitored in the region between 1500 and 4000 cm\(^{-1}\) (Fig. S10). Due to difficulties in differentiating vibrations of PS bilayers from those of the surrounding water [45], differences in OH vibration modes (3000–3500 cm\(^{-1}\) and 1600–
Fig. 6. Representative cryo-TEM images of MLVs of PS, LPNs, and LPNs/PS combinations. Non-modified PLGA nanoparticles (A), non-modified PLGA nanoparticles with MLVs of PS (B), 15 % (w/w) L_{5}N_{12}-modified LPNs (C), 50 % (w/w) L_{5}N_{12}-modified LPNs (D), 15 % (w/w) L_{5}N_{12}-modified LPNs with MLVs of PS (E), and 50 % (w/w) L_{5}N_{12}-modified LPNs with MLVs of PS (F). Red arrows refer to the interaction area between LPNs and PS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Influence of experimental conditions reflecting pathological microenvironments on the interaction between LPNs and PS. Zeta potential of Curosurf® SUVs and LPNs dispersed in different buffers (A), pH 7.4/ionic strength 30 mM (B), pH 7.4/ionic strength 150 mM (C), and pH 5.4/ionic strength 150 mM (D). For A, bars represent mean values ± SD, n = 3. Significantly different results are indicated: **** p < 0.0001.
1700 cm\(^{-1}\), respectively) are difficult to interpret. Therefore, the discussion below focuses on: (i) the hydrocarbon vibrational peaks (between 2800 and 3010 cm\(^{-1}\)), corresponding to the symmetric and antisymmetric stretching modes of the different C-based groups, and (ii) the stretching mode of the C=O group [46,47].

The kinetic evolution of these spectral features was monitored during LPN injection and incubation with PS at pH 5.4/ionic strength 150 mM. The peaks in the range of 2800–3010 cm\(^{-1}\) (L\(_2\)N\(_8\) aliphatic chain) and 1720–1780 cm\(^{-1}\) (stretching vibration of the C=O bond from PLGA) were identified from separate FTIR-ATR experiments with PLGA, L\(_2\)N\(_8\), and LPNs (Fig. S11). Consistent with the QCM-D findings showing that non-modified PLGA nanoparticles display no or only very weak interactions with PS, no IR spectral differences were observed, neither for the carbonyl band (Fig. 8A, Fig. S12) nor for the acyl chain (Fig. 8B, Fig. S12).

In contrast, and in line with the QCM-D results, L\(_2\)N\(_8\)-modified siRNA-loaded LPNs binding to PS gave rise to an increased C=O signature peak (1770 cm\(^{-1}\)) upon injection of LPNs (Fig. 8A, Fig. S13). Pronounced interaction of L\(_2\)N\(_8\)-modified LPNs with PS was observed in both the CH region (around 2850–3000 cm\(^{-1}\)) and in the C=O region during incubation and buffer rinsing (Fig. 8A and B, Fig. S13). Signals arising from the LPNs increased during buffer rinsing, which suggest rearrangement of LPNs in the PS layer, i.e., insertion of L\(_2\)N\(_8\) from the LPNs into the PS bilayers. This would result in a more complex, disordered, and dissipative structure, in line with the pronounced increase in dissipation observed for the QCM-D measurements. Taken together, these results are in line with the QCM-D findings for the LPN-PS interactions, which demonstrate that a pathological microenvironments enhances LPN deposition onto PS.

4. Discussion

PS forms a continuous and dynamic lining layer between the aqueous layer of liquid coating the airways and the air [48]. The interfacial surfactant film is formed by a single interfacial monolayer of amphiphilic molecules at the air–liquid interface and a network of interconnected membranes between the interfacial film and surface-associated multilayer structures, which act as reservoirs of surface-active molecules [49]. The mechanical properties of PS layers are determined by the mechanics of both monolayers and bilayers, as well as by the strong connection between them [49,50]. Modelling in vitro the deposition of nanoparticles onto the hydrophobic tail region of PS monolayers in the lungs is a complex challenge, which has not been addressed in the present study. In addition to the water-exposed surfactant membrane models used in the present study, we are also studying the interaction of LPNs with PS at the air/liquid interface using a PS monolayer model (data not shown). Hence, due to the difficulty in mimicking PS films with the multi-bilayer stacks connected to the interfacial monolayer, our focus was to investigate the interaction between PS and LPNs after rapid translocation into the subphase.

In order to investigate the interaction mechanisms between nanoparticles and PS, supported Curosurf® bilayers were used as model systems to simulate the PS layer [31]. However, there are substantial differences between a single supported bilayer and multilayered systems [38]. Furthermore, the PS layer is viscoelastic with specific dynamic and rheological properties, including dilatational viscosity and in-plane compressibility/expansivity [3]. Therefore, supported phospholipid bilayers have been used as model systems to simulate PS layers [18] and study the differences between a single supported bilayer and a multilayer system, like the one formed by PS at the respiratory air–liquid interface [38]. Rigid single supported PS bilayers may therefore represent an oversimplification of the behavior of the interfacial structures of PS as they exist in vivo. As shown in the cryo-TEM experiments, which were conducted in a liquid environment, PS forms MLVs, as reported previously [51]. In the QCM-D study, multilayers were deposited on a solid substrate. PS membrane multilayers exhibit folds and interlamellar swapping, consistent with the poly-l-lysine and surfactant protein forming extensive membrane–membrane contacts, which are similar to structures found in PS in a liquid environment. In addition, the change of Δf and ΔD suggest the formation of highly viscoelastic and intact multilayers. We suggest that the PS membrane multilayer model represents a more suitable model than the PS bilayer model, because PS membrane multilayers more closely reflect the PS multilayer stacks present in the alveoli. PS multilayers are more prone to interact with LPNs with respect to lipid removal/exchange after LPN deposition. However, the multilayered PS model display limitations, e.g., different inter-layer interactions of multilayered structure due to the presence of poly-l-lysine. The negatively curved structure of MLVs formed by the introduction of LPNs, as displayed in the cryo-TEM images, will not be present in the QCM-D model due to different inter-layer interactions.

The single PS membrane bilayer model is an indispensable supplement to the multilayer model, and it should be combined with the multilayer model to comprehensively investigate the interaction between PS and LPNs. A single PS membrane bilayer model reflects well the behavior of more complex multilayer systems, e.g., with respect to binding affinity and interaction strength, which

---

**Fig. 8.** Increase in carbonyl band (A, ν(C=O), 1720–1780 cm\(^{-1}\)) and acyl chain (B, ν(–CH\(_2\)), 2800–3000 cm\(^{-1}\)) absorbance upon injection of L\(_2\)N\(_8\)-modified LPNs and non-modified PLGA nanoparticles, respectively, to PS (10 min), subsequent incubation (60 min), and buffer rinsing (20 min), normalized with respect to the final absorbance measured for the carbonyl band region of the L\(_2\)N\(_8\)-modified LPNs. (I), (II) and (III) refer to the three distinct steps of these experiments, i.e., (I) exposure of PS to LPNs for 10 min, followed by (II) incubation for 1 h, and (III) subsequent rinsing with the corresponding buffer for 20 min. Data points represents mean values ± SD, n = 2.
underlines its usefulness as a model for comparing the influence of variables formulations or microenvironment conditions. Due to the difficulties in forming PS multilayers, multilayered systems can be used in formulations with complex behavior. Therefore, the PS membrane bilayer model is suitable for formulation optimization, while the multilayered model is more precise for obtaining comprehensive information of LPNs.

The design, synthesis, and characterization of natural and synthetic lipids for RNA delivery have been the subjects of extensive research. The properties of the individual lipid components determine the physicochemical properties of the resulting RNA-loaded nanoparticles, which influence their capability for drug delivery, e.g., serum stability, drug loading, and drug release [52]. The physicochemical properties of nanoparticles, including hydrophobicity, membrane fluidity, and surface charge, influence their intrapulmonary fate by determining the interaction between nanoparticles and lung tissue [53–55]. However, little is known about how the physicochemical properties of nanoparticles influence their interaction with PS. Hence, obtaining such knowledge is crucial for our understanding of the fate of inhaled nanoparticles.

We found that nanoparticles may become embedded into PS layers with or without disrupting the structure of the lipid layer, resulting in modification of the NP surface with PS components. However, depending on their surface properties, LPNs may also show limited interaction with PS. (Fig. 9). In particular, the aliphatic chain length of the lipid component is an important factor that governs the bilayer packing properties and the stability of LPNs. Therefore, L5 analogues with chain lengths of 8, 12, 14 and 16 carbon atoms, respectively, were studied to examine the effect of aliphatic chain length and fluidity. For LPNs intended for pulmonary drug delivery, hydrophilic nanoparticles have previously been found to display advantages for systemic drug delivery due to their rapid translocation to secondary organs and tissues [56]. In contrast, hydrophobic nanoparticles display a relatively long retention time [57]. The enhanced interaction strength with PS may assist drug distribution over the respiratory surface, if the nanoparticles are deposited in the upper or conductive airways, thus assisting in reaching target sites in the deep lungs. Nanoparticles of higher fluidity, e.g., LPNs modified with L5Na16, interact stronger with PS, and they can be trapped at the surfactant lining layer, which consequently lead to prolonged retention. However, nanoparticles with lower fluidity display higher uptake in macrophages [43], which might be optimal for chronic respiratory diseases with lung inflammation, where macrophages represent the target cells. We also found that fluidity influences the interaction strength between LPNs and PS. However, additional factors, e.g., in vitro cytotoxicity and transfection efficiency, and in vivo efficacy should also be considered when designing inhaled LPNs.

The LPNs have been engineered into solid dosages forms as dry powder inhaler formulations intended for inhalable alveolar delivery to silence the TNF-α expression in macrophages [24,25]. The L5 content significantly influences the physicochemical properties of LPNs, i.e., the surface charge, which is one of the physicochemical properties that influences particle uptake by macrophages [58]. In addition, the surface charge also plays an important role for the interaction between LPNs and PS. We found that negatively charged L5Na16-modified LPNs display limited interaction with the PS layer. In contrast, LPNs with a near-neutral surface charge, e.g., non-modified PLGA nanoparticles and 15 % L5Na12-modified LPNs, display a corona by binding of SP components to the LPN surface, which might facilitate the translocation of LPNs from the air to the alveolar fluid. However, the effect of surface modification with SP needs further investigation with focus on the uptake of nanoparticles by cells. A higher positive surface charge of LPNs, e.g., as for 25 % and 50 % LPNs investigated here, reportedly leads to a higher binding affinity to PS via attractive electrostatic interactions. The cationic charge of LPNs may not only affect the performance of the nanoparticles after deposition, but may also influence the biophysical properties of PS, which is crucial to study when investigating the toxicological profile. As demonstrated

![Fig. 9](Image 120x98 to 474x338)

**Fig. 9.** Schematic representation of the fate of nanoparticles upon inhalation into the alveoli. The nanoparticles interact with pulmonary surfactant in various ways, depending on the surface properties (hydrophobicity, membrane fluidity, and surface charge) of the nanoparticles. (i, ii) Hydrophilic nanoparticles display high permeability in the surfactant layer, which results in rapid translocation. However, hydrophobic nanoparticles can interact with PS to prolong the retention time, and the hydrophobic surface of nanoparticles can be modified by hydrophobic SPs. (iii, iv) Nanoparticles with a higher fluidity can be trapped at the surfactant lining layer and therefore lead to prolonged retention. (v, vi, vii) Negatively charged LPNs display limited interaction with the PS layer. Neutral LPNs are covered with a PS corona. In contrast, positively charged LPNs significantly disrupt the PS layer (drawn using BioRender.com).
using cryo-TEM, cationic LPNs disrupt the structure of PS, which eventually may give rise to toxicity when applied at a relatively high dose. To avoid structure-disruptive effects of cationic nanoparticles and to promote interaction with PS (section 3.3), near-neutral nanoparticles with a compact structure and a surface lipidoid modification, e.g., 15 % L\(_5\)N\(_{12}\)-modified LPNs, might be the most efficient and safest for pulmonary drug delivery in the treatment of lung inflammation diseases. Moreover, we found that the threshold ensuring complete coating of the polymer core is 15 %, because higher binding affinity and faster interaction kinetics were observed above this lipid content. Due to the fact that the surface charge of LPNs increases as the content of lipid increases, it is not necessary to exceed the threshold defining a complete lipid coating surface.

Although not investigated in the present study, the mucus layer in the respiratory tract represents yet another biological barrier, which considerably impedes the transport of nanoparticles used for pulmonary delivery [59,60]. Mucus is a complex aqueous mixture composed of mucins, lipids, proteins, enzymes, and salts covering the epithelial surfaces of the proximal part of the respiratory tract. Interactions between mucus and nanoparticles also play an important role for the fate of particulate drug delivery systems [2].

5. Conclusion

Improved understanding of the underlying interaction mechanisms between nanoparticles and PS may help in optimizing the design of nanocarriers intended for inhalation therapy. Here, we investigated multiple aspects of the interaction mechanisms between nanoparticles and PS using models mimicking nanoparticle deposition in the airways with focus on the effect of the PS model and the physicochemical properties of the nanoparticles. Based on our biophysical studies of different formulations, we found that PS membrane multilayers were more susceptible to disruption than single membrane bilayer systems upon interaction with LPNs with respect to lipid/SPs removal, but PS bilayers and multilayers exhibited similar behavior with respect to frequency and dissipation. In addition, the physicochemical properties of LPNs, including hydrophobicity, membrane fluidity, and surface charge, significantly influence interaction between nanoparticles and PS. In particular, the interaction strength is closely correlated to the fluidity and surface charge of the LPNs. Furthermore, pathological microenvironments (i.e., pH 5.4 and an ionic strength of 150 mM, corresponding to inflammatory conditions in vivo) promoted binding of LPNs to PS. Future experiments should focus on the effect of LPNs on the biophysical function and lateral structure of PS, as well as the translocation ability of LPNs through PS. The relationship between PS interaction and nanoparticle biodistribution (or efficacy) in vivo also needs further investigation.

Funding

We gratefully acknowledge the support from the Innovative Medicines Initiative Joint Undertaking under grant agreement No. 115,363 resources, which are composed of financial contribution from the European Union’s Seventh Framework Programme (FP7/2007–2013) and EFPIA companies’ kind contribution. This project has received funding from the European Union’s Seventh Framework Programme for research, technological development and demonstration under grant agreement No. 600207. We acknowledge the Drug Research Academy Graduate Programme in Pharmaceutical Sciences, University of Copenhagen, for funding the Small-Scale Powder Dispenser. We are also grateful to the Lundbeck Foundation – Denmark (Grant No. R219-2016-908), the Novo Nordisk Foundation – Denmark (Grant No. NNF17OC0026526 to C.F.), Independent Research Fund Denmark (Grant No. 4184-00422 and 9041-00198B to C.F.), and Hørslev Fonden – Denmark for financial support. O.C., B.G.-A. and J. P.-G. acknowledge support from the Spanish Ministry of Science and Innovation (Grant No. PID2021-124932OB100) and the Regional Government of Madrid (Grant No. P2108/NMT-4389). We acknowledge the China Scholarship Council (CSC) for the Scholarship to You Xu (Grant No. 201906210064).

CRediT authorship contribution statement


Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Abhijeet Girish Lokras and Akash Chakravarty for synthesizing L\(_5\)N\(_{12}\).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2022.11.059.

References

