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Biophysical inhibition of synthetic vs. naturally-derived pulmonary surfactant preparations by polymeric nanoparticles

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ABSTRACT

Reasonable suspicion has accumulated that inhaled nano-scale particulate matter influences the biophysical function of the pulmonary surfactant system. Hence, it is evident to provide novel insights into the extent and mechanisms of nanoparticle–surfactant interactions in order to facilitate the fabrication of safe nanomedicines suitable for pulmonary applications.

Negatively- and positively-charged poly(styrene) nanoparticles (diameters of ~100 nm) served as model carriers. Nanoparticles were incubated with several synthetic and naturally-derived pulmonary surfactants to characterize the sensitivity of each preparation to biophysical inactivation. Changes in surface properties (i.e. adsorption and dynamic surface tension behavior) were monitored in a pulsating bubble surfactometer.

Both nanoparticle formulations revealed a dose-dependent influence on the biophysical behavior of all investigated pulmonary surfactants. However, the surfactant sensitivity towards inhibition depended on both the carrier type, where negatively-charged nanoparticles showed increased inactivation potency compared to their positively-charged counterparts, and surfactant composition. Among the surfactants tested, synthetic mixtures (i.e. phospholipids, phospholipids supplemented with surfactant protein B, and Venticute®) were more susceptible to surface-activity inhibition as the more complex naturally-derived preparations (i.e. Alveofact® and large surfactant aggregates isolated from rabbit bronchoalveolar lavage fluid).

Overall, nanoparticle characteristics and surfactant constitution both influence the extent of biophysical inhibition of pulmonary surfactants.

1. Introduction

Nanomedicine represents a valuable drug delivery platform for the treatment of lung disorders following inhalation [1]. Among the numerous potential carrier systems, polymeric nanoparticles (NP) enable controlled drug release and targeting properties and thus, optimized the pharmacokinetic [2–4] and dynamic [5,6] profile of the encapsulated drug within the respiratory tract. However, the safety assessment of nano-scale drug delivery vehicles is currently a subject of intense research [7,8], especially after pulmonary challenge [9–11]. So far, evidence has accumulated that physicochemical (i.e. size and surface charge) and material (i.e. degradability) properties of polymeric NP as well as their concentration at the target site determine inflammatory responses within the lung [12,13]. Another toxicological aspect of lung-delivered NP arises from their direct interaction with the pulmonary surfactant system, a research field where only scant information is available [14–18].

Pulmonary surfactant, a mixture of ~90% of lipids (mainly phospholipids (PL)) and ~10% of proteins (mainly surfactant associated proteins (SP)), that covers the alveolar region of the lung prevents collapse of the alveoli by a drastic reduction in surface tension and concurrent promotion of the gaseous exchange [19,20]. A complex interaction between PL and SP enables the formation of films highly enriched with PL at the air–water interface [21]. During surface film compression (expiration), SP aid to purify the monolayer by removing the less surface active components into the bulk phase [22]. Upon inspiration (expansion of the alveolar surface area), SP facilitate a rapid re-entry and re-spreading of surfactant compounds located in the bulk phase.

Abbreviations: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; c_{NP}, concentration of polymeric nanoparticles; DLS, dynamic light scattering; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; γ_{ads}, surface tension after film adsorption; γ_{min}, minimum surface tension during film oscillation; IC_{50}, half maximal inhibitory nanoparticle concentration; LDA, laser Doppler anemometry; LSA, large surfactant aggregates; MWCO, molecular weight cut-off; NP, nanoparticles; p, probability value; PA, palmitic acid; PBS, pulsating bubble surfactometer; PDI, polydispersity index; PL, phospholipids; PLM, phospholipid mixture; PLM-B, phospholipid mixture supplemented with surfactant protein B; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol); PS, poly(styrene); PS100-, negatively-charged poly(styrene) nanoparticles (nominal diameter: 100 nm); PS100+, positively-charged poly(styrene) nanoparticles (nominal diameter: 100 nm); S.D., standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SP, surfactant associated protein; rSP, recombinant surfactant associated protein; TEM, transmission electron microscopy

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Hence, factors affecting surfactant biophysical characteristics might have severe outcome [15]. Pulmonary surfactant function can be dramatically influenced by particulate matter as demonstrated for inorganic (i.e. gold [23,24], titanium dioxide [25], and hydroxyapatite [26]), composite (i.e. AmorSil [27,28]) and polymeric (i.e. gelatin [29–31], poly(n-butyl cyanoacrylate) [32], poly(styrene) [25,33], poly(lactide-*co*-glycolide) [33] and EUDRAGIT® E100 [33]) NP. However, it remains challenging to provide definite conclusions on the extent of surfactant inhibition by NP from the existing in vitro studies, due to the employment and combination of diverse NP and pulmonary surfactant (i.e. synthetic [23,24,27–32] and naturally-derived [24–26,33]) preparations and experimental setups (i.e. film balance [24,26–32], captive- [23] and pulsating bubble surfactometer (PBS) [25,33]).

Therefore, this study aimed at characterizing biophysical interactions between lung surfactants and two standardized polymeric NP formulations to provide systematic insights into the NP-surfactant interplay. NP were characterized for size, size distribution, morphology, and ζ-potential by dynamic light scattering, transmission electron microscopy, and laser Doppler anemometry. Next, the surface properties of the synthetic and naturally-derived pulmonary surfactants with or without polymeric NP were examined by monitoring the equilibrium (γ_{ads}) and dynamic (γ_{min}) surface tension behavior in a PBS. Finally, the extent of biophysical inhibition was analyzed from the obtained dose-effect curves (e.g. half maximal inhibitory NP concentration value (IC_{50})). We hypothesized that the sensitivity of the respective pulmonary surfactant preparation to biophysical inhibition by polymeric NP is significantly affected by both the NP characteristics and surfactant composition.

2. Materials and methods

2.1. Materials

Negatively- and positively-charged poly(styrene) NP with nominal diameters of 100 nm (PS100− and PS100+) were purchased from Polysciences (Eppelheim, Germany) and Invitrogen (Darmstadt, Germany), respectively. Alveofact® was obtained from Lyomark (Oberhaching, Germany). Venticute® was from Nycomed (Konstanz, Germany). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt) (POPG) and palmitic acid (PA) were acquired from Sigma-Aldrich (Steinheim, Germany). All other chemicals and solvents used in this study were of the highest analytical grade commercially available.

2.2. Methods

2.2.1. Preparation and characterization of NP

PS100− and PS100+ were purified by dialysis against distilled water (MWCO: 50,000 Da, Spectra/Por® 6, Breda, Netherlands) to remove additives (i.e. surface active stabilizers and preservatives). The actual NP concentration in suspension was assessed gravimetrically after lyophilization (ALPHA 1–4 LSC, Christ, Osterode, Germany). The particle size (i.e. hydrodynamic diameter) and size distribution (i.e. polydispersity index (PDI)) of NP were measured by dynamic light scattering (DLS), and their ζ-potential was determined by laser Doppler anemometry (LDA) (Zetasizer NanoZS/ZEN3600, Malvern Instruments, Herrenberg, Germany). The morphology of NP was investigated by transmission electron microscopy (TEM) (JEM-3010 TEM, JEOL, Eching, Germany).

2.2.2. Isolation of SP-B

SP-B was isolated from Alveofact® by means of LH60-chromatography as described previously [34–36]. The purity amounted to 95% as assessed by SDS-PAGE [36,37]. The concentration of the purified SP-B was determined using a protein assay [38].

2.2.3. Bronchoalveolar lavage (BAL) and isolation of large surfactant aggregates (LSA) from BAL fluid (BALF)

Male rabbits (body weight: 2.5–3.5 kg) were sacrificed by intravenous application of a lethal dose of pentobarbital/ketamine. A catheter was immediately placed into the trachea and lungs were lavaged three times with 50 ml of ice-cold isotonic saline. After filtration through sterile gauze and sedimentation of cells (300 *g*, 15 min, 4 °C), supernatants were pooled and stored at −80 °C until further processing. LSA were isolated from BALF by high speed centrifugation (48,000 *g*, 60 min, 4 °C; Sorvall centrifuge (SS34 rotor), DuPont, Bad Homburg, Germany) [39,40].

2.2.4. Determination of PL content

Lipids were extracted from the surfactant preparations according to Bligh and Dyer [41]. PL were quantified by means of a colorimetric phosphorus assay [42].

2.2.5. Preparation of surfactant materials for biophysical studies

A synthetic PL mixture (PLM) was prepared by dissolving DPPC (69.0 wt.%), POPG (22.0 wt.%) and PA (9.0 wt.%) [43] in a mixture of chloroform/methanol (2/1 (v/v)) followed by sample drying under nitrogen gas. Supplementation of the PLM with SP-B (PLM-B) was achieved by adding the hydrophobic SP dissolved in chloroform/methanol to the organic PLM stock solution before drying [36]. Venticute® and Alveofact® were supplied as lyophilized powders, while LSA was isolated by high-speed centrifugation from rabbit BALF. All surfactant preparations were adjusted to a final PL concentration of 50 mg/ml.

2.2.6. Biophysical studies

Surface activity of samples was assessed by the oscillating bubble technique using a PBS (Electronetics Corp., Amherst, USA) as previously described [33,44]. The technique provided read-outs of the surface tension after film adsorption (γ_{ads}, static measurement) and at a minimum bubble radius during film oscillation (γ_{min}, dynamic measurement). Measurements of the prepared surfactants were performed at a constant PL concentration of 2 mg/ml in isotonic NaCl solution containing 2 mM Ca^{2+} at 37 °C (samples were fabricated from pulmonary surfactant stock suspensions). The concentration of polymeric NP (c_{NP}) added (from polymeric NP stock suspensions) to the surfactant preparation was adjusted between 0.2 and 5 mg/ml prior to the surface activity experiments. Briefly, after a 30 min incubation period at 37 °C (without shaking), samples of 30 μl were transferred to the disposable sample chamber, and adsorption rate was measured. Therefore, a bubble of minimal radius (0.4 mm) was created and while maintaining the bubble at that minimal size without pulsation, pressure difference across the air/liquid interface was monitored. Next pulsation was started by sinusoidally oscillating the bubble radius between 0.4 and 0.55 mm. The cycling rate was set to 20 cycles/min. The pressure differences across the air/liquid interface were recorded continuously. Using the Young–Laplace equation, the surface tension was calculated with a microprocessor. γ_{ads} and γ_{min} values were read after 12 and 300 s, respectively. Dose-effect curve characteristics were calculated using a sigmoidal dose-response function (GraphPad Prism 5, GraphPad Software, La Jolla, USA).

2.2.7. Statistics

All measurements were carried out in triplicate and values are presented as the mean ± S.D. unless otherwise noted. To identify statistically significant differences, one-way ANOVA with Bonferroni's post *t*-test analysis was performed (SigmaStat 3.5, STATCON, Witzenhausen, Germany). Probability values of $p < 0.05$ were considered significant.

3. Results

3.1. NP characteristics

NP formulations (PS100− and PS100+) were of spherical shape, had a mean size of ~100 nm with narrow size distributions (Fig. 1A, B, Table 1). The corresponding ζ-potential of PS100− was ~−46 mV and for PS100+ a value of ~60 mV was obtained (Fig. 1C, Table 1).

The equilibrium surface tension and dynamic surface tension behavior under pulsation were investigated for the solvent (i.e. isotonic NaCl solution containing 2 mM Ca^{2+}) and both NP formulations at a c_{NP} of 2 mg/ml using the PBS technique (Table 1). The solvent alone revealed no signs of surface activity. The γ_{ads} and γ_{min} value remained at 72.1 ± 2.2 and 70.5 ± 2.7 mN/m (mean ± S.D., $n = 7$), respectively. Similar to the pure solvent, both NP formulations displayed any substantial surface activity. No decrease in surface tension was observed during the adsorption phase and under bubble pulsation for PS100− and PS100 + .

3.2. Characteristics of surfactant preparations

Based on origin and composition, available surfactants are generally divided into simplified synthetic and more complex naturally-derived surfactants from animal sources [45]. Five pulmonary surfactant preparations with diverse biochemical composition and biophysical characteristics were employed in the current study (Table 2), among them synthetic (i.e. PLM, PLM-B, and Venticute®) as well as naturally-derived (i.e. Alveofact® and LSA) surfactants.

The PLM was prepared according to Tanaka et al. [43], comprising a phospatidylcholine (DPPC), a phosphatidylglycerol (POPG), and a fatty acid (PA). PLM-B was prepared by supplementing the PLM with SP-B. The synthetic surfactant Venticute® was composed of DPPC, POPG, PA, $CaCl_2$, and recombinant SP-C (rSP-C) [48]. The naturally-derived pulmonary surfactant Alveofact® was isolated from bovine BALF by chloroform/methanol extraction. Hence, Alveofact® contains all of the PL of natural lung surfactant, including mainly phosphatidylcholines and phosphatidylglycerols, both hydrophobic SP, and a minor amount of diverse lipids (e.g. fatty acids and cholesterol) [45–47,50]. LSA, which were obtained from rabbit BALF by differential centrifugation, represent the predominant surfactant fraction of natural lung surfactant, containing hydrophilic (e.g. SP-A) and a high content of hydrophobic (e.g. PL and SP-B and SP-C) constituents [39,49].

The surface properties of the diverse pulmonary surfactant preparations displayed rapid adsorption ($\gamma_{ads} < 30$ mN/m ($n = 10$)) and near-zero minimal surface tension values ($\gamma_{min} \leq 3$ mN/m ($n = 10$)) when tested in the PBS at a PL concentration of 2 mg/ml (Table 2). Among the diverse formulations, only the PLM revealed significantly reduced surface activity during adsorption and bubble oscillation ($\gamma_{ads} = 54.0 \pm 8.9$ mN/m (mean ± S.D. ($n = 10$); $\gamma_{min} = 17.3 \pm 3.9$ mN/m (mean ± S.D. ($n = 10$)).

3.3. Biophysical inhibition of surfactant preparations by polymeric NP

The diverse surfactant preparations were investigated for biophysical activity (i.e. adsorption facilities (γ_{ads}) (Fig. 2) and minimal surface tension behavior under bubble pulsation (γ_{min}) (Figs. 3 and 4, Table 3)) in the presence of PS100− and PS100+, respectively. Therefore, surfactant preparations (2 mg PL/ml) were incubated with increasing c_{NP} ranging from 0.2 to 5.0 mg/ml.

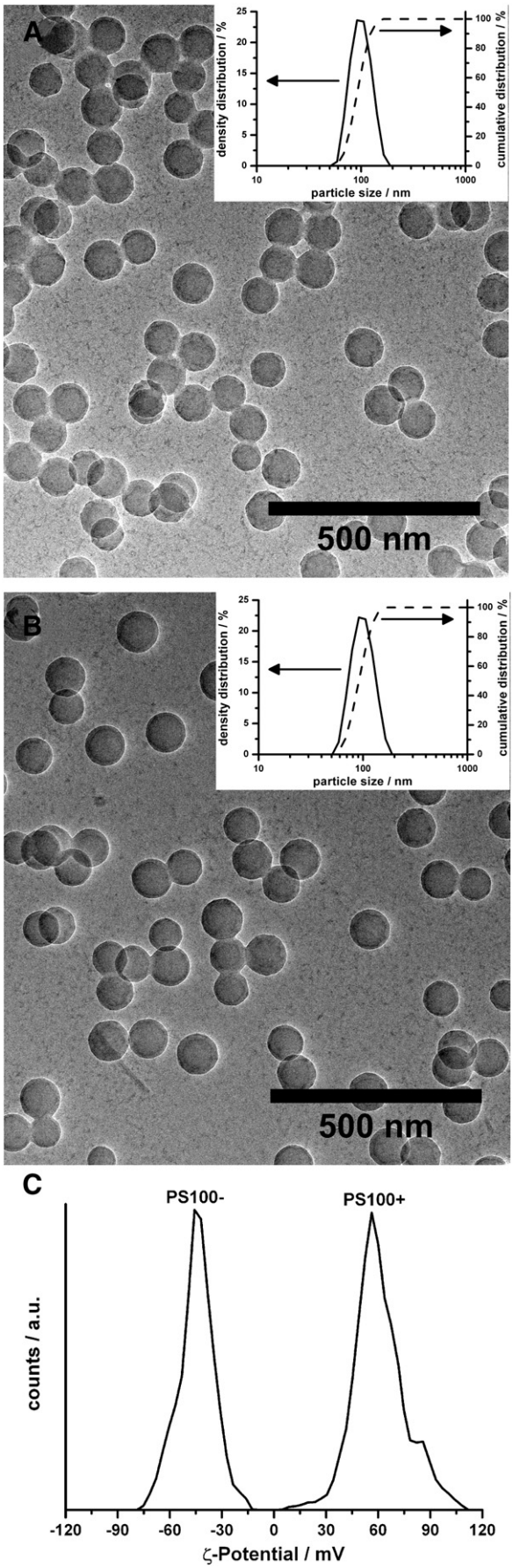

Fig. 1. Representative TEM images of PS100− (A) and PS100+ (B). The insets in (A) and (B) reveal typical particle size distribution curves from DLS analysis. The solid lines represent the particle size density distribution, and the dashed lines represent the cumulative particle size distribution. Typical ζ-potential distribution curves for the NP formulations from LDA analysis are illustrated in (C).

Table 1
Physicochemical characteristics of polymeric NP formulations.

Formulation	Size/nm	PDI	ζ-Potential/mV	γ_{ads}[a] / mN/m	γ_{min}[a] / mN/m
PS100−	96.8 ± 0.3	0.022 ± 0.010	−46.3 ± 3.3	68.8 ± 1.4	65.1 ± 0.6
PS100+	94.8 ± 1.1	0.019 ± 0.006	59.0 ± 2.5	70.9 ± 2.1	69.5 ± 1.6

Values are presented as the mean ± S.D. ($n \geq 3$).
[a] As determined by the PBS technique at a c_{NP} of 2 mg/ml.

γ_{ads} of the PLM, PLM-B, Venticute®, Alveofact®, and LSA were not adversely affected by addition of a "low" dose of PS100− and PS100+ (i.e. 0.5 mg/ml) (Fig. 2). Higher concentrations of polymeric NP (i.e. 5.0 mg/ml) led to significantly decreased adsorption properties for all surfactants tested (except LSA incubated with PS100+), however, at considerably different final γ_{ads} values. Both naturally-derived pulmonary surfactants (i.e. Alveofact® and LSA) exhibited significantly lower sensitivity to changes in γ_{ads} values upon NP addition than the synthetic surfactant mixtures. LSA revealed the significantly highest stability, when tested in the presence of PS100− or PS100+ (5 mg/ml). Moreover, adsorption studies disclosed elevated surfactant sensitivity to PS100− compared to PS100+.

The influence of PS100− and PS100+ on the diverse pulmonary surfactant preparations was also obvious during measurements of γ_{min} (Fig. 3). Under bubble oscillation NP caused a dose-dependent effect on γ_{min} of all surfactants, with similar characteristics regarding curve progression, yet at substantially different sensitivities. Following γ_{min} values, which remained indistinguishable from the control values displayed in Table 2 for "low" NP concentrations (i.e. <0.7 mg/ml), a sudden elevation of γ_{min} was observed upon contact with higher doses of polymeric NP, ending up in stable minimum surface tension plateaus. Dose-effect curve characteristics were then calculated using a sigmoidal dose-response function (Fig. 4, Table 3). Here, IC_{50} values ranging from 0.9 ± 0.2 to 1.7 ± 0.3 mg/ml (mean ± S.D., $n \geq 4$) and 1.0 ± 0.1 to 2.1 ± 0.1 mg/ml (mean ± S.D., $n \geq 4$) (Fig. 4A), γ_{min} plateau values ranging from 55.8 ± 6.7 to 12.7 ± 1.7 mN/m (mean ± S.D., $n \geq 4$) and 46.1 ± 5.0 to 7.8 ± 1.7 mN/m (mean ± S.D., $n \geq 4$) (Fig. 4B) and NP concentrations required to increase the γ_{min} to values of ≥ 5 mN/m ranging from 0.7 ± 0.1 to 1.4 ± 0.1 mg/ml (mean ±S.D., $n \geq 4$) and 0.9 ± 0.2 to 2.7 ± 0.8 mg/ml (mean ± S.D., $n \geq 4$) (Table 3) were determined for the diverse pulmonary surfactant preparations incubated with PS100− and PS100+, respectively.

The overall stability of the pulmonary surfactant preparations towards minimum surface tension changes by polymeric NP during bubble pulsation agreed well with observations from adsorption experiments (Fig. 2). Among the synthetic surfactants, PLM-B demonstrated a lower sensitivity to inhibition than Venticute® (i.e. PLM supplemented with rSP-C). Other than for the synthetic surfactants, significantly increased NP doses were necessary to inhibit the naturally-derived pulmonary surfactants (i.e. Alveofact® and LSA). LSA revealed the considerably highest stability of all tested surfactant preparations. Apart from the type of surfactant, the surface charge of NP was identified as another factor influencing the γ_{min}, where PS100− showed increased inactivation potency compared to PS100+.

4. Discussion

4.1. NP characteristics

Inhalative drug delivery has gained considerable attraction for the treatment of numerous lung diseases [51]. In general, the advantages of inhalation therapy depend on the fate of the delivered medication (mechanism and rate of elimination) in the respiratory tract. To overcome the short-comings of "conventional" inhalation therapy (i.e. rapid decay of pulmonary drug concentration), more sophisticated pulmonary drug delivery systems, which provide controlled and targeted drug release within the lung, need to be introduced [1]. Among them, polymeric NP reveal several advantages for the treatment of respiratory diseases, like drug release in a predetermined manner and targeting of specific sites or cell populations [2–6]. Consequently, the number of necessary daily inhalation maneuvers and undesirable side effects will be reduced, which in turn will clearly improve the therapeutic benefit as well as the convenience and compliance of patients.

Model formulations (i.e. PS100− and PS100+) exhibited physicochemical characteristics similar to frequently lung-delivered polymeric nanocarriers [2–6] with respect to size (i.e. ~100 nm) and surface chemistry (i.e. negative and positive surface charge) (Fig. 1, Table 1). As expected, no surface activity was obtained for the solvent and two polymeric NP formulations when tested in the PBS (Table 1) [33,52].

4.2. Characteristics of surfactant preparations

Pulmonary surfactant is an essential element of the terminal airspaces [15,20]. The surface-active lining material stabilizes the respiratory region by reducing the surface tension, thereby facilitating the gaseous exchange. Numerous studies have addressed the impact of composition and complex interaction of surfactant constituents that enable rapid adsorption and a decrease in surface tension to values near 0 mN/m during compression/expansion cycles [19,53]. It is evident that both PL and hydrophobic SP (i.e. SP-B and SP-C) are mandatory for adequate surfactant function in the lung [19–22]. Single PLM, which depicts only an approximation of pulmonary surfactant PL [43,45], displayed limited efficacy in lowering the surface tension to appropriate values (Table 2). By contrast, supplementation of synthetic PLM with hydrophobic SP (i.e. PLM-B and Venticute®) led to rapid adsorption (γ_{ads} < 30 mN/m) and near-zero minimal surface tension (γ_{min} < 3 mN/m) under pulsation. LSA represent the predominant fraction of natural lung surfactant, which are assumed to be the precursor of the alveolar surfactant film [39,40]. Depletion of hydrophilic surfactant constituents by organic BALF extraction, as employed for the preparation of Alveofact®, resulted in a pulmonary surfactant preparation highly enriched in PL and hydrophobic SP [45–47,50]. As a

Table 2
Biochemical composition and biophysical characteristics of the employed pulmonary surfactants.

Preparation	PL (wt.%)	SP (wt.%)	Miscellaneous (wt.%)	γ_{ads}[b] / mN/m	γ_{min}[b] / mN/m
PLM	DPPC (69.0) POPG (22.0)	–	PA (9.0)	54.0 ± 8.9	17.3 ± 3.9
PLM-B	DPPC (69.0) POPG (22.0)	SP-B (1.8)	PA (7.2)	27.2 ± 2.6*	2.8 ± 1.0*
Venticute®	DPPC (63.4)[a] POPG (27.8)[a]	rSP-C (1.8)[a]	PA (4.5)[a] $CaCl_2$ (2.5)[a]	26.1 ± 1.8*	2.7 ± 1.3*
Alveofact®	diverse PL (~90.0)[a]	SP-B, SP-C (~2.0)[a]	Fatty acids[a] Cholesterol[a]	25.7 ± 1.0*	3.0 ± 1.5*
LSA	diverse PL (~90.0)[a]	SP-A, SP-B, SP-C (~5.0)[a]	Fatty acids[a] Cholesterol[a]	25.3 ± 1.4*	2.5 ± 1.2*

[a] Values obtained from the literature [39,45–50].
[b] Values are presented as the mean ± S.D. ($n = 10$). The (*) denotes statistically significant differences compared to the surface properties of the PLM.

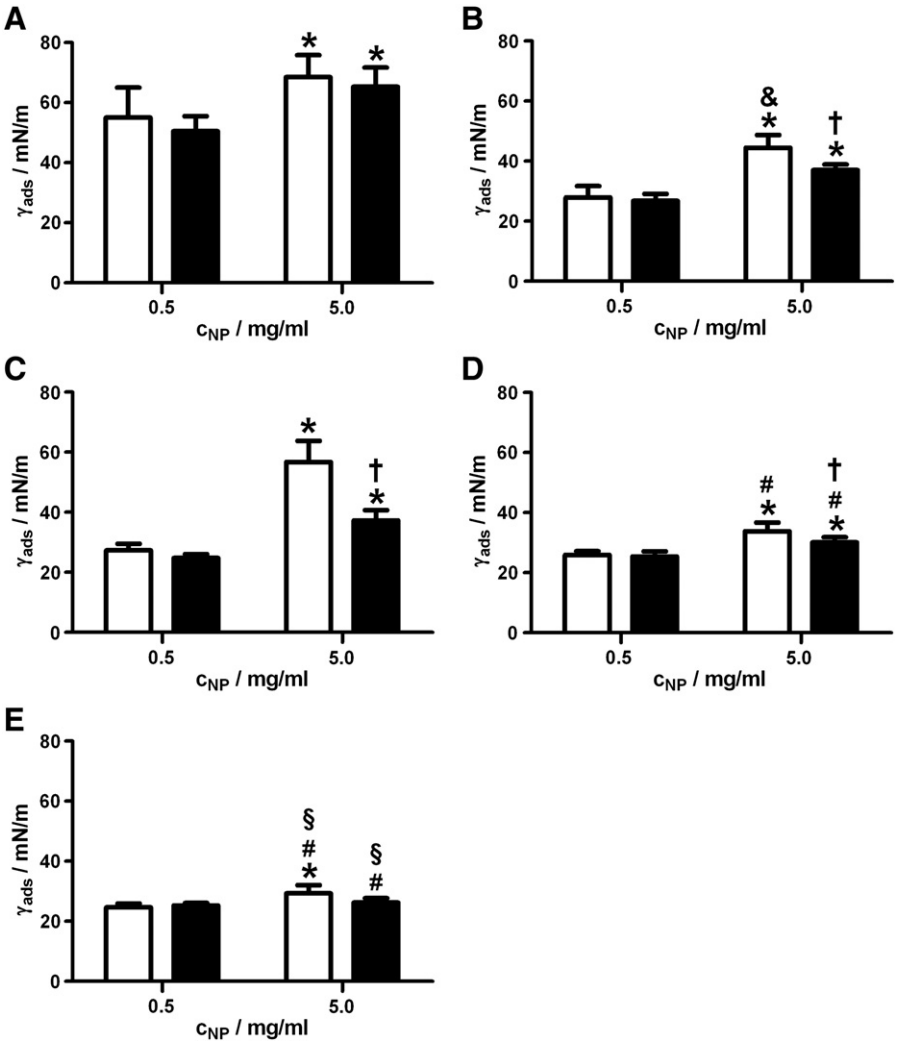

Fig. 2. Dose-related effect of PS100− (open bars) and PS100+ (closed bars) on γ_{ads} of the PLM (A), PLM-B (B), Venticute® (C), Alveofact® (D), and LSA isolated from rabbit BALF (E). Values are presented as the mean ± S.D. ($n \geq 4$). Statistically significant differences ($p < 0.05$): (*) surfactant preparations incubated with NP vs. blank surfactant preparations (Table 2); (&) PLM-B vs. Venticute®; (#) Alveofact and LSA vs. all synthetic surfactant preparations; (§) LSA vs. all other surfactants; (†) PS100+ vs. PS100−.

consequence, the complex pulmonary surfactants from natural source revealed biophysical activities comparable to those obtained for SP-enriched PLM, which are all consistent with results measured in vivo [15].

4.3. Biophysical inhibition of surfactant preparations by polymeric NP

A basic concern in the field of nanomedicine is the development of safe drug delivery vehicles causing negligible adverse effects on the biological environment present at the target site [7,54]. However, the same properties that make polymeric NP attractive as drug delivery vehicles evoked toxicity concerns after pulmonary application [9–13]. Following deposition in the respiratory region of the lung, NP get in direct contact with the pulmonary surfactant interface, leading to potential biophysical interactions that could have biocompatible or bioadverse outcomes [14,17]. An insistent problem in the development of surfactant compatible NP formulations is the lack of studies, which address the extent and mechanisms of NP–surfactant interactions. However, probing these interactions would facilitate the development of predictive structure–activity relationships between the applied formulation and the pulmonary environment [55].

In the current study, polymeric NP (i.e. PS100− and PS100+) caused a dose-dependent effect on the biophysical properties of all investigated pulmonary surfactants. Although the adsorption (γ_{ads}) and dynamic surface tension lowering (γ_{min}) facilities were not adversely affected by "low" doses of polymeric NP (i.e. <0.7 mg/ml), dose escalations led to distinct surfactant inhibition (Figs. 2 and 3). Thus, the total NP concentration appears to be an important parameter for actual surfactant inhibition. NP concentrations that were shown to be effective in vitro are on the upper end of what will be reached in vivo after a single inhalative administration. However, accumulation of polymeric NP might be observed when frequent dosing is necessary (e.g. chronic diseases), which would worsen the NP/surfactant balance. Furthermore, the sensitivity of the diverse preparations was significantly affected by the surface charge (ζ-potential) of the polymeric NP. Accordingly, the ability to inhibit the surface activity was more pronounced for negatively-charged NP (i.e. PS100−) than for their positively-charged counterparts (i.e. PS100+). These findings are in general agreement with a previous study, where NP properties (e.g. surface charge) as well as their concentration impacted the biophysical performance of the pulmonary surfactant system [33]. Surfactant dysfunction provoked by nano-scale particulate matter occurs through a direct interaction with individual surfactant constituents (e.g. SP) [23,25,26,33]. In these studies, the ability of NP to adsorb the hydrophobic, positively-charged SP (i.e. SP-B and SP-C) was offered as a putative mechanism for the altered surfactant function. Moreover, adsorption of hydrophilic SP (e.g. SP-A) to metal and polymeric NP was shown to trigger their uptake by alveolar macrophages [17,56–58]. Protein adsorption processes

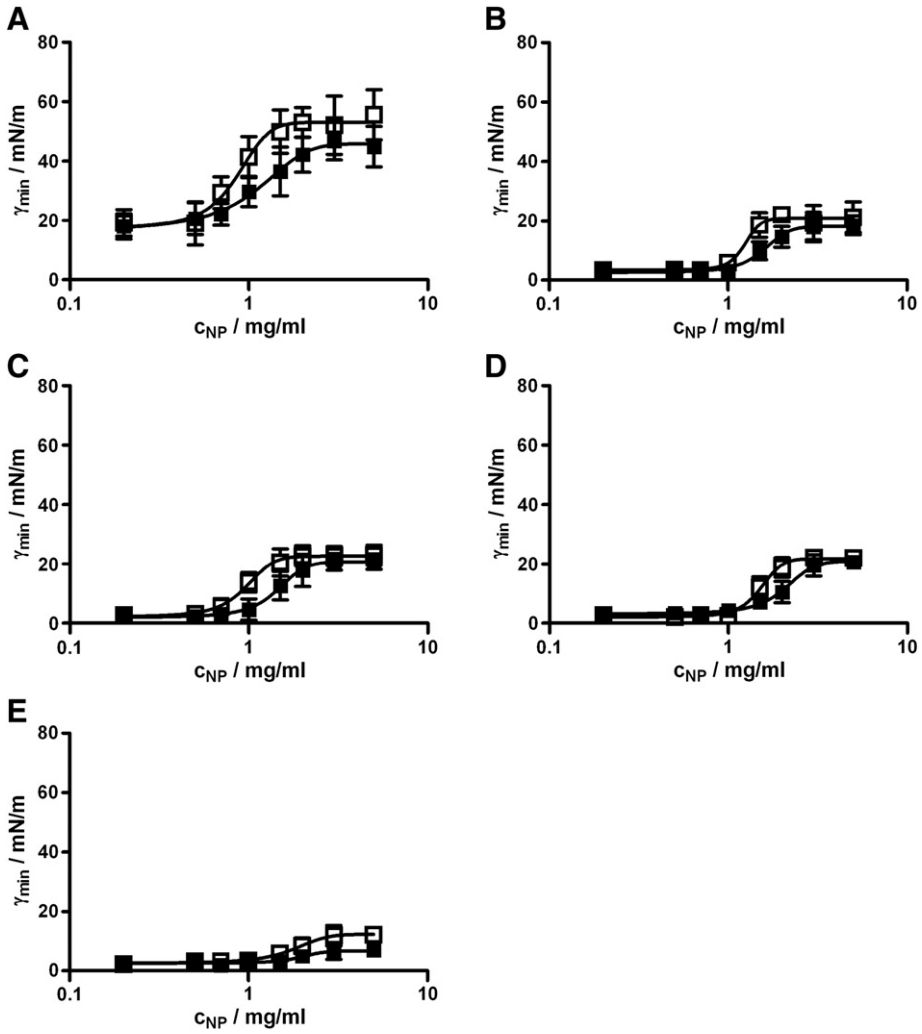

Fig. 3. Dose-related effect of PS100− (open symbols) and PS100+ (closed symbols) on γ_{min} of the PLM (A), PLM-B (B), Venticute® (C), Alveofact® (D), and LSA isolated from rabbit BALF (E). Values are presented as the mean ± S.D. ($n \geq 4$). The continuous lines in (A)–(E) represent sigmoidal dose-response fits of the experimental data ($R^2 \geq 0.93$).

to polymeric surfaces are normally governed by a fine balance between hydrophobic and Coulomb forces [33,59]. Therefore, SP-B and SP-C should reveal different affinity to the surfaces of both NP types, presuming a favored electrostatic interaction with PS100− compared to PS100+, as indirectly documented by the impact of these formulations on surfactant function (Figs. 2, 3 and 4, Table 3).

The type of pulmonary surfactant preparation was identified as another determinant for the extent of NP-conditioned surfactant inhibition. It is well known that the composition of pulmonary surfactants strongly influences their structural properties (i.e. ultrastructure) [47,60,61], interfacial behavior (i.e. surface tension and viscosity) [47,50,60] and sensitivity to biophysical inhibition [15]. As an example, the sensitivity of pulmonary surfactants to biophysical inhibition by plasma proteins (e.g. fibrinogen) was shown to correlate with surfactant constitution, where synthetic PLM and PLM with added rSP-C were the most sensitive preparations, PLM-B and naturally-derived preparations being the least [15,46,62,63]. Similar results were made in the current study (Figs. 2, 3 and 4, Table 3). Here, the employed PLM was very sensitive to inhibition by polymeric NP. Supplementation with SP-B (i.e. PLM-B) or rSP-C (i.e. Venticute®) partly shifted the sensitivity of the PLM. However, Venticute® displayed markedly higher susceptibility to inactivation than PLM-B. In contrast, when naturally-derived pulmonary surfactants were applied a significantly decreased sensitivity to inhibition by polymeric NP was noticed. Several aspects may predetermine the rank order of surfactant sensitivity to NP-conditioned biophysical inhibition, most likely variations in the PL profile and SP nature and content [15,16]. Moreover, the more complex surfactant preparations (including for instance SP-A) reveal a higher level of aggregation and membrane–membrane contacts, which could prevent accessibility of polymeric NP to interact with the "delicate" constituents of the surface activity "machinery", in a similar manner to how those preparations are also the more resistant to other inhibitory substances. Overall, our findings and speculations prompt further investigation on the interactions between polymeric NP and individual surfactant components (i.e. PL and SP) and the effect of polymeric NP on the (ultra)structure of pulmonary surfactant preparations to fully explain the physiological relevance of adsorption and dynamic surface tension alterations.

5. Conclusion

Controlled drug release and targeting properties are prerequisites for the fabrication of suitable polymeric nanocarriers for lung administration. However, nano-scale particulate matter was shown to interact with the essential pulmonary surfactant system. Consequently, relevant carrier characteristics need to be identified, which enable the fabrication of surfactant-compatible polymeric NP formulations. In this regard, the current study underlined the significance of NP characteristics (i.e. surface charge) and surfactant composition on the extent of biophysical inhibition. The ability of polymeric NP to adsorb SP was regarded as a

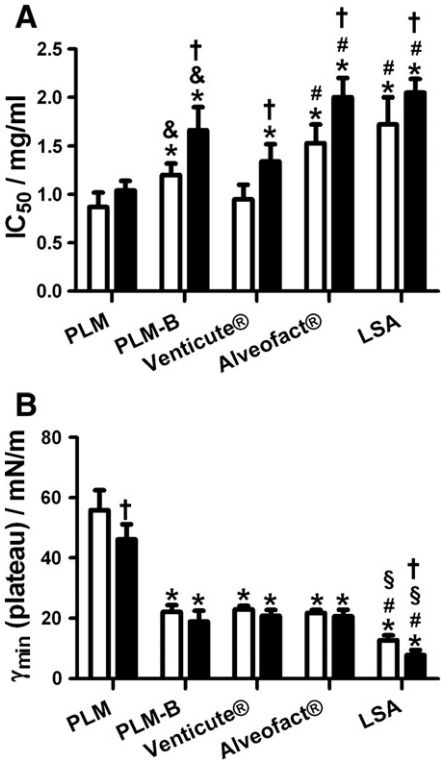

Fig. 4. Calculated dose-effect curve characteristics for biophysical inhibition experiments (PS100− (open bars) and PS100+ (closed bars)). Values were obtained from sigmoidal dose-response fits displayed in Fig. 3 and are presented as the mean ± S.D. ($n \geq 4$). Statistically significant differences ($p < 0.05$): (*) all surfactant preparations vs. PLM; (&) PLM-B vs. Venticute®; (#) Alveofact and LSA vs. all synthetic surfactant preparations; (§) LSA vs. all other surfactants; (†) PS100+ vs. PS100−.

key factor for their interference with pulmonary surfactant function. Surface characteristics of the NP were shown to contribute actively to interactions with the biological environment. The overall stability of the employed pulmonary surfactants towards biophysical inhibition by polymeric NP was also dependent on preparation origin and composition and could be ranked in the following order: PLM < Venticute® < PLM-B < Alveofact® < LSA. Overall, the findings from the current study represent a basis for the rational design of nanomedicines suitable for pulmonary drug delivery applications.

Conflict of interest

The authors disclose that no conflicting interests associated with the manuscript exist.

Table 3
Concentrations of PS100− and PS100+ required to increase the γ_{min} of the employed pulmonary surfactants to values ≥ 5 mN/m.

Preparation	c_{PS100-} / mg/ml	c_{PS100+} / mg/ml
PLM	n.a.	n.a.
PLM-B	$0.9 \pm 0.1^{\&}$	$1.1 \pm 0.1^{\dagger}$
Venticute®	0.7 ± 0.1	$0.9 \pm 0.2^{\dagger}$
Alveofact®	$1.2 \pm 0.1^{\#}$	$1.3 \pm 0.2^{\#}$
LSA	$1.4 \pm 0.1^{\#\S}$	$2.7 \pm 0.8^{\#\S\dagger}$

n.a. = not applicable.
Values were calculated from Fig. 3 and are presented as the mean ± S.D. ($n \geq 4$). Statistically significant differences ($p < 0.05$): (*) all surfactant preparations vs. PLM; (&) PLM-B vs. Venticute®; (#) Alveofact and LSA vs. all synthetic surfactant preparations; (§) LSA vs. all other surfactants; (†) PS100+ vs. PS100−.

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