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ORIGINAL PAPER

Influence of surfactant protein C on the interfacial behavior of phosphatidylethanolamine monolayers

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Abstract In the current work we study with monolayer tensiometry and Brewster angle microscopy (BAM) the surface properties of Dipalmitoleoylphosphatidylethanolamine (DPoPE) films at the air/water interface in presence and absence of specific surfactant protein C (SP-C). DPoPE is used, as it readily forms both lamellar (L_α) and non-lamellar inverted hexagonal (H_{II}) phases and appears as a suitable model phospholipid for probing the interfacial properties of distinct lipid phases. At pure air/water interface L_α shows faster adsorption and better surface disintegration than H_{II} phase. The interaction of DPoPE molecules with SP-C (predeposited at the interface) results in equalizing of the interfacial disintegration of the both phases (reaching approximately the same equilibrium surface tension) although the adsorption kinetics of the lamellar phase remains much faster. Monolayer compression/decompression cycling revealed that the effect of SP-C on dynamic surface tensions (γ_{max} and γ_{min}) of mixed films is remarkably different for the two phases. If γ_{max} for L_α decreased from the first to the third cycle, the opposite effect is registered for H_{II} where γ_{max} increases during cycling. Also the significant decrease of γ_{min} for L_α in SP-C presence is not observed for H_{II} phase. BAM studies reveal the formation of more uniform and homogeneously packed DPoPE monolayers in the presence of SP-C.

Keywords DPoPE · Surfactant protein C · Lipid phase state · Lamellar and non-lamellar phases · Phospholipid monolayers

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Introduction

Phosphatidylethanolamines (PEs) are important constituents of natural biomembrane phospholipids (Cullis et al. 1985; Seddon and Templer 1995). Despite their lower content compared to phosphatidylcholines and phosphatidylglyceroles, PEs are supposed to be of key functional importance because of their phase polymorphic behavior. Numerical observations show that under physiological conditions PEs form wide variety of structures ranging from lamellar bilayer (L_α) phase to non-lamellar inverted hexagonal (H_{II}) phase which assembles into large cylinder aggregates. These non-lamellar structures take part in various cell processes such as cell signal transduction (Jensen and Schutzbach 1984), membrane fusion (Cullis et al. 1985; Seddon 1990; Siegel 1993), transmembrane movement of ions and large molecules (Litzinger and Huang 1992), etc.

Lung surfactant (LS) is a complex lipid–protein mixture that reduces surface tension at the air/water interface of the alveolus thus stabilizing them during respiration (Hills 1988; Lalchev et al. 2008). In the water hypophase of the alveolus LS exists as a dispersion of nano-sized aggregates:

(1) lamellar bilayer or multilayer structures (liposomes, multilamellar vesicles, planar bilayers) formed by the main phospholipid components of the surfactant (DPPC, DPPG, etc.) and (2) non-lamellar inverted phase aggregates (Lalchev et al. 2008; Perkins et al. 1996). According to some authors bulk aggregates of tubular myelin which have major role in LS surface activity also display structural organization similar to phospholipid inverted phases (Palaniyar et al. 1999). It is supposed that during expiration significant amount of lipid and protein molecules that compose LS surface film are pulled into the alveolar subphase and remain adherent with the monolayer. The readsorption of the "squeezed-out" surfactant compounds to the interface during subsequent inspiration is of crucial importance for the normal lung function. Previous studies indicate that non-bilayer inverted phase lipids are required to connect these compounds (forming vesicles and multilayers in the subphase) with the monolayer at the air/water interface in order to facilitate their readsorption and reintegration (Biswas et al. 2007; Rüdiger et al. 1998). A model is proposed assuming that unsaturated inverted-phase phospholipids (like some PEs) stabilize negatively curved structures which connect the vesicles to the interface, analogously to the stalk intermediate considered as a crucial step in the fusion of two bilayers (Chernomordik et al. 1999; Kinnunen 1992; Siegel et al. 1989). The model correlates with the old finding of Yu et al. (1984) that artificial pulmonary surfactants based on H_{II}-forming PEs perform as well as PG-based surfactants. Thus lipid polymorphic phase behavior may have an important role in the effective functioning of LS.

The successful (re)adsorption and (re)spreading of the bulk LS aggregates and the phase coexistence in the resulting 2D film are regulated by the interaction of phospholipids with the hydrophobic specific surfactant proteins SP-B and SP-C (Ding et al. 2003; Wang et al. 1996). In the mammalian species SP-C consists of 33–35 amino acids including the hydrophobic valine, leucine and isoleucine and two cysteine-linked palmitoyl chains at the N-terminal segment (Weaver and Conkright 2001). This specific composition makes SP-C the most hydrophobic protein known to exist in nature. Model LS films composed of lipids and SP-C display a reversible transition from a monolayer to surface-associated multilayers upon compression and expansion at the air/water interface. It is shown that multilayer formation is not detected in the absence of SP-C (Wang et al. 2005). The result could be attributed to the capability of the surfactant protein to interact with and stabilize non-lamellar structures and to regulate their surface adsorption and disintegration (Biswas et al. 2007). However, studies comparing the interfacial behavior of non-lamellar and lamellar phospholipid phases in presence of SP-C are still rare.

Dipalmitoleoylphosphatidylethanolamine (DPoPE) is suitable model phospholipid for studying the role of lipid phase state in lipid-surfactant protein interactions at air/water interface, as it readily forms both lamellar (L_α) and non-lamellar inverted hexagonal (H_{II}) phases (Fig. 1) at/and near physiological temperatures (Jordanova et al. 2003).

As LS in vivo lowers surface tension in a 2D dynamically compressed monomolecular film at the air/alveolar interface, Langmuir monolayers provide a relevant model membrane system for studying the effects of specific surfactant proteins on the interfacial properties of phospholipids (Nag et al. 1996a, b, 1997; Ross et al. 2002).

The aim of the present work is to study by monolayer tensiometry the influence of SP-C on the interfacial behavior

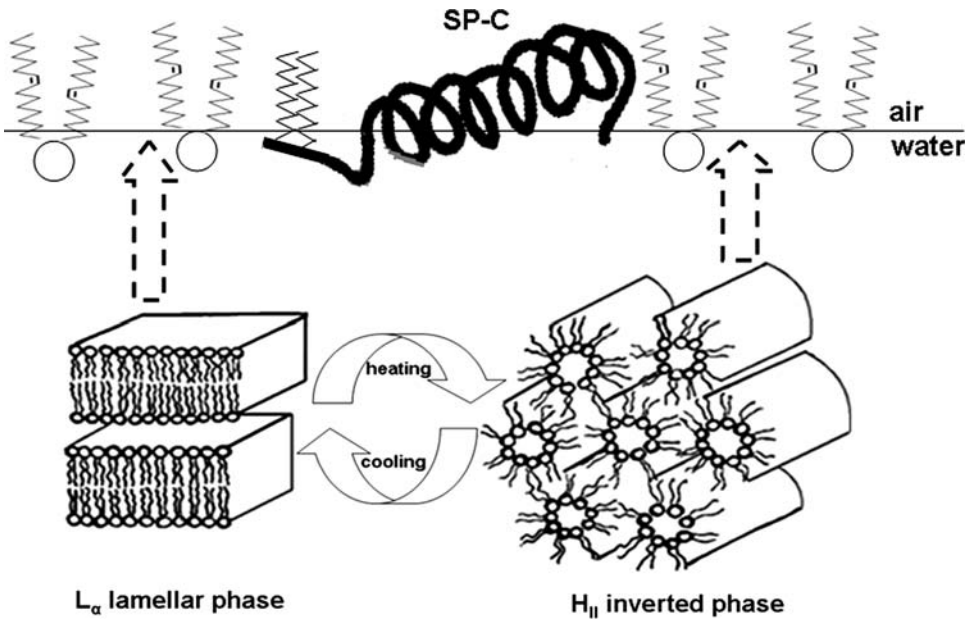

Fig. 1 A simplified scheme of the performed experiment. The lamellar (L_α) or non-lamellar inverted hexagonal (H_{II}) lipid phases (assembled in large cylinder aggregates) are injected in the monolayer subphase. The type of the phases depends on DPoPE dispersions thermal history as previously described by Jordanova et al. 2003 (see "Materials and methods"). The phase aggregates adsorb (*dashed arrows*) and release monomers at the air/water interface which is pure or with predeposited SP-C. For details see text

of lamellar bilayer (L_α) and non-lamellar inverted hexagonal (H_{II}) phases of DPoPE. Two types of tensiometric experiments are performed with DPoPE adsorption films (pure or in presence of SP-C): (1) registration of the kinetics of surface tension decrease until reaching equilibrium value (γ_{eq}) and (2) measurement of the dynamic (at interfacial compression/decompression) surface tensions: γ_{max} and γ_{min}. The time necessary for reaching γ_{eq} and its value are common criteria for evaluation of adsorption kinetics and degree of interfacial disintegration of lipid bulk structures (Hills 1988). Minimal surface tension (γ_{min}) realized at the final stage of interfacial compression depends on the maximal surface packing density achieved in the adsorption film. Maximal surface tension (γ_{max}) observed after interfacial expansion reflects the degree of surface readsorption and rearrangement of the film (Birdi 1989). The influence of SP-C/DPoPE interaction on the phase heterogeneity of insoluble phospholipid monolayers is characterized by Brewster angle microscopy (BAM).

Materials and methods

Preparation of L_α and H_{II} phases

1,2-Dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPoPE) was purchased from Avanti Polar Lipids (Alabaster, USA) and used as received. Lipid dispersions of L_α and H_{II} phases were prepared by mechanical agitation in 0.15 M NaCl (Merck) solution at different temperatures. Fully hydrated DPoPE forms a lamellar liquid crystalline phase L_α over a broad temperature range, from a melting transition taking place at about −35°C up to 39°C, where the dispersion transforms into the H_{II} phase. As proven in our previous study by DSC (Jordanova et al. 2003) $L_\alpha \rightarrow H_{II}$ transition is reversible with a hysteresis of ca. 15°C during cooling and due to that hysteresis after the first heating–cooling cycle the dispersions of DPoPE at 37°C reside in stable (for days) supercooled H_{II} phase. In order to avoid possible oxidation of the lipid molecules or destabilization of H_{II} phase the dispersions (of L_α or H_{II} phases) were kept on dark in closed glass tubes and were freshly used within 2–3 h after their preparation. DSC shows no changes in dispersion thermal behavior after such storage period.

Isolation of LS hydrophobic proteins

Lung surface active material was obtained by washing porcine lungs with 0.15 M NaCl. The lavage fluid was subjected to a series of centrifugations (Ng et al. 1983). The purification was performed by sucrose gradient centrifugation (0.68 and 0.33 M sucrose, 78,000*g*, 60 min, 4°C) and the obtained fraction was used for extraction of LS hydrophobic proteins by diisopropyl ether/*n*-butanol under conditions in which SP-B and SP-C remained in the aqueous phase and the lipids resided in the organic phase (Cham and Knowles 1976; Christova et al. 1998). The aqueous phase was evaporated under a stream of nitrogen and the dry residue was extracted by diethyl ether/ethanol. After centrifugations at 18,000*g* for 30 min at 4°C a pellet was obtained, which was extracted by mixture chloroform/methanol/0.1 M HCl (1/1/0.04 vol/vol). The lower chloroform layer contains SP-C. Phospholipid content of purified SP-C using the method of Shin (Shin 1962) was determined to be <4 mol lipid/mole SP-C.

Separation of extracted SP-C by SDS-PAGE

The chloroform layer, obtained in the last step of separation, was collected and evaporated under a stream of nitrogen. Then the content of SP-C in the dry residuum was analyzed by SDS-PAGE. The protein was visualized by silver staining and Coomassie brilliant blue R-250. The molecular weight markers (SeaBlue) were within 4–250 kDa.

Formation of adsorption lipid monolayers at interface with predeposited SP-C and at pure air-water interface

Monolayers of SP-C were formed by spreading of certain volume of the pure protein solution (corresponding to 1 μg SP-C) over the subphase of 0.15 M NaCl in a Langmuir trough (area 1,710 mm^2; volume 25 ml) provided with movable Teflon barrier for surface compression–decompression. The surface tension γ (mN/m) was followed with time by the Wilhelmy plate method, using an Automatic Wilhelmy tensiometer (Biegler Electronic, Austria). The trough was provided with a thermostat maintaining the temperature at 37 ± 0.5°C. After about 20 min the equilibrium value of surface tension (γ_{eq}) for pure SP-C monolayer was reached.

Then certain amounts of DPoPE dispersions in different phases (L_α and H_{II}) were injected through the bottom of the trough into the subphase to final volume concentration of 0.5, 1 and 5 μg DPoPE/ml. The decrease of surface tension with time due to DPoPE adsorption and interaction with SP-C in the mixed films at the air/water interface was detected until equilibrium surface tension values were reached.

Further the resulting mixed films composed by the predeposited SP-C and the adsorbed DPoPE molecules were subjected to area compression/decompression between 100 and 20% of the initial trough area. The time of compression/decompression cycle was 3 min. Dynamic surface tensions were measured: $\gamma_{min,}$ the surface tension after compression to 20% of the initial film area; and γ_{max}, the

surface tension after decompression of the film to its initial (100%) area.

Control experiments at pure air/water interface (i.e., without deposition of SP-C) were also performed for evaluation of the adsorption kinetics and γ_{eq} of DPoPE (in both phase states) and for registering γ_{max} and γ_{min} of the resulting adsorption films during compression/decompression.

Brewster angle microscopy of insoluble monolayers

Brewster angle microscopy provided a well-suited approach for visualization of lateral domains formation in monolayers without use of fluorescent probes (Henon and Meunier 1991; Honing and Mobius 1991). BAM-images were recorded using Micro BAM2 (Nima Technology Ltd, Coventry, UK) equipped with laser diode (659 nm, 30 mW at laser aperture). In case of adsorption films it was not possible to obtain sufficient contrast (sufficient difference in the reflectivity coefficients) between the interface and the subphase containing bulk lipid aggregates (L_α and H_{II}). For the purpose experiments with insoluble monolayers were performed. Insoluble DPoPE monolayers on the air/water interface of Langmuir trough were formed by spreading of phospholipid molecules dissolved in chloroform over 0.15 NaCl subphase. At least 20 min was provided for chloroform evaporation. Then monolayers were compressed to surface tension of 33 mN/m (similar to γ_{eq} obtained by adsorption of DPoPE at pure air/water interface). At that point SP-C (dissolved in chloroform) was deposited on the film surface. Experiments with monolayers obtained by spreading of mixed solutions of DPoPE and SP-C in chloroform were also performed. The temperature of the subphase was controlled at 37 ± 0.5°C. The polarizer and analyzer were set to p-polarization and incoming laser light was limited to an angle of incidence of $53 \pm 2°$ (Brewster angle for aqueous solutions). The obtained images were analyzed and processed using the integrated software. The surface tension of the insoluble monolayers was measured by the Wilhelmy plate method as described above.

Results

The results from SDS-PAGE of the extracts of SP-B and SP-C are shown on Fig. 2. Figure 2a, start 1 both hydrophobic proteins SP-B and SP-C are identified before their separation. In the last fraction there is only SP-C, which can be seen in Fig. 2b, starts 1 and 2. The fraction of purified SP-C (3.9 kDa molecular weight) is used in our further experiments with monolayers at air/water interface.

At pure air/water interface lamellar bilayer (L_α) phase showed faster adsorption and more complete interfacial disintegration compared to the non-lamellar inverted hexagonal (H_{II}) phase for all bulk concentrations of DPoPE. This is manifested by the great difference for the three used lipid concentrations (0.5, 1 and 5 μg DPoPE/ml) in the kinetics of surface tension decrease and in the measured γ_{eq} values (Fig. 3a; Table 1). Like example at 5 μg DPoPE/ml the measured values of equilibrium surface tension are 34.3 mN/m for L_α and 44.0 mN/m for H_{II} phases.

Figure 3b displays the dependence of surface tension (γ) on time for SP-C film pure and in presence of DPoPE molecules adsorbed from the subphase. The initial part of the curve shows γ decrease after spreading of SP-C over the interface by deposition of few droplets concentrated protein solution in chloroform. The use of volatile hydrophobic solvent is necessary due to the high hydrophobicity of the purified SP-C making it insoluble in water electrolyte solutions (Serrano and Perez-Gil 2006; Simatos et al. 1990). After γ drops to a plateau value of 53.4 mN/m aliquots of

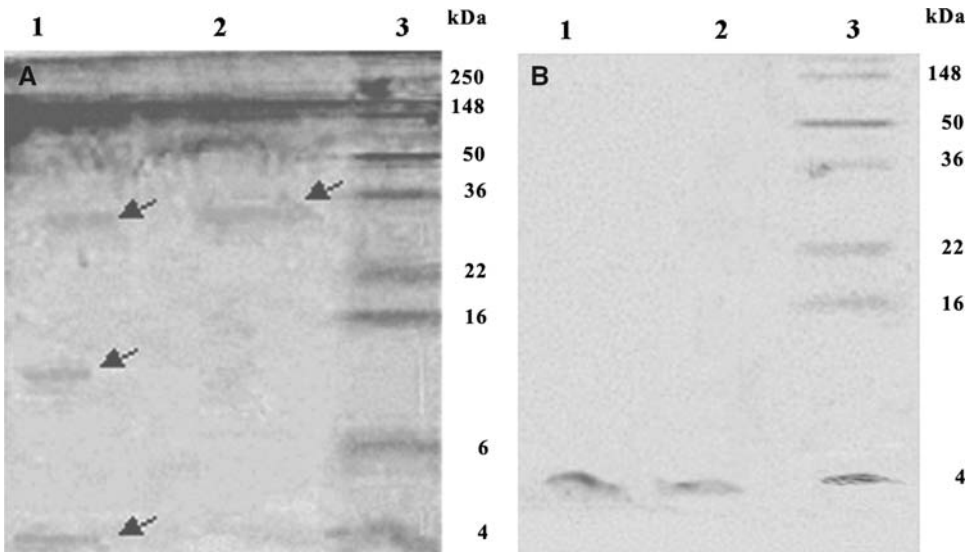

Fig. 2 Electrophoretical analysis of surfactant proteins. **a** *Lane 1* fractions from pig lavages enriched in both surfactant proteins: SP-B—dimmer and monomer and SP-C (shown with *arrows*); *2* pellet fraction, enriched only in SP-B dimmer; *3* SeaBlue molecular weight markers (4–250 kDa). **b** *1* and *2* SP-C (3.9 kDa MW) enriched fractions from pig lavages; *3* SeaBlue molecular weight markers (4–250 kDa)

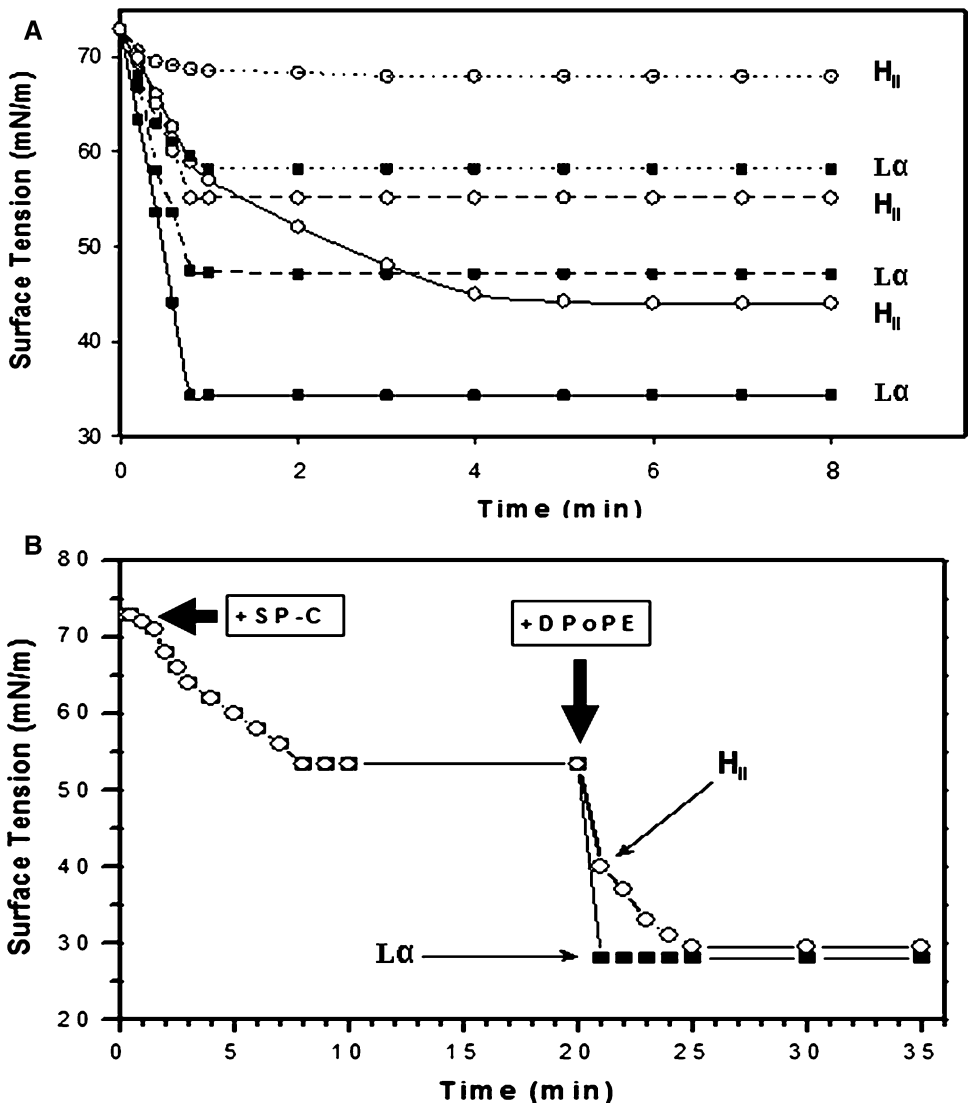

Fig. 3 Surface tension γ as a function of time for pure adsorption films of DPoPE (**a**) and for mixed films from SP-C and DPoPE (**b**). **a** Concentrations of DPoPE are 0.5 μg/ml (*fine dotted lines*), 1 μg/ml (*large dotted lines*) and 5 μg/ml (*thick lines*). **b** *Thick arrows* point the moment of addition of SP-C and DPoPE; only the data for 5 μg/ml DPoPE are presented. Symbols denote lamellar L_α (*filled square*), inverse hexagonal H_{II} (*open circle*) phases of DPoPE and SP-C (*rectangle with open diamond*). Experiments are performed at 0.15 M NaCl subphase, T = 37°C

DPoPE dispersions, in lamellar L_α and non-lamellar H_{II} phases, are injected in Langmuir trough subphase. It can be seen that the adsorption and interfacial disintegration of L_α phase remains much faster (within seconds) while in H_{II} phase equilibrium surface tension value is reached within 5 min. However, both phases display one and the same equilibrium value of surface tension of approximately 29 mN/m. Only the data for 5 μg DPoPE/ml are represented at Fig. 3b, as at this lipid concentration are performed the monolayer compression/decompression experiments described below. The effect of SP-C on the adsorption of lipid phases for the lower DPoPE concentrations (0.5 and 1 μg/ml) is analogous. The interfacial disintegration of L_α and H_{II} became identical and they decrease γ to one and the same equilibrium value (58 mNm at 0.5 μg DPoPE/ml and 47 mN/m at 1 μg DPoPE/ml) but the difference in the adsorption kinetics of the phases is preserved (data not shown).

Figure 4 and Table 1 summarize the dependences of dynamic surface tensions of the mixed lipid–protein films on the cycle number of compression/decompression between 100% (γ_{max}) and 20% (γ_{min}) of the initial area. For adsorbed films from pure DPoPE lamellar phase γ_{max} slightly increases from 34.3 to 36.8 mN/m between the first and the third compression/decompression cycle, respectively (Fig. 4a). In contrast in presence of SP-C γ_{max} decreases from 28.1 mN/m (for first cycle) to 23.1 mN/m (for third cycle). For adsorption films of pure DPoPE in inverted hexagonal phase γ_{max} significantly elevates from 44.0 to 50.9 mN/m during cycling. Although the addition of SP-C decreases the values of γ_{max} compared to the data at pure "protein-free" air/water interface, the tendency for increase of maximal surface tension between the first and the third cycle (from 29.6 to 36.2 mN/m) is preserved.

A comparison of γ_{min} values is shown in Fig. 4b. For pure DPoPE in L_α phase γ_{min} changes slightly (from 27.1 to

Table 1 Equilibrium γ_{eq} and dynamic (γ_{max} and γ_{min}) surface tension values (mN/m) of spread SP-C monolayer, of adsorption monolayers from pure DPoPE (in L_α and H_{II} phase states) and of mixed SP-C + DPoPE monolayers

Composition of monolayer	γ_{eq} (mN/m)	Values of γ_{max} and γ_{min} during successive compression/decompression cycles
Pure SP-C	53.4	53.4/25.0
		66.2/24.6
		67.8/25.3
Lα phase of DPoPE	34.3	34.3/27.1
		35.9/27.1
		36.8/26.5
Mixed monolayers SP-C + L_α phase	28.1	28.1/25.6
		25.0/19.0
		23.1/17.5
H_{II} phase of DPoPE	44.0	44.0/27.8
		47.8/24.6
		50.9/22.8
Mixed monolayers SP-C + H_{II} phase	29.6	29.6/25.0
		34.3/25.3
		36.2/24.0

26.5 mN/m). Mixed DPoPE L_α phase/SP-C films display lowest value of γ_{min}: it decreases from 25.6 to 17.5 mN/m between the first and the third cycle. Adsorption films from inverse hexagonal DPoPE phase show opposite tendency compared with the L_α phase: γ_{min} drops significantly for pure DPoPE H_{II} phase, while mixing with SP-C initially decreases γ_{min} but its value remains almost constant during consecutive cycling.

In order to visualize the changes in phase coexistence of DPoPE film due to interaction with SP-C, insoluble phospholipid monolayers are observed through BAM. When the film is compressed surface tension continuously lowers (Fig. 5a).

At high degree of compression (at less than 44 Å^2 per molecule) a change in the isotherm slope is realized and a plateau γ(A)-trace is registered.

At these conditions large condensed domains (250–300 μm length) in the pure phospholipid monolayer are observed (Fig. 6a).

The addition of SP-C (at the same concentration as in the previous experiments) results in drop of surface tension with 6 mN/m (dashed line in Fig. 5b) and rapid (within seconds) disaggregation of the condensed phase (Fig. 6b). Finally much smaller domains (approx. 10 μm) homogeneously distributed through the monomolecular film area are formed (Fig. 6c). Then the morphology of the film remained constant in time (monitored for at least 2 h). Analogous film appearance at the same phospholipid packing densities is observed when SP-C and DPoPE are mixed together and spread simultaneously on the trough surface. Control experiments are performed with pure SP-C spread at the interface where no domains are observed.

Discussion

In the current work we study the effects of the interaction of SP-C with DPoPE on the adsorption and interfacial disintegration of phospholipid lamellar and non-lamellar phases and on the phase coexistence in insoluble DPoPE monomolecular films. The formation of lamellar or non-lamellar inverted hexagonal phase structures (Fig. 1) in DPoPE dispersions depends on sample thermal history as revealed by our previous study (Jordanova et al. 2003).

Interfacial behavior of adsorption films by L_α and H_{II} phases of DPoPE formed at pure air/water interface

In absence of SP-C the adsorption and interfacial disintegration of the non-lamellar inverted hexagonal phase are much slower and incomplete in comparison with the lamellar bilayer phase as it can be seen from the transients and

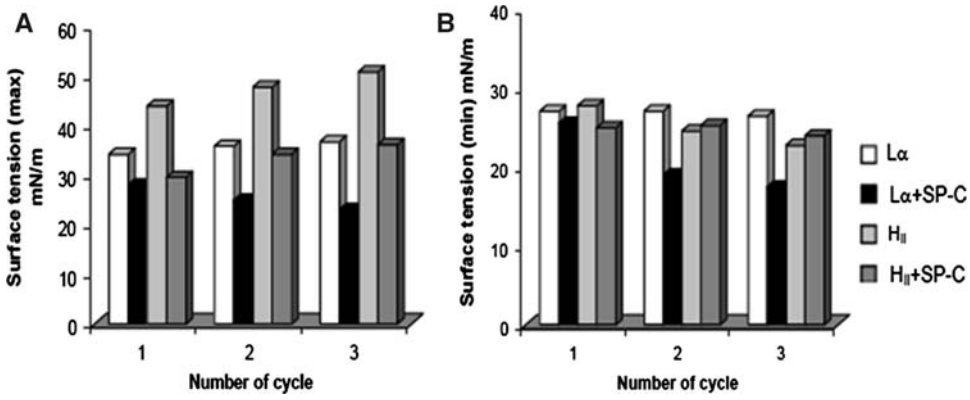

Fig. 4 Dynamic characteristics of pure (DPoPE) and mixed (DPoPE + SP-C) monolayers were studied by measuring the surface tension γ during compression/decompression between 100% (γ_{max}) (**a**) and 20% (γ_{min}) (**b**) of the initial monolayer area. The compression/decompression rate was 3 min per cycle. The monolayer surface tension was monitored up to the third cycle in our studies

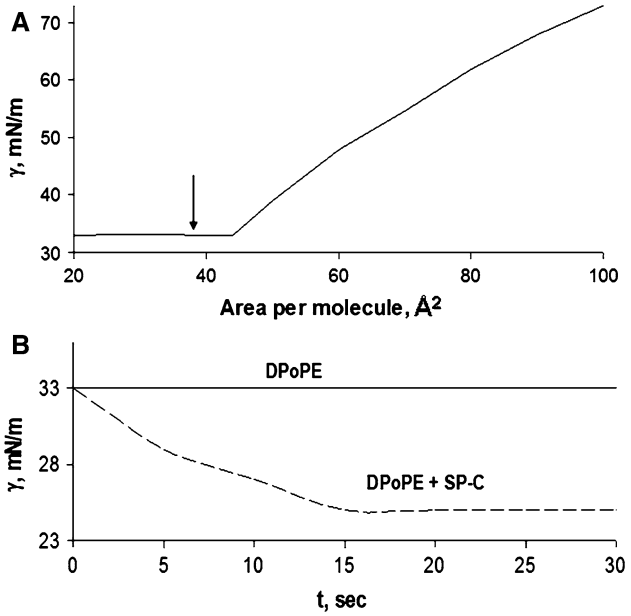

Fig. 5 **a** Compression isotherm of DPoPE insoluble monolayer showing the dependence of surface tension (γ) on the area per molecule (Å^2). *Arrow* points the region of the isotherm where phase coexistence was observed and where SP-C was added (see Fig. 6). **b** *Dashed line* shows the drop of surface tension after addition of SP-C to the compressed DPoPE monolayers (*solid line* denotes the transient of surface tension for pure DPoPE films). The experiments were performed at 0.15 M NaCl subphase, $T = 37°C$

equilibrium values of surface tension obtained at pure air/water interface (Fig. 3a; Table 1).

For all studied bulk concentrations of DPoPE, two main differences can be noticed between the surface tension transients of L_α and H_{II} phases: (1) equilibrium surface tension values are always reached faster for the lamellar phase and (2) at equal concentrations of DPoPE in the subphase, γ_{eq} for L_α phase is approximately 10 mN/m lower than γ_{eq} for H_{II} phase. If the faster establishment of equilibrium for the lamellar phase means lower energetic barrier for the adsorption of L_α compared to inverted hexagonal phase, the differences in γ_{eq} values point to the different amount of lipid molecules adsorbed at the interface in both cases. This indicates that not only the kinetics of adsorption of H_{II} phase is slower but also its degree of interfacial disintegration is significantly lower compared to the lamellar phase. Thus at equal DPoPE concentrations in the subphase the structure of the bulk phospholipid aggregates proves to be of major importance for the interfacial disintegration of the two studied phases (L_α and H_{II}) and for DPoPE surface activity. Similar results demonstrating the influence of bulk lipid state on the adsorption of liposomes are already reported. For example, in the classic work of Schindler (Schindler 1980) it was found that at equal bulk lipid concentration the adsorption of small unilamellar vesicles improves with the increase of the liposome radius. Other studies demonstrated that adsorption of phospholipid vesicles strongly depend on the physical state (gel or melted) and on the initial hydration of the bilayer (Pattus et al. 1978; Gugliotti and Politi 2001). Thus as the experiments performed in our study are well above DPoPE chain melting transition temperature, the major difference between the normal lamellar and inverted hexagonal aggregates remains the structural organization (Fig. 1), which should be responsible for the significant difference observed between the adsorption behavior of the two phases. Our results are also in agreement with previous works showing lower surface activity (i.e., higher surface tension values) of dispersions composed entirely by the inverted phase lipid aggregates (Jordanova et al. 2003) when compared to dispersions containing phospholipid bilayer phases. This phenomenon correlates with the data that phospholipids with negative spontaneous curvature hardly stabilize planar interfaces as is the case of the Langmuir monolayer system (Seddon and Templer 1995).

The higher values of γ_{max} for H_{II} compared to L_α during dynamic compression/decompression cycling and the roughly three-times difference in maximal surface tension increase between the first and the third cycle ($\Delta\gamma_{max} = 2.5$ mN/m for L_α and $\Delta\gamma_{max} = 6.9$ mN/m for H_{II} phase) additionally point to lower readsorption and interfacial

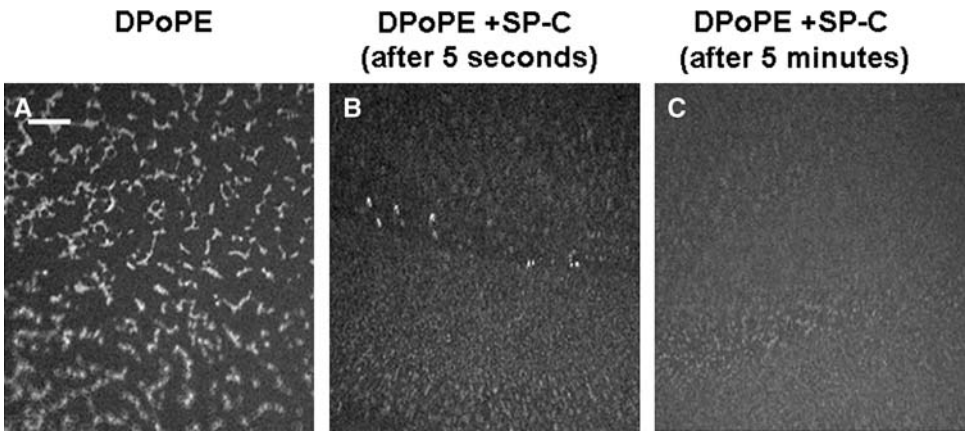

Fig. 6 BAM images of insoluble DPoPE monolayers pure (**a**) and 5 s (**b**) and 5 min (**c**) after addition of SP-C. The size of the bar is 400 μm. Experiments were performed at 0.15 M NaCl subphase, T = 37°C

rearrangement of films by the non-lamellar phase in juxtaposition to the lamellar one (Fig. 4a; Table 1). The fact that the differences in the properties of the two phases are preserved in the course of consecutive compression/decompression cycles suggest that inverted hexagonal aggregates in the monolayer subphase remained stable during the surface deformation and do not convert to structures more susceptible to interfacial disintegration (like the lamellar phase).

Still despite these differences in the performance of both phases at the pure air/water interface, adsorption films by both L_α and H_{II} aggregates display practically identical γ_{min} value of approx. 27 mN/m for all cycles. The latter indicates that the maximal surface packing density at 20% of the initial surface area for DPoPE molecules alone is practically independent of the bulk phase state of the phospholipid. This finding correlates with earlier works showing that fast non-equilibrium compression (analogous to the one in the alveolus in vivo) of the interfacial phospholipid film results in standardization of the adsorbed molecules orientation and surface packing (Hills 1988).

Interfacial behavior of adsorption films by L_α and H_{II} phases of DPoPE formed at air/water interface with predeposited SP-C monolayer

The interaction of DPoPE molecules with SP-C (predeposited at the air/water interface) results in equalizing of the interfacial disintegration of both phases (reaching roughly the same γ_{eq} value of 29 mN/m) although the adsorption kinetics of the lamellar phase remained much faster (Fig. 3b).

The fact that SP-C equalizes the interfacial disintegration arising from the adsorption of DPoPE lamellar and inverted hexagonal phases represents a major finding of the current study. Such a result might be of physiological relevance as it suggests a possible mechanism as to how the non-lamellar PEs existing in LS dispersions in vivo are "inserted" in the pulmonary surface film. A probable explanation of the observed phenomenon is that SP-C interacts with the lipid aggregates that adsorb to the air/water interface and promotes their disintegration to smaller structures or to individual molecules. The proposed mechanism is supported by our experiments with BAM imaging of insoluble DPoPE monolayers (Fig. 6a). When DPoPE films are subjected to high compression (corresponding to less than less than 44 $Å^2$ per DPoPE molecule) so that surface tension of 33 mN/m to be obtained (i.e., similar to γ_{eq} registered in the studies of L_α adsorption to pure air/water interface) large condensed domains are observed in the film plane. However, after addition of SP-C the domains disintegrated to much smaller structures (Fig. 6b, c) and more uniform monolayers are formed (for detailed discussion of BAM results see the subheading below).

The presence of SP-C in the mixed films resulted in decrease of γ_{max} and γ_{min} for the two phases (Fig. 4; Table 1). However, the behavior of the two dynamic parameters notably differs from L_α to H_{II} phase. It is to note that in the case of DPoPE L_α phase the interaction with SP-C resulted in 5 mN/m decrease of the maximal surface tension value during cycling. This indicates that there is no loss of surface active material during continuous compression/decompression as it is in the case of pure lamellar phase and that the film is enriched with surfactant molecules. As the measured γ_{max} of less than 28 mN/m is lower than the value for pure SP-C film (>50 mN/m) the effect can be clearly attributed to increased number of DPoPE molecules at the interface. Such finding agrees with the proposed role of SP-C during the dynamic compression of LS in vivo: to maintain association of lipid–protein complexes (in the alveolus subphase) with the interface at the most compressed state (end of expiration) and to enhance the readsorption and interfacial rearrangement of these complexes (Serrano and Perez-Gil 2006; Perez-Gil 2002). Studies of compressed phospholipid/protein films containing SP-C revealed that the protein really enhances formation of attached membrane patches during compression (Wang et al. 2005; Serrano and Perez-Gil 2006; Malcharek et al. 2005). SP-C-promoted attachment would then facilitate the ulterior reinsertion of surface active molecules from the reservoirs during reexpansion. Recent molecular dynamic simulations also support the idea for the presence of SP-C in the bilayer folds formed in the water subphase during LS monolayer compression (Baoukina et al. 2007).

In case of the non-lamellar phase the presence of SP-C has an opposite effect compared to the one on L_α phase. The maximal surface tension increases with 6.6 mN/m from the first to the third cycle (identical to $\Delta\gamma_{max}$ registered when H_{II} phase adsorbs to pure air/water interface) which indicates loss of surface active material during cycling.

The interaction of SP-C with DPoPE molecules in adsorption films by both phase aggregates, results in less than 2 mN/m decrease of γ_{min} compared to the corresponding values at pure air/water interface. Still if for H_{II} phase the minimal surface tension value remains constant during cycling, the presence of SP-C in L_α films resulted in 8.1 mN/m decrease of γ_{min}. As γ_{min} value is a measure of the density of DPoPE molecular packing at maximal compression, the result confirms our suggestion that SP-C improves the readsorption and interfacial rearrangement of lamellar structures.

In the reported study SP-C is predeposited to pure air/water interface, where it spreads and reaches γ of 53.4 mN/m. We accept that no irreversible surface denaturation of the protein takes place as previous FT-IR spectroscopy measurements prove that although after spreading at high initial surface tension (69 mN/m) SP-C molecule unfolds at

the interface subsequent increase in the packing density of the surrounding lipid film (as occurs in our experiments during the adsorption of DPoPE molecules) completely recovers the native α-helix conformation of the protein (Shanmukh et al. 2002). Our studies with preformed phospholipid insoluble monolayers (known to prevent surface denaturation of inserted protein molecules, Nag et al. 1996a, b) also indicate that the native functional conformation of the surfactant protein is attained in the saturated DPoPE adsorption films. The addition of SP-C to DPoPE insoluble monolayers (Fig. 5b) with similar surface tension (33 mN/m), i.e., similar molecular packing densities at the interface compared to adsorption films of pure L_α phase (Fig. 3a), resulted in γ decrease to 27 mN/m (of the same order as $\gamma_{eq} = 29$ mN/m for the mixed SP-C/DPoPE films obtained in adsorption studies—Fig. 3b).

Effect of SP-C/DPoPE interaction on the morphology of insoluble monolayers

The constant slope of surface tension (surface area) isotherm up to 44 Å^2 area per molecule (Fig. 5) indicates that monolayer remains in gaseous/liquid phase in this range of packing densities. This can be explained with the structure of DPoPE: a phospholipid with two unsaturated acyl chains that will hardly fit in tightly packed surface structures (Adamson and Gast 1997; Gaines 1966). Only at high compression (corresponding to less than 44 Å^2 per DPoPE molecule) a plateau in the isotherm (indicating phase coexistence in the film) is realized and interfacial lipid condensates are formed (Fig. 6a). It is noteworthy that in this plateau region of the compression isotherm the surface tension is 34 mN/m. That value is of the same order as γ_{eq} (Fig. 3a) of films by pure DPoPE L_α phase (i.e., corresponding to the packing density realized in the saturated phospholipid adsorption films).

When SP-C is added to DPoPE monolayers (Fig. 5b) surface tension drops to 27 mN/m (i.e., similar to γ_{eq} of mixed SP-C/DPoPE adsorption films). BAM studies reveal disintegration of the large (250–300 μm) condensed domains to much smaller 2D structures homogeneously distributed in film plane (Fig. 6b, c). Thus the drop in surface tension in presence of SP-C can be attributed both: (1) to the inclusion of SP-C in the surface film resulting in less available area for the lipid molecules and (2) to the interfacial disintegration of the large domains leading to increased number of DPoPE monomers spread at interface. The observation is in accordance with the literature data that SP-C alters the lipid packing in the monolayer, resulting in both a reduced size and increased number density of condensed-phase lipid domains with lower intrinsic order than the condensates formed in pure lipid monolayers upon increasing the monolayer pressure (Kruger et al. 1999; Nag et al. 1996a, b, 1997). Thus it seems that the interaction of SP-C with the phospholipid does indeed lead to a softening of the monolayer material, which has been postulated to be one major role of the protein in LS system. The homogenization of the molecular packing in the monolayer is also able to explain the decrease of dynamic surface tension values in mixed films when compared to pure DPoPE ones (Fig. 4; Table 1). As the cohesive interaction between the phospholipid monomers is decreased in the fluidized (due to DPoPE/SP-C interaction) film, it will become much easier for DPoPE molecules to respond to dynamic compression/decompression by faster readsorption and rearrangement at the interface (Hills 1988).

In earlier works the effect of SP-C on the integrity and size of liquid-condensed domains in insoluble binary DPPC/DPPG monolayers is related partially to the electrostatic interaction between the anionic PG head group and the positively charged residues at the protein N-terminus (Kruger et al. 2002, 1999). At the physiological pH of 6.8–7.0 used in our experiments PE head group is zwitterionic. It is known that at high surface packing densities strong hydrogen bonding and electrostatic interaction occurs between the PO_4^- and N^+H_3 moieties of adjacent PE molecules and there are practically no free charges in the head group region of the monolayer (Eibl and Woolley 1979; Mansour and Zografi 2007). Therefore the disintegration of the large (250–300 μm) domains induced by SP-C is most probably not related with electrostatics but with hydrophobic interactions occurring between the protein α-helix and the phospholipid molecules. It should be kept in mind also that the large aggregates observed in Fig. 6a are obtained at high compression (less than 44 Å^2 per DPoPE molecule) and can be considered more as collapsed structures than as liquid-condensed domains. The registered effect of SP-C on the morphology of the insoluble monolayers might also be related to the protein content in the film. The pictures shown in Fig. 6b, c are observed at ≈6–7 mol% SP-C. That amount is above the SP-C physiological concentration of 1 mol% applied in (Kruger et al. 2002) but still it is below the protein content of 10–20 mol% implemented in numerous previous works (Lipp et al. 1996, 1997; Nag et al. 1996a, b).

The interaction between the molecules of SP-C and DPoPE at the air/water interface deserves further study. As the main task of our work is to investigate the adsorption of DPoPE molecules in lamellar and non-lamellar inverted-hexagonal phase states, in most of the experiments (Figs. 3, 4; Table 1) aliquots of the lipid dispersions are injected in the subphase while SP-C is predeposited at the interface. Thus it is not possible to determine the number of DPoPE molecules adsorbed at the interface, and the mol-to-mol ratio between the lipid and SP-C. The dissolution of DPoPE in volatile spreading solvents (like chloroform or chloroform/MetOH

mixtures) will prevent the formation of the bulk lipid aggregates, and is beyond the main scope of the current work. However, in our future research we intend to study the changes in surface pressure of insoluble monolayers when varying SP-C/DPoPE molar ratio. Such experiment might provide information about the interaction between the surfactant protein and the PE molecules when they are finally adsorbed at the interface.

Conclusions

In the present work we find that the interaction of specific surfactant protein C with DPoPE molecules strongly influences the interfacial properties of the phospholipid adsorption film. The effect of SP-C depends on the phase structure of DPoPE aggregates (lamellar or inverted phase non-lamellar ones).

A major finding of the current study is that SP-C equalizes the equilibrium surface tension (i.e., the degree of interfacial disintegration) resulting from the adsorption of L_α and H_{II} phases, although the adsorption kinetics of the lamellar phase remains much faster. This effect might be of physiological relevance as it suggests possible protein-mediated mechanism for the interfacial disintegration of non-lamellar PE-enriched phases at the alveolar surface. The finding correlates well with the effect of the protein on the morphology of compressed insoluble monolayers where more uniform and homogeneously packed mixed films are seen.

Compared to pure air/water interface the inclusion of specific surfactant protein C results in lower dynamic surface tension values of the adsorption films. It is noteworthy that the effect of SP-C on the values of γ_{max} and γ_{min} during compression/decompression cycling is remarkably different for the two phases. If the maximal surface tension for L_α phase decreased from the first to the third cycle, the opposite effect is registered for H_{II} where γ_{max} increases during cycling. Also the significant decrease of γ_{min} for L_α in presence of SP-C is not observed in case of inverted hexagonal phase.

A potential application of this study might be in the field of design of surfactant replacement preparations, as the inclusion of unsaturated non-lamellar lipids to surfactant formulations results in pharmaceutically applicable dispersions able to rapidly adsorb and to maintain low-surface tension values (Biswas et al. 2007) at relatively constant surface viscosity (Rüdiger et al. 2005).

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