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Multilayer Formation upon Compression of Surfactant Monolayers depends on Protein Concentration as well as Lipid Composition: an Atomic Force Microscopy Study

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Abstract

Pulmonary surfactant forms a surface-active layer of lipids and proteins at the alveolar air/liquid interface, consisting mainly of a monolayer at end-inspiration, and changing into multilayer structures upon expiration. From the latter structures, surface-active material can readily be reinserted into the monolayer upon inspiration. The determinants for the formation of multilayers were investigated by compressing films, beyond the squeeze-out plateau, to a surface tension of 22 mN/m. Atomic force microscopy (AFM) was used to visualize the topography of lipid films containing varying amounts of native SP-B. These films were compared with films containing synthetic peptides based on the N-terminus of human SP-B: monomeric mSP-B\textsubscript{1-25} or dimeric dSP-B\textsubscript{1-25}. The formation of typical hexagonal network structures as well as the height of protrusions were shown to depend on the concentration of SP-B. Protrusions of bilayer height were formed from physiologically relevant concentrations of 0.2 - 0.4 mol\% SP-B upwards. Much higher concentrations of SP-B\textsubscript{1-25} peptides were needed to obtain network structures, and protrusion heights were not equal to those found for films with native SP-B. A striking observation was that while protrusions formed in films of DPPC/DPPG (80/20) had single bilayer thickness, those formed in DPPC/POPG (80/20) had various heights of multilayers, whereas those seen in DPPC/POPC/DPPG (60/20/20) were mainly of bilayer height. For the first time direct observations by AFM show i) that a certain minimal concentration of SP-B is required for the formation of layered protrusions upon film compression, ii) that protrusion height depends on whether the phospholipids contain an unsaturated fatty acyl chain, and iii) that protrusion height also depends on whether the unsaturated acyl chain is present in phosphatidylcholine or in phosphatidylglycerol. Synthetic SP-B\textsubscript{1-25} based surfactants to be used clinically will therefore have to contain, in addition to DPPC, a high concentration of SP-B\textsubscript{1-25} peptides as well as unsaturated phospholipids, preferentially unsaturated PG.

1. Introduction

Pulmonary surfactant is a mixture of lipids and proteins, synthesized and secreted into the alveolar fluid by the alveolar type II epithelial cells. Its main function is to reduce the surface tension at the alveolar air/liquid interface, thus preventing the alveoli from collapsing at end-expiration and making breathing at minimal effort possible. This is achieved by the formation of a surface-active film that consists of a lipid monolayer highly enriched in dipalmitoylphosphatidylcholine (DPPC) and bilayer or multilayer structures (‘surface-associated reservoir’) closely attached to the monolayer. From such multilayer structures surfactant material can be readily incorporated into the monolayer film upon inspiration. The existence of a layered film has recently been visualized \textit{in vitro} by atomic force and fluorescence light microscopy [13,163,206] and \textit{in vivo} by electron microscopy [12]. Film compression during expiration might lead to a squeeze-out of non-DPPC [169,170], film
expansion during inspiration to selective adsorption of DPPC [12], or they might just lead to an alteration of structure rather than a change in composition [171,172,206,13].

Administration of exogenous surfactant is a successful strategy in treating premature infants suffering from respiratory distress syndrome (RDS) [207]. Moreover, surfactant therapy is also considered promising for adults with acute respiratory distress syndrome (ARDS) [10,11,208]. Presently, the majority of clinically used surfactants is derived from animals. In an effort to circumvent the possibility of zoonotic infections and to reduce the considerable costs of surfactant production, the use of surfactants containing artificial proteins and lipids is currently under consideration. With respect to the proteins, the hydrophobic surfactant protein B (SP-B) is known to fulfill a crucial role in the lung, since respiratory distress is always observed in SP-B deficient humans [209,19] and in homozygous SP-B knock-out mice [20]. SP-B is a 79 amino acid amphipathic protein, active as an 18 kDa dimer [35], and has a net positive charge that is thought to be essential for its interaction with negatively charged phospholipids such as phosphatidylglycerol (PG) [72,173,56]. The SP-B amino acid sequence among mammals has been highly conserved [210]. Because of the importance of SP-B for proper surfactant activity, synthetic peptides based on its sequence have been developed: mSP-B_{1-25} is a monomeric synthetic peptide based on the N-terminal segment of human SP-B, while dSP-B_{1-25} is the dimeric form of mSP-B_{1-25} (Fig. 1). The structure and surface activity of these peptides have been investigated thoroughly, both in vitro and in vivo. The conformation of mSP-B_{1-25} was found to be α-helical [211,212]. In vitro comparison of the surface activity of mSP-B_{1-25} with that of dSP-B_{1-25} in a captive bubble surfactometer (CBS) revealed that both peptides reduced the surface tension, with the dimeric peptide expressing better ability to lower surface tension than the monomeric peptide [53]. In vivo experiments showed that SP-B_{1-25} peptides improved lung function in two animal models of surfactant deficiency [213-216]. With respect to the lipids (discussed in [176]), it has long been recognized that DPPC (40 to 50 wt% of the surfactant lipid pool) is responsible for keeping the surface tension near zero during expiration. Negatively charged PG (5 to 10 wt%) is likely to interact with the positive charges of SP-B. Lipids with unsaturated fatty acids are thought to be important for fluidizing the surfactant film.

Various established methods, among which captive bubble surfactometry, are available to obtain information on surfactant surface activity. In addition to these methods, growing interest has recently emerged in atomic force microscopy (AFM) [217], which yields information on surface topography, thereby providing new insight into the action of surfactant proteins and lipids during the breathing cycle. Although it has been found that upon compression material is squeezed-out of the monolayer to form protrusions connected to the monolayer [13,163], it is so far not clear which surfactant components determine the size and height of the protrusions. Therefore, in this study we used AFM to visualize the determinants for protrusion formation. For this purpose, we investigated monolayer films containing either bovine SP-B or an SP-B_{1-25} peptide in the fully saturated lipid system DPPC/DPPG (80/20, mol/mol), or in the partially unsaturated mixtures DPPC/POPG (80/20) or DPPC/POPC/DPPG (60/20/20).
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Native human SP-B (residues 1-25):

\[
\text{NH}_2 - FPIPLPYCWL\text{RALIKRIQAMIPKG- X}
\]

Native bovine SP-B (residues 1-25):

\[
\text{NH}_2 - FPIPLPYCLEL\text{RTLIKIQAVIPKG- X}
\]

Monomeric mSP-B\text{1-25}:

\[
\text{NH}_2 - FPIPLPYCWA\text{RALIKRIQAMIPKG- COOH}
\]

Dimeric dSP-B\text{1-25}:

\[
\text{NH}_2 - FPIPLPYCWL\text{A RA L I KRIQAMIPKG - COOH}
\]

Figure 1. Amino acid sequences of native SP-B and of SP-B derived synthetic peptides.
The differences in amino acid sequence between the various peptides are depicted in bold font. X represents the rest of the amino acids of native SP-B, which consists of 79 amino acids per monomer and is active as a dimer.

2. Materials and Methods

2.1 Materials

1,2-dipalmitoyl-\text{sn}-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-\text{sn}-glycero-3-(phospho-rac-(1-glycerol)) (DPPG), 1-palmitoyl-2-oleoyl-\text{sn}-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-\text{sn}-glycero-3-phospho-rac-(1-glycerol) (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA); chloroform (CHCl\text{3}) and methanol (MeOH) from Labscan (Dublin, Ireland) were HPLC grade.

2.2 Biochemical assays

Bovine SP-B obtained from lung lavage was isolated and characterized according to standard procedures [158]. The protein concentration was determined by fluorescamine assay [182]. Concentrations of phospholipids were determined according to Rouser \textit{et al.} [183]. The monomeric peptide mSP-B\text{1-25} was synthesized based on the N-terminal 25 amino acids of human SP-B with Cys-11 substituted for Ala [215] (Fig. 1). The dimeric version of the
peptide, dSP-B$_{1-25}$, was obtained by linking two monomer peptides through their only remaining cysteine, Cys-8 [53].

2.3 Surface pressure - area (Π - A) diagrams

Π - A curves were obtained using a home-built teflon trough with an operational area of 630 cm$^2$. Surface tension was measured with a platinum Wilhelmy plate connected to a microbalance (Cahn2000, Ankersmit, Oosterhout, the Netherlands). Films, composed of DPPC/DPPG (80/20, molar percentages), DPPC/POPG (80/20) or DPPC/POPC/DPPG (60/20/20) plus varying amounts of bovine SP-B or one of the SP-B$_{1-25}$ peptides, were formed by spreading aliquots in CHCl$_3$/MeOH onto the water subphase at 20 ± 3 °C. After the solvent had been allowed to evaporate for at least 5 min, films were compressed at a rate of 13.8 % of the operational area per min (28.8 Å$^2$/molecule lipid · min) until film collapse (usually at a surface tension of 10 - 15 mN/m). Repeated measurements gave identical diagrams.

2.4 Atomic Force microscopy

For Langmuir-Blodgett transfer, films were prepared on a home-built teflon trough with an operational area of 66.5 cm$^2$. Surface tension was measured as described above. Before film spreading, a freshly cleaved mica sheet was dipped vertically into a subphase of demineralized water at room temperature (20 ± 3 °C). Films, having the same composition as described for the surface pressure - area curves, were formed by spreading aliquots onto the subphase. The films were compressed at a rate of 8.6 % of the operational area per min until a surface tension of 22 mN/m was reached. Subsequently, the films were transferred onto a disc of mica (14 mm in diameter) at a rate of 2.0 mm/min at constant surface tension.

For AFM measurements, transferred films were mounted on the J-type scanner (150 μm × 150 μm scan range) of a Nanoscope III Multimode microscope (Digital Instruments, Santa Barbara, CA, USA) operating in contact mode in air. Scanning was performed using oxide-sharpened Si$_3$N$_4$ tips with a spring constant of 0.12 N/m. The force with which the tip scanned the sample was set such that it was as small as possible while the image was stable and clear, which was usually at a force of 15 nN. Samples were checked for possible tip-induced deformation by zooming out after a region had been scanned. Since scanning in tapping mode did not give better images compared to those obtained in contact mode, we used contact mode because of its ease of handling.

2.5 Statistics

The computer program SPSS version 9.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis by ANOVA with Bonferroni’s post-hoc test. Differences were considered significant at $P < 0.05$. 

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Figure 2. Pressure - Area isotherms of DPPC/DPPG films with and without native SP-B or SP-B$_{1-25}$ peptide. Compression isotherms of monolayers containing DPPC, DPPG or DPPC/DPPG (80/20, mol/mol) (A) plus 0.02 - 4 mol% bovine SP-B (B), 2 - 20 mol% mSP-B$_{1-25}$ (C), or 2 - 20 mol% dSP-B$_{1-25}$ (D) on a water subphase at 21 °C. The squeeze-out plateaus observed for bovine SP-B are indicated by arrows (B).
3. Results

Pressure- area isotherms were recorded to obtain information about the surface tension at which protrusion formation occurred. Lipid/protein monolayers were compressed by movement of a barrier in a Langmuir-Wilhelmy trough, which leads to a decrease in the area available to the film. Consequently, film surface pressure (\(\pi\)) was increased, and film surface tension (\(\gamma\)) was lowered. The relationship between \(\pi\) and \(\gamma\) is given by the equation \(\pi = 72.5 - \gamma\), where 72.5 mN/m represents the surface tension of pure water at 21 °C. A typical pressure-area isotherm of pure lipid monolayers is depicted in Fig. 2A. The isotherm of DPPC/DPPG (80/20) shows phase behavior similar to that of pure DPPC. When bovine SP-B was added to DPPC/DPPG (Fig. 2B), two plateau regions were observed: the first at a surface pressure of approximately 23 mN/m and a second one, that was much more pronounced, at \(\pi = 40\) mN/m. These plateaus were seen most clearly at higher protein concentrations and are in line with data found for porcine SP-B [163]. For monolayers containing 4 mol% SP-B the squeeze-out plateau was elongated and the monolayer could be compressed to very low areas, indicating massive squeeze-out. No additional plateaus were observed above \(\pi = 48\) mN/m until onset of film collapse (at approximately \(\pi = 59\) mN/m in our experimental setup), indicating that no extra squeeze-out occurred. For mSP-B\(_{1-25}\) containing monolayers, isotherms of films with 2 mol% or less peptide were identical to isotherms of DPPC/DPPG (Fig. 2C). Furthermore, at increasing peptide concentrations no plateau like that observed at \(\pi = 40\) mN/m for bovine SP-B was seen, but instead a region with a decreased slope was observed between surface pressures of 8 and 32 mN/m, comparable to the first faint plateau observed for bovine SP-B. The region with decreased slope was most clearly visible for monolayers containing as much as 20 mol% of the peptide. If protrusion formation occurs, it can be expected to be in this region. A similar region with decreased slope was seen for dSP-B\(_{1-25}\) peptide containing monolayers (Fig. 2D). From Fig. 2 it is apparent that the compressibility of bovine SP-B containing monolayers is considerably higher than those containing SP-B\(_{1-25}\) peptides at the same concentration. Since squeeze-out had fully occurred above a surface tension of 24 mN/m (i.e. below a surface pressure of 48 mN/m), determinants for protrusion formation were studied using monolayers compressed beyond this plateau to a surface tension of 22 mN/m.

Lipid/protein films, compressed to the desired surface tension, were deposited on mica and the topography was subsequently visualized by atomic force microscopy. Fig. 3 shows the effect of surface tension on film structure, using films of DPPC/DPPG/SP-B (80/20/4, mol/mol/mol). Films deposited at \(\gamma = 62\) mN/m, i.e. when squeeze-out has not yet occurred, showed brighter islands (i.e. having a higher surface) surrounded by darker (i.e. lower) regions (Fig. 3A). According to other AFM studies [163,206] the bright islands at this surface tension correspond to liquid condensed (LC) phase while the dark regions consist of liquid expanded (LE) phase. The LC domains showed dark spots (readily seen at higher magnification, Fig. 3B), probably consisting of trapped LE phase. Upon compression of the monolayer (i.e. decreasing the surface tension) the amount of LC phase was increased (Fig. 3C). The
difference in height between both lipid phases was found to be $1.2 \pm 0.1$ nm, which is similar to that found by others [163,206]. Film topography altered dramatically when films were compressed through the second plateau region of the isotherm to a surface tension of 22 mN/m (Fig. 3D). At this surface tension protrusions were formed that appeared as bright mountains amongst dark valleys consisting of monolayer. The height of the protrusions was $4.1 \pm 1.1$ nm. It has been shown for the same lipid/protein mixture that while the protrusions consist of proteins as well as lipids in LE phase, the lipid monolayer is in LC phase [163].

![AFM topography images](image)

**Figure 3.** AFM topography of DPPC/DPPG films containing native SP-B compressed to varying surface tensions.
Films of DPPC/DPPG/bovine SP-B (80/20/4, molar percentages) were transferred onto mica at $\gamma = 62$ mN/m (scan area = 10 × 10 μm in (A) and 2 × 2 μm in (B)), $\gamma = 42$ mN/m (C) (scan area = 10 × 10 μm), and $\gamma = 22$ mN/m (D) (scan area = 10 × 10 μm).

To learn more about the origin and development of the protrusions, we made a concentration curve of bovine SP-B in DPPC/DPPG (80/20) films compressed to a surface
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Figure 4. AFM topography of DPPC/DPPG films containing low to moderate amounts of native SP-B. Films were transferred onto mica at $\gamma = 22$ mN/m, containing DPPC/DPPG (80/20, mol/mol) plus low to moderate bovine SP-B concentrations of 0.02 mol% (A), 0.1 mol% (B), 0.2 mol% (C), or 0.4 mol% (D); scan areas were $10 \times 10 \mu m$. Furthermore, a zoomed-in image of DPPC/DPPG/SP-B (80/20/0.4) is shown (E) at a scan area of $3 \times 3 \mu m$.

tension of 22 mN/m (Figs. 4 and 5). Although indications for protrusion formation were observed even at very low SP-B concentration (0.02 mol%), protrusion height differed
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statistically significant through the concentration curve: at very low concentration (0.02 mol%) heights were 0.5 ± 0.1 nm; at low to moderate concentration (0.1 - 0.2 mol%) heights were 2.0 ± 0.9 nm overall; at moderate to high concentrations (0.4 - 8 mol%) heights were 4.1 ± 1.1 nm overall; see Table 1 for protrusion heights of individual concentrations. Furthermore, upon increasing the protein concentration the protrusion regions became connected and started forming networks of small circular domains (readily observed as white dots in Fig. 4E). The typical hexagonal shape of the cells of the networks is best seen at a physiologically relevant SP-B concentration of 0.2 - 0.4 mol%. Although AFM determinations in the x and y direction are not as accurate as in the z direction, widths of samples scanned with the same tip can be roughly compared to each other. Protrusions were found to be disc-like in shape with a typical diameter of approximately 35 nm that did not change over the range of SP-B concentrations studied. An interesting observation was the presence of large circles of protruded material captured inside a hexagonal cell, most clearly seen in films containing 0.2 mol% SP-B (Fig. 4C). The height of these protrusions was 0.7 ± 0.1 nm, suggesting that they represent domains with different lipid orientation. One can speculate that these large circles consist of lipids with extended acyl chains, that have an increased height compared to the lipids around the circles, either because the latter have tilted chains, or because they are in a fluid state like the lipids in the networks. The large circles were not observed at high protein concentrations (2 - 8 mol%, Fig. 5) at which the monolayer domains were much smaller. Increasing the SP-B concentration led to a higher amount of protruded material until the monolayer had almost vanished and mostly protruded material was seen (Fig. 5E and F). Meanwhile, the network structures lost their typical hexagonal appearance. Importantly, when films contained no protein or peptide, protrusions were not observed upon compression to a surface tension of 22 mN/m (not shown).

AFM images of films containing SP-B$_{1-25}$ peptides differed dramatically from those containing bovine SP-B, in the sense that i) much higher peptide concentrations were needed to obtain the same kind of network structures, and ii) the height of the protrusions was considerably lower than that found for the native protein. Interestingly, monomeric mSP-B$_{1-25}$ and dimeric dSP-B$_{1-25}$ were found to have similar film topography (compare Figs. 6 and 7). Although protrusions were visible at 2 mol% of the SP-B$_{1-25}$ peptides (Figs. 6A and 7A), the first indication for structured networks is seen at a peptide concentration of 8 mol% (Figs. 6B and 7B). At an SP-B$_{1-25}$ concentration as high as 20 mol% hexagonal structures comparable to those of 0.4 mol% bovine SP-B were observed, although the size of the hexagonal cells were markedly larger in the case of dSP-B$_{1-25}$ than in that of mSP-B$_{1-25}$ or native SP-B (compare Fig. 7C with Figs. 6C and 4D). The overall height of the protrusions was 2.7 ± 1.3 nm for films containing 2 mol% or 8 mol% mSP-B$_{1-25}$ or 2 mol% dSP-B$_{1-25}$; see Table 1 for protrusion heights at individual concentrations. Surprisingly, protrusion height of films containing 20 mol% mSP-B$_{1-25}$ was significantly lower than that found at lower mSP-B$_{1-25}$ concentrations and for films containing dSP-B$_{1-25}$. Films containing 8 mol% or 20 mol% dSP-B$_{1-25}$ had overall protrusion heights of 3.5 ± 1.7 nm. Protrusion heights of films containing the SP-B$_{1-25}$ peptides were significantly lower than those containing 0.4 mol%
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bovine SP-B, except for films with high concentrations (8 and 20 mol%) of dSP-B$_{1:25}$. Finally, the tendency to form the large circles of protruded material inside the network, as observed for 0.2 mol% bovine SP-B (Fig. 4C), was also seen for films containing 20 mol% dSP-B$_{1:25}$ (Fig. 7C and D).

![Image of AFM topography of DPPC/DPPG films containing high amounts of native SP-B.](image)

**Figure 5.** AFM topography of DPPC/DPPG films containing high amounts of native SP-B. Films were transferred onto mica at $\gamma = 22$ mN/m, containing DPPC/DPPG (80/20, mol/mol) plus high concentrations of bovine SP-B of 2 mol% at a scan area of 10 × 10 μm (A) and 2 × 2 μm (B), 4 mol% at a scan area of 10 × 10 μm (C) and 2 × 2 μm (D), and 8 mol% at a scan area of 10 × 10 μm (E) and 2 × 2 μm (F).
Figure 6. AFM topography of DPPC/DPPG films containing varying concentrations of monomeric SP-B_{1-25} peptide.
Films were transferred onto mica at γ = 22 mN/m, containing DPPC/DPPG (80/20, mol/mol) plus the monomeric peptide mSP-B_{1-25} at 2 mol% (A), 8 mol% (B), and 20 mol% (C), scanned at 10 × 10 μm. Furthermore, an image of DPPC/DPPG/mSP-B_{1-25} (80/20/20) is shown at a scan area of 30 × 30 μm (D).

The influence of lipid unsaturation on film topography was investigated by substituting DPPG by POPG. For these experiments, protein or peptide concentrations used were those previously observed to give clear network structures in the DPPC/DPPG (80/20) mixtures, i.e. 0.4 mol% bovine SP-B and 20 mol% of the SP-B_{1-25} peptides. Compression isotherms (Fig. 8) clearly showed squeeze-out plateaus at approximately π = 40 mN/m (γ = 32 mN/m) for DPPC/POPG films containing either native SP-B or SP-B_{1-25} peptide. Film appearance (Fig. 9) was similar for lipid mixtures containing either saturated or unsaturated PG (compare Fig. 9 with Figs. 4D, 6C and 7C). The size of the hexagonal cells was again larger in the case of dSP-B_{1-25} than for mSP-B_{1-25}, albeit that the difference was less extreme than in the fully saturated lipid system. DPPC/POPG films containing low amounts of peptides, like 2 mol% mSP-B_{1-25} or 1 mol% dSP-B_{1-25}, did not show network structures (Fig. 9D and E). A spectacular difference was found for the height of the protrusions: whereas in the case of a fully saturated lipid system compressed material always formed protrusions with a height of...
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approximately 4 nm, the exchange of DPPG for POPG resulted in the presence of protrusions of up to 24 nm. This was found for bovine SP-B as well as the SP-B\textsubscript{1-25} peptides. Mostly, protrusions of 4, 8, 12, 16, 20 and 24 nm were found. Occasionally, higher protrusions of up to 60 nm were seen.

Table 1. AFM measurements of protrusion height of compressed films containing DPPC/DPPG (80/20) plus varying amounts of native SP-B or SP-B\textsubscript{1-25} peptides. The number of protrusion height determinations is denoted by \( n \).

| protein or peptide (mol\%) | protrusion height (nm) |
|----------------------------|------------------------|
| bovine SP-B                |                        |
| 0.02 (\( n=12 \))         | 0.5 ± 0.1 \( ^a \)     |
| 0.1 (\( n=18 \))          | 2.0 ± 1.0 \( ^b \)     |
| 0.2 (\( n=10 \))          | 2.1 ± 0.8 \( ^b \)     |
| 0.4 (\( n=21 \))          | 4.3 ± 1.5 \( ^c \)     |
| 2 (\( n=9 \))             | 3.6 ± 0.8 \( ^c \)     |
| 4 (\( n=19 \))            | 4.1 ± 1.1 \( ^c \)     |
| 8 (\( n=20 \))            | 4.3 ± 0.9 \( ^c \)     |
| mSP-B\textsubscript{1-25} |                        |
| 2 (\( n=18 \))            | 2.4 ± 0.9 \( ^d,^f \)  |
| 8 (\( n=59 \))            | 2.8 ± 1.4 \( ^d,^f \)  |
| 20 (\( n=75 \))           | 2.0 ± 0.9 \( ^e,^f \)  |
| dSP-B\textsubscript{1-25} |                        |
| 2 (\( n=34 \))            | 2.7 ± 1.3 \( ^d,^f \)  |
| 8 (\( n=46 \))            | 3.5 ± 2.0 \( ^d \)     |
| 20 (\( n=39 \))           | 3.4 ± 1.4 \( ^d \)     |

\( ^a \) Value differs significantly from those observed for all other concentrations of native SP-B (\( P < 0.05 \)).
\( ^b \) Values differ significantly from those observed for other concentrations of native SP-B (\( P < 0.05 \)), but not from each other.
\( ^c \) Values differ significantly from those observed for other concentrations of native SP-B (\( P < 0.05 \)), but not from each other.
\( ^d \) Value does not differ significantly from those observed for other SP-B\textsubscript{1-25} peptides, except for 20 mol\% mSP-B\textsubscript{1-25}.
\( ^e \) Value differs significantly from that for other SP-B\textsubscript{1-25} peptides (\( P < 0.05 \)).
\( ^f \) Values differ significantly (\( P < 0.05 \)) from that observed for 0.4 mol\% bovine SP-B.
Figure 7. AFM topography of DPPC/DPPG films containing varying concentrations of dimeric SP-B1-25 peptide.
Films were transferred onto mica at γ = 22 mN/m, containing DPPC/DPPG (80/20, mol/mol) plus the dimeric peptide dSP-B1-25 at 2 mol% (A), 8 mol% (B), and 20 mol% (C), scanned at 10 × 10 μm. Furthermore, an image of DPPC/DPPG/dSP-B1-25 (80/20/20) is shown at a scan area of 30 × 30 μm(D).

In a subsequent set of experiments the topography of films consisting of DPPC/POPC/DPPG (60/20/20, molar percentages) instead of DPPC/POPG (80/20) was investigated. In this way it was studied whether the increased height of the protrusions was dependent on the presence of the unsaturated acyl chain in phosphatidylglycerol, or could also be brought about by an unsaturated acyl chain in an equal number of the phosphatidylcholine molecules. Compression isotherms of DPPC/POPC/DPPG films containing proteins or peptides (Fig. 10) did not show the pronounced plateaus as observed for films of DPPC/POPG at the same protein or peptide concentration (Fig. 8), although to some degree isotherm flattening was observed, albeit at higher surface pressure (at π = 42 mN/m) than in the films of the other lipid mixtures. DPPC/POPC/DPPG films containing 20 mol% SP-B1-25 peptides (Fig. 11) appeared different from those formed in the two other lipid mixtures at the same peptide concentration, but resembled films of lower peptide concentration. Moreover, network structures were not visible in films with 20 mol% mSP-B1-25. Surprisingly, it was found for
DPPC/POPG films with 0.4 mol% bovine SP-B and dimeric peptide. Heights of 8 - 32 nm were also observed, but this was less common and was seen only for films containing bovine SP-B and dimeric peptide.

4. Discussion

Surfactants based on synthetic peptides are of growing interest to clinical use, because of their low risk of containing biohazardous contaminants and their relative ease of production. Before clinical application of artificial surfactant, detailed knowledge about biophysical activity of their synthetic peptides and lipids is required. Here we describe the effect of synthetic peptides based on the N-terminal 25 amino acids of human SP-B on the topography of supported DPPC/DPPG (80/20) films and compare it with the effect of bovine SP-B, which is present in a large number of commercially available surfactants. Moreover, the effect of lipid acyl chain unsaturation and the effect of the nature of the phospholipid that contains an unsaturated acyl chain on the formation of multilayered surfactant protrusions was investigated using films with DPPC/POPG (80/20) or DPPC/POPC/DPPG (60/20/20) as lipid components. Our findings suggest that the molecular composition of mixed lipid/protein monolayers plays an important role in surfactant film topography.

Compression isotherms of monolayers of DPPC/DPPG (80/20) with bovine SP-B (0.02 - 4 mol%) showed two squeeze-out plateaus of which the plateau starting at...
Figure 9. AFM topography of DPPC/POPG films containing native SP-B, mSP-B₁₋₂₅ or dSP-B₁₋₂₅.
Films were transferred onto mica at $\gamma = 22 \text{ mN/m}$, containing DPPC/POPG (80/20, mol/mol) plus 0.4 mol% bovine SP-B (A), 20 mol% mSP-B₁₋₂₅ (B), 20 mol% dSP-B₁₋₂₅ (C), 2 mol% mSP-B₁₋₂₅ (D), or 1 mol% dSP-B₁₋₂₅ (E), scanned at 10 $\times$ 10 $\mu$m. The height trace is that of the white line in image A. The arrowheads on the height trace show the approximate height difference, which was 15.7 nm.

$\gamma = 32 \text{ mN/m}$ was broadened as protein concentration increased (Fig. 2). It should be noted that we define squeeze-out as exclusion of fluid lipid and protein from the monolayer into the
DPPC/POPC/DPPG films with and without native SP-B or SP-B_{1-25} peptide. Compression isotherm of monolayers containing POPC, or DPPC/POPC/DPPG (60/20/20, mol/mol/mol) with 0.4 mol% bovine SP-B, 20 mol% mSP-B_{1-25}, or 20 mol% dSP-B_{1-25}.

Figure 10. Pressure - Area isotherms of DPPC/POPC/DPPG films with and without native SP-B or SP-B_{1-25} peptide.

Compression isotherm of monolayers containing POPC, or DPPC/POPC/DPPG (60/20/20, mol/mol/mol) with 0.4 mol% bovine SP-B, 20 mol% mSP-B_{1-25}, or 20 mol% dSP-B_{1-25}.

surface-associated reservoir, and not as exclusion out of layered structures and into an aqueous subphase. AFM measurements showed that increasing concentrations of bovine SP-B in DPPC/DPPG films resulted in the formation of more protrusions. At SP-B levels of 0.2 - 0.4 mol% the protrusions appeared as small disc-like domains which formed network structures, which is in agreement with results from AFM studies in which porcine SP-B was used [163]. Interestingly, similar SP-B concentrations were found to show optimal activity in vitro, as measured by CBS (0.5 - 0.75 mol%) [57,185], spreading trough (0.2 mol%) [57], and lipid mixing assays (0.2 mol%) [173,218,219]. Moreover, it is comparable to the amount of SP-B reported in bronchoalveolar lavage fluid, ranging from 0.02 mol% [57] to 0.9 mol% [220]. Ultimately, addition of more protein will stop leading to formation of extra protrusions, due to the fact that there is no more lipid available. This probably occurs in films with more than 4 mol% SP-B.

The protrusion height in experiments with films containing DPPC/DPPG (80/20) and a concentration of native SP-B $\geq$ 0.4 mol% was 4.0 - 4.3 nm (Table 1). This is lower than in AFM studies using porcine SP-B in DPPC/DPPG (80/20), in which protrusions with a height of 6 - 7 nm after film compression to a surface tension of 22 mN/m were reported [163]. Although the same lipid mixture and protein were used, and although both in our study and in the study by [163] contact mode AFM in air was used, subtle differences in film compression rate, temperature, brand of microscope and force of scanning may result in differences in measured protrusion height. In another study using contact mode AFM,
Figure 11. AFM topography of DPPC/POPC/DPPG films containing native SP-B, mSP-B$_{1-25}$ or dSP-B$_{1-25}$.
Films were transferred onto mica at $\gamma = 22$ mN/m, containing DPPC/POPC/DPPG (60/20/20, mol/mol/mol) plus 0.4 mol% bovine SP-B (A), 20 mol% mSP-B$_{1-25}$ (B), or 20 mol% dSP-B$_{1-25}$ (C), scanned at $10 \times 10$ μm.

compression of mSP-B$_{1-25}$ in a lipid mixture of DPPG/POPG (3/1), transferred at $\gamma = 18$ mN/m, was found to result in protrusions of 10 - 40 nm, in steps of 5.0 nm [206]. Furthermore, X-ray diffraction determination of bilayer thickness showed heights of 3.7 nm [204] to 4.3 nm [328] for DPPC in fluid liquid crystalline state, and 4.7 nm for DPPC in gel state [328]. Since our observed heights were found to be reproducible along the concentration curve from 0.4 mol% bovine SP-B upwards, we believe that the height of 4 nm represents the dimensions of a protruded bilayer. Since protrusion heights of $\leq 2$ nm were found for SP-B concentrations of $\leq 0.2$ mol%, these protrusions are not made up of bilayers. This means that low amounts of protein relative to lipid can not induce and sustain protrusions of bilayers, but will probably partly reorient the lipids and lift them out of the monolayer. The molecular organization of such protruded material is not clear. We suggest that surfactants used for therapy containing SP-B as the sole protein should contain more than 0.2 mol% of the protein in order to ensure sufficient formation of surface-associated reservoir.
Compression isotherms of DPPC/DPPG monolayers containing the peptides mSP-B$_{1-25}$ and dSP-B$_{1-25}$ (Figs. 2C and D) did not have the pronounced squeeze-out plateau observed for bovine SP-B at $\pi = 40$ mN/m (Fig. 2B), even when they were used at high concentrations, but showed a region of decreased slope comparable to the faint plateau of bovine SP-B observed at $\pi = 25$ mN/m. The absence of a plateau suggests that squeeze-out in SP-B$_{1-25}$ peptide containing films took place in a different way than in films with the native protein. The compressibility of DPPC/DPPG films containing native SP-B (Fig. 2A) was higher than that observed for films containing SP-B$_{1-25}$ peptides (Figs. 2C and D). This may reflect a more pronounced fluidizing effect of full-length SP-B compared to SP-B$_{1-25}$ peptides. Alternatively, it could mean that native SP-B is better able to stack the lipids in multilayers. This observation was confirmed by AFM measurement which showed that much higher SP-B$_{1-25}$ peptide concentrations were needed to form structures similar to those seen with native SP-B. Furthermore, the height of the protrusions was significantly smaller than that obtained with native SP-B, except when high concentrations (8 and 20 mol%) of dimeric peptide were included in the film. From this we conclude that, although SP-B$_{1-25}$ peptides are able to form network structures, they are not as effective in doing so as native SP-B is.

Interestingly, structures observed for films of DPPC/DPPG (80/20) containing mSP-B$_{1-25}$ peptides and those containing dSP-B$_{1-25}$ of roughly the same weight% (i.e. at roughly the same amount of N-termini) were not the same (compare for instance 20 mol% mSP-B$_{1-25}$ (Fig. 6C) with 8 mol% dSP-B$_{1-25}$ (Fig. 7B)). Similar structures for mSP-B$_{1-25}$ and dSP-B$_{1-25}$ were only observed when compared at equimolar peptide concentrations, both in films of DPPC/DPPG and in films of DPPC/POPG. This indicates that dimerization of mSP-B$_{1-25}$ does not result in an increased tendency to form protrusions. However, in DPPC/POPC/DPPG films the dimeric peptide did show a higher tendency to form protrusion networks than the monomeric peptide did (compare Fig. 11B with Fig. 11C). We have no explanation for this observation.

We found that the presence of unsaturated lipid acyl chains resulted in formation of multilayered structures. Multilayers were previously observed in studies using electron microscopy [12,221]. These findings are in accordance with a recent AFM study, using monomeric SP-B$_{1-25}$ in DPPG/POPG (3/1) [206], in which multilayered structures were reported as well. Presumably, unsaturated lipids are able to easily form curved protrusions during surface compression, because of their fluid and flexible character, while mixtures containing only saturated lipids (e.g. DPPC/DPPG) will be tightly packed and resist squeeze-out into protrusions. It has been shown in a number of studies that SP-B specifically interacts with PG [72,219,173,56,222]. The high number of multilayered protrusions found in DPPC/POPG lipid mixtures compared with the single bilayer protrusions found in DPPC/POPC/DPPG suggests that SP-B preferentially interacts with POPG rather than with POPC or DPPG.

It is of interest to consider whether surface activity measured by CBS is correlated with surface topography visualized by AFM. Our AFM observation that a higher concentration of SP-B$_{1-25}$ peptide than of native SP-B was needed for the formation of network structures is in
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In line with a recent CBS study [53]. In that study it was shown that spread DPPC/POPG (80/20) films containing SP-B1-25 peptides were in general less surface active than those containing native human SP-B. This suggests that surface topography measured by AFM can give an indication for surface activity measured by CBS. However, in the same CBS study it was reported that spread DPPC/POPG (80/20) films containing 1 mol% dSP-B1-25 i) had a better surface activity (i.e. lower surface tensions) than spread films containing 2 mol% of mSP-B1-25, and ii) reached equally low minimum surface tensions as spread films containing 0.5 mol% native human SP-B. Those CBS findings can not be readily correlated to the AFM results described in this article, since we found that the appearance of DPPC/POPG films containing 1 mol% dSP-B1-25 (Fig. 9E) was similar to that of films containing 2 mol% mSP-B1-25 (Fig. 9D), but was very different from that of films containing 0.4 mol% native bovine SP-B (Fig. 4D). On the other hand, differences between both SP-B1-25 peptides were seen in DPPC/POPC/DPPG films, since films containing mSP-B1-25 (Fig. 11B) did not have network structures, while those containing dSP-B1-25 did (Fig. 11C). These data suggest that there may not always be a clear correlation between surface topography measured by AFM and surface activity measured by CBS. However, it should be kept in mind that the CBS experiments were performed at 37 °C, while our AFM experiments were carried out at room temperature. This may have led to differences in lipid fluidity between films in the two types of measurements, possibly affecting surfactant activity or topography. Obviously, to make a firmly founded comparison between AFM and CBS results, the experimental conditions should be kept the same.

We conclude i) that proteins are required to form protrusions of material that is squeezed out of the surfactant monolayer upon compression, and that protrusions of bilayer height are formed at a physiologically relevant concentration of 0.4 mol% SP-B, ii) that peptides based on the first 25 amino acids of the N-terminus of SP-B are also able to induce protrusion formation, but only at much higher concentrations, and iii) that determinants for protrusion height are lipid unsaturation as well as lipid headgroup, since in the presence of unsaturated lipid, protrusions of multiples of bilayers are found to be formed most clearly in the presence of POPG. Synthetic SP-B1-25 based surfactants to be used clinically will therefore have to contain, in addition to DPPC, a high concentration of SP-B1-25 peptides as well as unsaturated phospholipids, preferentially unsaturated PG.

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