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ARTICLE

# Surfactant protein C and lung function: new insights into the role of α-helical length and palmitoylation

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**Abstract** Surfactant protein C (SP-C) is known to be essential for lung function and the formation of a surface confined reservoir at the alveolar interface. The structural features relevant for the peptide's extraordinary ability to form extended three-dimensional structures were systematically investigated and are summarized in the present paper. The influence of palmitoylation was studied for full length SP-Cs as well as truncated variants with the N-terminal residues 1–17 and 1–13, respectively. The combined results from film balance measurements, fluorescence microscopy (FLM) and scanning force microscopy (SFM) reveal a fine-tuned balance between the influence of the palmitoyl chains and α-helical length. Native SP-C added to DPPC/DPPG monolayers (molar ratio 80:20) induced the formation of the surface confined reservoir independent of its palmitoylation degree. However, topographic images revealed that only bilayers and not multilayers where formed when the acyl chains were missing. The influence of palmitoylation increased when α-helical length was considerably reduced to 17 or even 13 amino acid residues. In these strongly truncated SP-C peptides palmitoyl chains increased monolayer stability and anchored the peptides in the lipid film. However, no multilayer formation was observed at all for all shortened peptides. The α-helix of SP-C seems to be a prerequisite for the formation of extended three-dimensional structures and obviously has to be able to span a lipid bilayer. Palmitoylation obviously mediates interactions between lipids and/or peptides not only within a protein/lipid film but also between neighbouring layers and induces a stacking of bilayers.

**Keywords** Lung surfactant protein C · Palmitoylation · Helical length · Film balance technique · Fluorescence microscopy · Scanning force microscopy · Langmuir–Blodgett transfer

**Abbreviations**

| | |
|---|---|
| DPPC | 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine |
| DPPG | 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoglycerol |
| Bodipy-PC | 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine |
| SP-C | Surfactant protein C |
| SP-B | Surfactant protein B |
| FLM | Fluorescence light microscopy |
| LB | Langmuir–Blodgett |
| SFM | Scanning force microscopy |
| ESI-MS | Electrospray ionisation mass spectrometry |
| IRRAS | IR reflection-absorption spectroscopy |

## Introduction

Pulmonary surfactant is a lipid/protein mixture lining the alveolar air/water interface and reducing the surface

Dedicated to Prof. K. Arnold on the occasion of his 65th birthday.

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tension to near-zero values. It is essential for the breathing process since it facilitates the work of breathing and prevents alveolar collapse (Schürch et al. 1976). The different components of pulmonary surfactant contribute in different ways to the extraordinary properties of the surfactant film. Phospholipids, especially however saturated ones such as the main lipid component 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), account for the monolayer stability of the surfactant film at high surface pressures during exhalation. Unsaturated phospholipids as well as hydrophobic surfactant proteins SP-B (8.7 kDa) and SP-C (4.2 kDa), which constitute approximately 1% by weight of surfactant, are essential for the dynamic properties of the surfactant film. They considerably enhance the rate of adsorption and rapid spreading of material secreted from type II cells (Oosterlaken-Dijksterhuis et al. 1991a, b; Perez-Gil et al. 1992b).

The described complementary properties of lipids and peptides provided a basis for the squeeze-out hypothesis which proposes the selective exclusion of fluidizing lipids and surfactant proteins during the breathing cycle. The remaining monolayer at the air/water interface is enriched in lipids that promote low surface tension (Bangham et al. 1979; Goerke 1998; Pastrana-Rios et al. 1994). It seems as if during this process multilaminated structures beneath the surfactant monolayer are formed in vivo (Schürch et al. 1998). Similar structures have been detected in surfactant model systems by means of scanning force microscopy (SFM). These studies revealed that SP-B and SP-C are essential for the formation of surface-associated reservoirs built up of lipid/protein multilayers. The topographic characteristics of the formed structures strongly depend on the protein present in the surfactant model system (Amrein et al. 1997; Galla et al. 1998; Krol et al. 2000; von Nahmen et al. 1997b) and the lipid composition (Malcharek et al. 2005).

In the case of SP-C it was shown that stacked bilayers were formed in the plateau region of lipid/protein monolayers, e.g. at high surface pressures. These multilayers mainly consisted of phospholipids and SP-C (Bourdos et al. 2000) and displayed height differences of 5.5–6.6 nm (Amrein et al. 1997; Krol et al. 2000; von Nahmen et al. 1997b). Although it has become clear from these studies that SP-C is necessary for the formation of multilayer structures during compression neither the exact mechanism of this process nor structure/function relationships relevant for the exclusion of lipids and/or proteins are known.

The main objective of this study was to explore the correlation between the grade of palmitoylation as well as α-helical length of SP-C and its functional activity. It has been reported that the palmitoyl groups of SP-C covalently linked to the two cysteine residues in positions 5 and 6 are required for the peptide to be functionally active (Creuwels et al. 1993; Qanbar et al. 1996; Wang et al. 1996). From a structural point of view it has been reported that the acyl chains either increase (Qanbar et al. 1996; Szyperski et al. 1998; Vandenbussche et al. 1992) or decrease (Creuwels et al. 1993; Shiffer et al. 1993) the helical content of the peptide. This discrepancy was independent of the environment used for analysis and might be attributed to different procedures employed to deacylate native SP-C. So far, however, the influence of depalmitoylation or truncation of the C-terminal part on the peptides ability to induce multilayer formation has not been elucidated.

In order to investigate the functional relevance of palmitoylation and the importance of α-helical length different peptides were systematically investigated with the film balance technique, fluorescence microscopy (FLM) and SFM. We here provide data which indicate that a sensitive balance exists between the influence of palmitoylation and the length of the N-terminal part. In full length SP-Cs acylation does not significantly influence the peptides ability to form a surface confined reservoir. However, palmitoylation does influence the topographical features of the observed three-dimensional structures. In extremely shortened peptides only containing the first 17 or 13 N-terminal residues of native SP-C, however, palmitoyl chains contribute considerably to monolayer stability and anchor the peptides to the surface film. Since the α-helices are too short to span bilayers multilayer formation did not occur upon film compression. We therefore conclude that palmitoylation as well as α-helical length of SP-C are important for lung function and that their influence is based on modulating different molecular interactions within the surfactant film.

## Materials and methods

### Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). 2-(4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (ß-BODIPY® 500/510 $C_{12}$-HPC, Bodipy-PC) was obtained from Molecular Probes (Eugene, OR). All lipids were used without further purification. Solvents were HPLC grade and obtained from Merck (Darmstadt, Germany).

Native porcine SP-C (SP-$C_{35+pp}$) was isolated by a method as described previously (Haagsman et al. 1987). A depalmitoylated form of human recombinant SP-C with the sequence GIPCCPVHLKRLLIVVVVVVLI VVVIVGALLMGL (SP-$C_{34-pp}$) was a generous gift from Byk-Gulden Pharmaceuticals (Konstanz, Germany). The identity of both peptides was checked by electrospray ionisation mass spectrometry (ESI-MS). A molecular mass of 4,186.4 and 3,548.7 Da confirmed the given primary structure and palmitoylation state of SP-$C_{35+pp}$ and SP-$C_{34-pp}$, respectively.

SP-C peptides with 17 amino acid residues (with and without palmitoylation at the cysteines 5 and 6) were synthesized as described previously by Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry (Plasencia et al. 2001). The sequence was LRIPCCPVNLKRLLVVV. The identity of the peptides was verified by ESI-MS and gave the expected molecular masses of 2,412.0 and 1,935.5 Da for SP-$C_{17+pp}$ and SP-$C_{17-pp}$, respectively.

Synthetic peptides consisting of the 13 N-terminal residues of SP-$C_{17}$ peptides with and without palmitoylation (SP-$C_{13+pp}$ and SP-$C_{13-pp}$) as well as the deacylated derivative with a L→W substitution at position 1 (SP-$C_{13L1W}$) were kindly provided by J. Perez-Gil, Madrid, Spain. The primary structure of all three peptides was checked with ESI-MS and gave molecular masses of 2,001.5, 1,525.0 and 1,598.0 Da for SP-$C_{13+pp}$, SP-$C_{13-pp}$ and SP-$C_{13L1W}$, respectively. All peptides were dissolved in chloroform/methanol (1:1, v/v).

### Film balance technique

Film balance experiments were performed on an analytical Wilhelmy-film balance (Riegler and Kirstein, Mainz, Germany) with an operational area of 144 cm². All surface pressure/area measurements were performed on a pure water subphase (purified with a Milli-$Q_{185}$ Plus system, Millipore GmbH, Eschborn, Germany) at a temperature of 20°C. Lipid/peptide monolayers were composed of DPPC and DPPG in a molar ratio of 80:20 and were supplemented with various concentrations of different types of SP-C. The lipid/protein mixtures were prepared in a chloroform/methanol solution (1:1, v/v) and spread onto the subphase. After 10–15 min of equilibration time the monolayers were compressed at a rate of 5.81 $cm^2$/min.

### Fluorescence light microscopy

As described previously (von Nahmen et al. 1997a) a setup consisting of an epifluorescence microscope (Olympus STM5-MJS, Olympus, Hamburg, Germany) equipped with a *xy*-stage and connected to a CCD camera (Hamamatsu, Herrsching, Germany) was used to obtain fluorescence micrographs of lipid/peptide mixtures at the air/water interface. All lipid/peptide systems were doped with 0.2 mol% Bodipy-PC. The measurements were performed at a temperature of 20°C on pure water.

### Langmuir–Blodgett (LB) transfer

For SFM investigations mica-supported phospholipid monolayers were prepared by Langmuir–Blodgett transfer. First, a freshly cleaved mica sheet (Electron Microscopy Science, Munich, Germany) was dipped into the subphase. Then lipid/peptide mixtures were spread from chloroform/methanol (1:1, v/v) solutions onto a pure water subphase of a Wilhelmy-film balance (Riegler and Kirstein, Mainz, Germany) with an operational area of 39 cm² at a temperature of 20°C. After an equilibration period of 10 min the film was compressed with a velocity of 1.79 $cm^2$/min until a surface pressure of 50–55 mN/m was reached. The monolayer was equilibrated for another 15 min at this surface pressure before transferring the film onto the mica sheet with a velocity of 0.7 mm/min.

### Scanning force microscopy

Surface images of the LB films were obtained at ambient conditions (20°C) using a Dimension 3000 scanning force microscope with a Nanoscope IIIa controller from Digital Instruments (Santa Barbara, CA, USA) operating in tapping mode. Silicon NanoProbe tips (BS-Tap 300, Nanoscience Instruments Inc., Phoenix, AZ, USA) with a spring constant of 40 N/m and a resonance frequency of 250–300 kHz were used for these investigations. The ratio of setpoint amplitude $A_{sp}$ to amplitude of vibration $r$ was set to 0.4–0.7 (moderate tapping).

## Results and discussion

### Phase behavior of pure peptide monolayers at the air/water interface

Film balance measurements with different SP-C variants were performed in order to understand the processes taking place within a pure protein monolayer. It was our aim to further study the influence of the palmitoyl chains on the surface properties of extremely truncated SP-C analogues with only 17 and 13 amino acid residues, respectively. Furthermore, a SP-C variant with only 13 amino acids and a substitution in the first position

(leucine to tryptophan) was studied (SP-$C_{13L1W}$) in order to investigate the influence of amino acid polarity.

First, the full length peptide, that is native dipalmitoylated SP-C, with 35 amino acids (SP-$C_{35+pp}$) and its unpalmitoylated counterpart (SP-$C_{34-pp}$) were studied. The isotherm of pure SP-$C_{35+pp}$ displays characteristic plateau regions at 25 and 33 mN/m, respectively (Fig. 1a). A collapse of the peptide monolayer is observed at a surface pressure of approximately 35 mN/m. These features display the general ability of SP-C to form monolayers at the air/water interface and result from its strong amphipathic character as has been reported previously (Post et al. 1995). The secondary structure of the peptide at the interface is highly α-helical (58–66%) as was determined with CD measurements (Creuwels et al. 1993). Interestingly, the α-helix content strongly depends on the surface pressure with SP-C molecules being more ordered at high surface pressure.

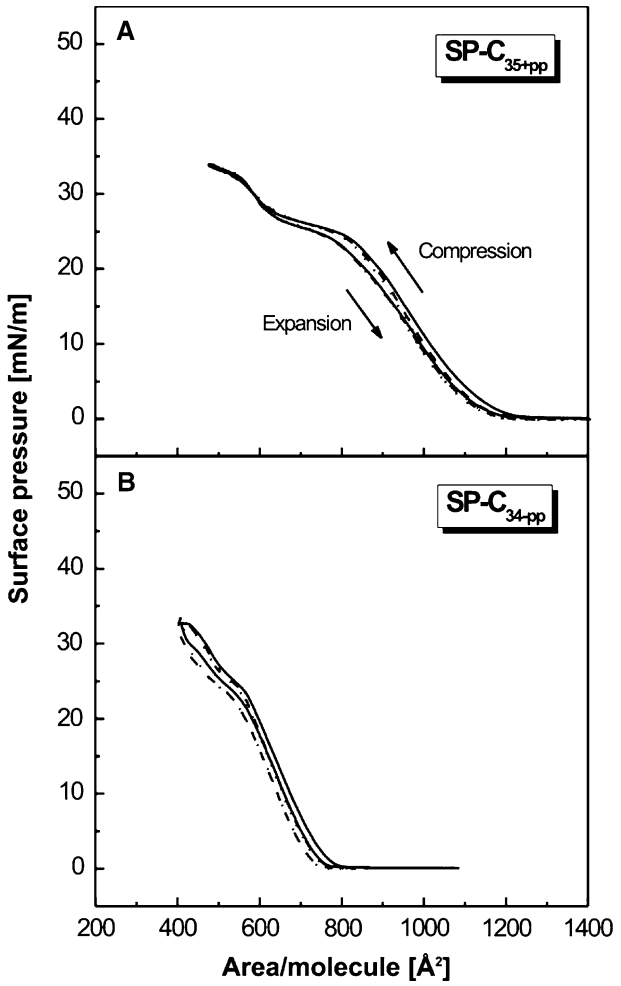


**Fig. 1** Pressure/area isotherms of native SP-$C_{35+pp}$ (**a**) and depalmitoylated SP-$C_{34-pp}$ (**b**). Three compression and expansion cycles were performed on pure water at 20°C: cycle 1 (*solid line*), cycle 2 (*dashed line*) and cycle 3 (*dotted line*)

In order to study the stability of the spread protein films consecutive compression/expansion isotherms were performed. The monolayer was compressed up to a surface pressure of 34 mN/m and then immediately expanded by inverting the direction of the barrier. As can be seen in Fig. 1a the compression and expansion isotherms are highly superimposable. We therefore conclude that there is no substantial material loss into the subphase for monolayers composed of native SP-C and that highly reversible processes occur within the monolayer.

Pressure/area isotherms of SP-C monolayers have been described in the literature and revealed similar phase behavior. Only the molecular areas of the plateau regions differed slightly. These differences can generally be attributed to differences in subphase composition (Perez-Gil et al. 1992a) or temperature (Oosterlaken-Dijksterhuis et al. 1991b) as well as possible discrepancies in the purification procedure.

The processes leading to the formation of the observed plateaus are not yet clear. Ellipsometric measurements revealed that the thickness of the layer almost doubles from the beginning to the end of the first plateau from 17.5 to 32.5 Å (Post et al. 1995). Further increasing the surface pressure lead to a continuous increase in layer thickness. This behavior is explicable by the formation of multiple layers as has already been described for α-helical peptides (Malcolm 1973) and/or by changes in protein conformation e.g. by increasing the amount of α-helical structures. It is also possible that the fatty acids contribute to the overall phase behavior of the peptide. However, since the plateau found at 25 mN/m is temperature independent (Creuwels et al. 1993) it is assumed that it is not caused by liquid to gel state transitions of the palmitoyl chains. Instead, it is possible that during compression the fatty acid chains get in direct contact to the hydrophobic parts of the protein and influence intermolecular interactions. The exact processes taking place in pure SP-C monolayers, however, are still not identified and remain to be elucidated.

When the palmitoyl chains are missing in the SP-C peptide different pressure/area isotherms are obtained (Fig. 1b). SP-$C_{34-pp}$ has a limiting area, which is significantly lower than the one for SP-$C_{35+pp}$ (800 $Å^2$ instead of 1,200 $Å^2$). First it has to be considered that the palmitoyl chains themselves may occupy some of the surface area for the +pp peptide explaining why this isotherm (Fig. 1a) is more expanded. Second the orientation of the two peptides may differ explaining some of the differences in the isotherms (Creuwels et al. 1993). Secondary structure differences do not seem to

be relevant since deacylation obviously does not significantly influence the α-helix content in a pure peptide monolayer (Creuwels et al. 1993). Finally the amino acid sequence differences between SP-$C_{35+pp}$ isolated from porcine lung and the unpalmitoylated SP-$C_{34-pp}$ being a recombinant human analogue has to be taken into account. The interesting and most important feature revealed by these measurements, however, is that SP-$C_{34-pp}$ generally displays similar phase behavior as its palmitoylated analogue despite the sequence differences. The isotherm of pure SP-$C_{34-pp}$ shows a plateau at a surface pressure of 24 mN/m and a shoulder at 34 mN/m. The collapse pressure is at around 35 mN/m and no significant hysteresis is observed. We therefore conclude that the removal of the palmitoyl chains does not significantly influence the general phase behavior of full length SP-C. The typical properties of these peptides seem to mainly result from the long hydrophobic α-helix.

When SP-C variants with only 17 amino acid residues were studied a prominent shift of the SP-$C_{17+pp}$ as well as SP-$C_{17-pp}$ isotherms to significantly smaller molecular areas was observed (Fig. 2). It is known from CD studies in lipid micelles that the α-helix content of truncated SP-$C_{17-pp}$ is approximately 40% and the onset of helical structure is at nearly identical sequence location as in native SP-C (Johansson et al. 1995). IRRAS studies of the palmitoylated form also confirm the existence of the helical structure (R. Mendelsohn, unpublished data) reason why we attribute the significant shift of the SP-$C_{17}$ isotherms to a considerate shortening of the α-helix and a reduction of the peptide area requirement.

Furthermore, palmitoylated SP-$C_{17+pp}$ only shows one plateau at a significantly lower surface pressure of 20 mN/m compared to the plateaus between 25 and 33 mN/m for SP-$C_{35+pp}$. The most interesting feature, however, is that palmitoylated SP-$C_{17+pp}$ has a significantly higher collapse pressure than full length SP-Cs or the non-palmitoylated variant (Fig. 2a). This striking difference can only be attributed to the acyl chains which seem to stabilize the peptide monolayer and might explain the lipid-like behavior. When successive compression/expansion cycles were performed, the isotherm of SP-$C_{17+pp}$ did not show any material loss during these cycles whereas the unpalmitoylated analogue was squeezed out with every new compression (Fig. 2b). These findings lead us to the assumption that the palmitoyl chains act as adhesive modules in the peptide stabilizing the monolayer. Most important, the acyl moieties seem to be pivotal for the prevention of an irreversible squeeze-out of the SP-C variants with only 17 residues.

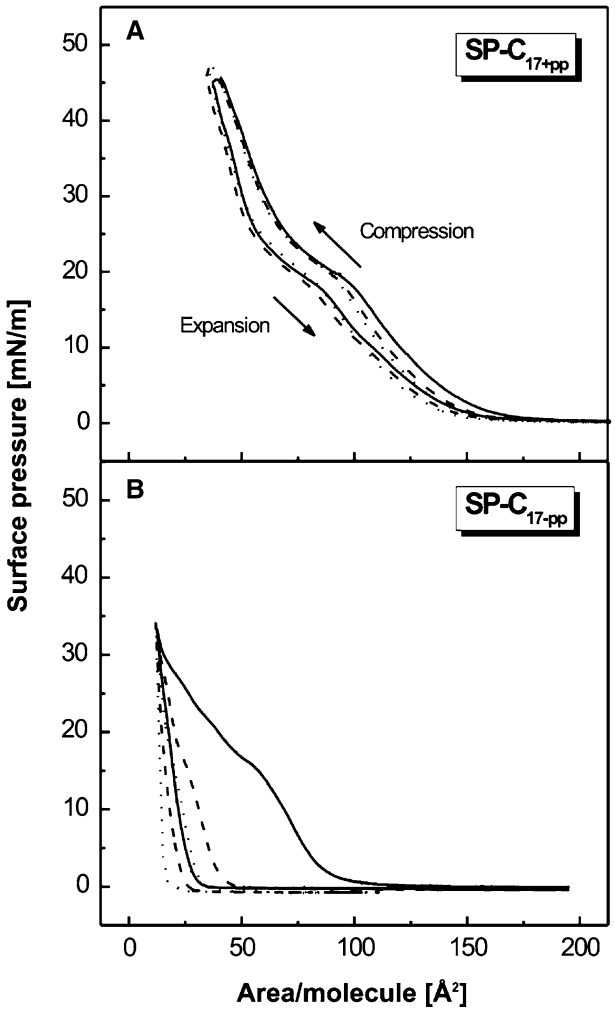


**Fig. 2** Pressure/area isotherms of truncated peptides with 17 amino acid residues: SP-$C_{17+pp}$ (**a**) and depalmitoylated SP-$C_{17-pp}$ (**b**). Three compression and expansion cycles were performed on pure water at 20°C: cycle 1 (*solid line*), cycle 2 (*dashed line*) and cycle 3 (*dotted line*)

A similar tendency was observed for peptides with only 13 residues. Whereas the palmitoylated variant could be compressed to surface pressures of around 50 mN/m, the unpalmitoylated peptides SP-$C_{13-pp}$ and SP-$C_{13L1W}$ already collapsed at 32 and 38 mN/m, respectively (Fig. 3). Similar results have been obtained on a buffered subphase and were attributed to less stable monolayes of deacylated peptides (Bi et al. 2002). Interestingly, palmitoylation of these peptides did not significantly change secondary structure. They all still formed a very short helix with 1.5 turns (Bi et al. 2002). However, palmitoylation had an influence on the location of the helical part which could be embedded either in a more hydrophobic or hydrophilic environment.

In contrast to SP-C peptides with 17 amino acids, however, palmitoylation could not completely prevent irreversible material loss into the subphase upon

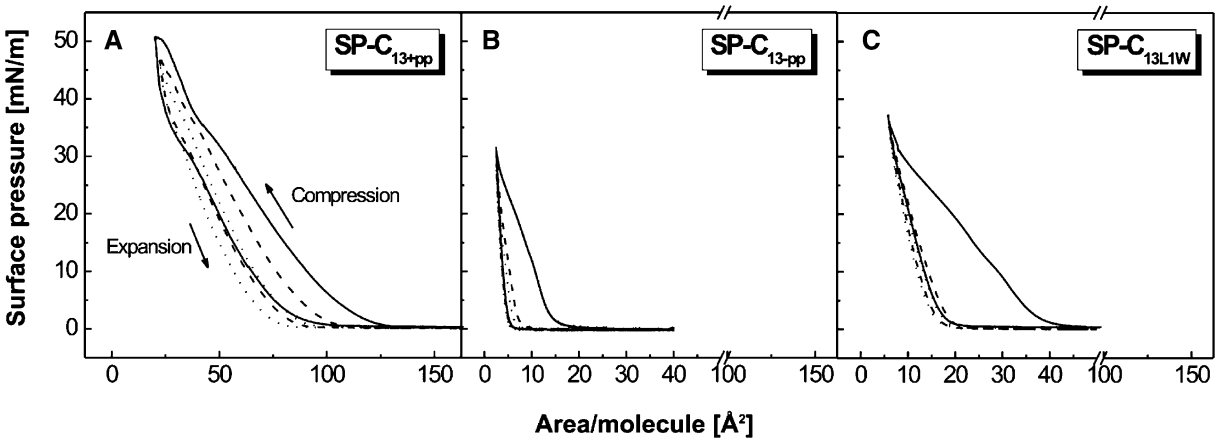


**Fig. 3** Pressure/area isotherms of truncated peptides with 13 amino acid residues: SP-$C_{13+pp}$ (**a**), depalmitoylated SP-$C_{13-pp}$ (**b**) and the deacylated derivative SP-$C_{13L1W}$ (**c**). Three compression and expansion cycles were performed on pure water at 20°C: cycle 1 (*solid line*), cycle 2 (*dashed line*) and cycle 3 (*dotted line*)

successive compression/expansion cycles (Fig. 3a). This material loss was more pronounced in the case of SP-$C_{13-pp}$ and SP-$C_{13L1W}$, therefore the barrier was expanded manually when reaching a molecular area of 5 Å$^2$ (Fig. 3b, c). Interestingly, the isotherm of SP-$C_{13L1W}$ is shifted to larger molecular areas by 15 Å$^2$ and displays a higher compressibility in the first cycle than SP-$C_{13-pp}$ (Fig. 3c). These differences might be due to a different location of specific amino acid residues with respect to the air/water interface and the area demanding ring of the tryptophan.

It becomes apparent from these sets of experiments that the influence of the palmitoyl chains increases when the α-helical part of the peptide is shortened. In the case of peptides containing 17 amino acids the monolayer is significantly stabilized by the acyl chains. Hydrophobic interactions between the acyl chains and the α-helix seem to be essential for monolayer stability since peptides containing only 13 amino acids displayed irreversible material loss upon repetitive compressions. SP-$C_{17}$ peptides still possess a significant α-helical part as was shown from NMR data and CD spectra of depalmitoylated SP-$C_{17-pp}$ in lipid micelles (Johansson et al. 1995). A peptide containing 13 amino acid residues, however, forms a considerably shortened helix (Bi et al. 2002) and thus does not possess enough hydrophobic amino acid residues which could properly interact with the palmitoyl chains.

## Phase behavior of lipid/peptide mixtures at the air/water interface

Ternary mixtures of native SP-$C_{35+pp}$, DPPC and DPPG have proven to be an adequate lung surfactant model system and have therefore been extensively studied in the past with powerful monolayer tools such as the film balance technique and, in particular, epifluorescence microscopy (Krol et al. 2000; Nag et al. 1996; Post et al. 1995; Taneva and Keough 1994; von Nahmen et al. 1997a) as well as infrared spectroscopy (IRRAS) (Wang et al. 2005). Some of the most prominent features of this model system shall be summarized here. They serve as a reference for the subsequent described results obtained for the truncated SP-C variants.

One of the most striking characteristics of DPPC/DPPG/SP-$C_{35+pp}$ monolayers with a molar ratio of 80:20:0.4 is the occurrence of a pronounced plateau at a surface pressure of 50–55 mN/m accompanied by a reappearance of fluorescence intensity (Fig. 4a). The extraordinary region of increased compressibility found at 50–55 mN/m is generally attributed to the formation of a surface-confined reservoir. It is assumed that SP-C triggers the generation of three-dimensional multilayer structures enriched with SP-C and specific lipids which was confirmed by detailed quantification of the FLM intensity profiles and scanning force microscopy (Krol et al. 2000; Post et al. 1995; von Nahmen et al. 1997a; von Nahmen et al. 1997b).

Although the topographical features of these stacked multilayers have been extensively studied by means of SFM the relevance of the amphipathic α-helix or the hydrophobic palmitoyl chains for the protrusion formation has remained unclear. With the approach described in this study we aim to reach a more refined understanding of the structure/function relationship decisive for the generation of the lipid/protein reservoir.

When DPPC/DPPG mixtures with the depalmitoylated variant SP-$C_{34-pp}$ were studied with the film balance technique the SP-C specific plateau at 55 mN/m was not visible until 1.5 mol% of the peptide were

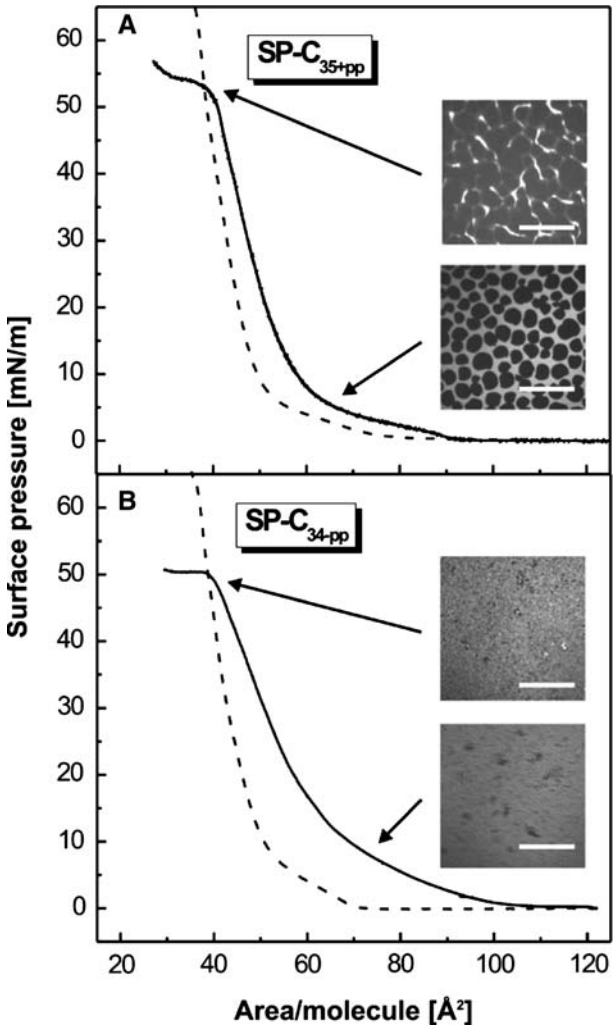


**Fig. 4** **a** Pressure/area isotherms of DPPC/DPPG (80:20, mol/mol) monolayers in the absence (*dashed line*) and presence of 0.4 mol% native SP-$C_{35+pp}$ (*solid line*). **b** Pressure/area isotherms of DPPC/DPPG (80:20, mol/mol) monolayers in the presence of 0.4 mol% (*dashed line*) and 5 mol% depalmitoylated SP-$C_{34-pp}$ (*solid line*). The *insets* show fluorescence images of lipid monolayers doped with 0.2 mol% Bodipy-PC and containing 0.4 mol% SP-$C_{35+pp}$ (**a**) and 5 mol% SP-$C_{34-pp}$ (**b**), respectively, at 5 mN/m and in the plateau region. The *scale bar* is 50 μm. All measurements were performed on pure water at 20°C

present in the monolayer. SP-$C_{34-pp}$ concentrations of 5 mol% were necessary to obtain a plateau similar to that of palmitoylated SP-$C_{35+pp}$ (Fig. 4b). It is not excluded that amino acid sequence differences lead to these concentration differences. However, and that is the interesting point, independent of the amino acid sequence the typical SP-C plateau appears in the absence of the palmitoyl chains. The fluorescence images, however, differed considerably from the ones of lipid monolayers containing SP-$C_{35+pp}$. These differences mainly result from the high amount of SP-$C_{34-pp}$ present in the monolayer leading to a considerable increase in fluidity.

In order to study the influence of palmitoylation in considerably truncated SP-C molecules peptides with 17 residues were investigated with the film balance technique and fluorescence microscopy. As expected, addition of SP-$C_{17+pp}$ or the depalmitoylated variant SP-$C_{17-pp}$ to the binary lipid mixture of DPPC/DPPG 80:20 (mol/mol) even up to a peptide content of 10 mol% did not lead to the appearance of the plateau characteristic for full length SP-C (Fig. 5a, b). At lower peptide concentrations the lipid/peptide mixtures displayed isotherms similar to that of pure lipid films. However, in the presence of 5 (or more) mol% SP-$C_{17+pp}$ or SP-$C_{17-pp}$ a noticeable plateau appeared at 29

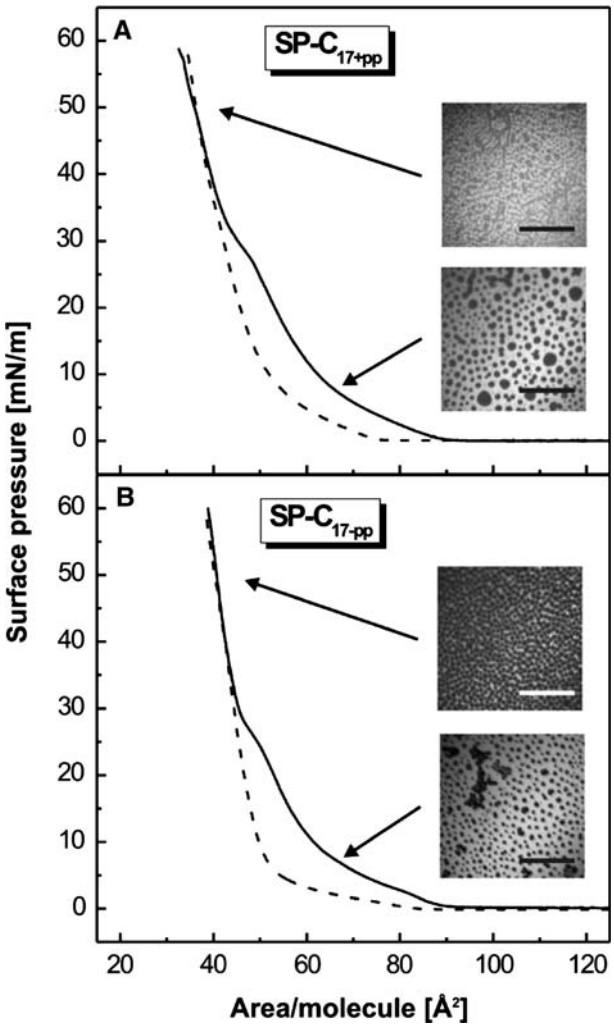


**Fig. 5** Pressure/area isotherms of DPPC/DPPG (80:20, mol/mol) monolayers containing SP-$C_{17+pp}$ (**a**) and SP-$C_{17-pp}$ (**b**). The peptide concentrations were 0.4 mol% (*dashed line*) and 10 mol% (*solid line*), respectively. The insets show fluorescence images of lipid monolayers doped with 0.2 mol% Bodipy-PC and containing 10 mol% of the SP-$C_{17}$ peptides obtained at 5 and 50 mN/m. The *scale bar* is 50 μm. All measurements were performed on pure water at 20°C

and 25 mN/m, respectively. It is unlikely that this plateau can be attributed to the formation of three-dimensional multilayers at the air/water interface. First of all, the isotherms obtained for SP-$C_{17+pp}$ or SP-$C_{17-pp}$ monolayers with different amounts of peptides coincide at surface pressures higher than the plateau pressure, e.g. at 30 mN/m. This is a clear indicator for material loss during compression. Second, the FLM images obtained for the lipid films containing either SP-$C_{17+pp}$ or SP-$C_{17-pp}$ did not display any fluorescence intensity increase in the plateau region around the rigid domains as was observed for native SP-C (von Nahmen et al. 1997a). In the case of palmitoylated SP-$C_{17+pp}$ dark liquid condensed (lc) domains with a diameter of 5–15 μm were visible at a surface pressure of 5 mN/m. These domains continuously decreased upon compression of the monolayer (Fig. 5a). In the case of unpalmitoylated SP-$C_{17-pp}$ the lc domains found at 5 mN/m were significantly smaller (2–8 μm) (Fig. 5b). Third, truncated SP-C peptides containing only 17 amino acid residues contain a considerably shortened α-helix with a length of approximately 13 Å and a hydrophobic part of 5 Å. These values were calculated taking into account that SP-$C_{17-pp}$ had a similar onset of helical structure as native SP-C (Johansson et al. 1995). Truncated SP-C molecules with only 17 amino acid residues will never be able to span a lipid double layer and thus stabilize a surface-confined surfactant reservoir. We therefore attribute the observed plateau to a mere exclusion of material from the monolayer.

Most strikingly, this substance loss from the monolayer is different for the two truncated SP-C analogues. In the case of SP-$C_{17+pp}$ the compressibility at surface pressures higher than 29 mN/m is larger than in pure DPPC/DPPG mixtures (Fig. 5a). Thus we assume that not only peptide but also lipid molecules are squeezed out of the monolayer. In the case of the depalmitoylated variant SP-$C_{17-pp}$ the isotherm slopes are identical to the ones found for pure lipid monolayers (Fig. 5b). This is a sign for a pure exclusion of peptide without a simultaneous loss of lipids. Such a behavior could be explained by palmitoyl chains acting as an anchor attaching the N-terminal region to the lipid layer by mediating hydrophobic interactions (Johansson et al. 1998).

Finally, mixtures of DPPC/DPPG with peptides containing only 13 amino acid residues were studied. In addition to the palmitoylated and unpalmitoylated variants a peptide containing a tryptophan instead of a leucine in the first position was analyzed. In all three cases monolayers containing peptide concentrations up to 10 mol% did not show any SP-C specific plateau at surface pressures above 50 mN/m (Fig. 6). Instead a faint plateau became apparent at 10 mN/m. As was discussed above for SP-$C_{17}$ peptides this plateau is most probably due to a squeeze-out of peptide. Similar assumptions have already been made for SP-$C_{13}$/lipid mixtures studied on a buffered subphase. Monolayers containing DPPC and SP-$C_{13+pp}$ revealed a plateau at 30–35 mN/m which was attributed to a portion of the α-helical region of the peptide being squeezed out of the hydrophobic areas (Bi et al. 2002). A pressure driven squeeze-out was also postulated for depalmitoylated SP-$C_{13}$ (Plasencia et al. 2001). Differences in plateau height are probably due to different subphase and monolayer compositions.

Since material loss in lipid/SP-$C_{13}$ mixtures occurs at lower surface pressures than in the case of peptides with 17 amino acid residues we conclude that monolayers containing SP-$C_{13}$ peptides are less stable. This is

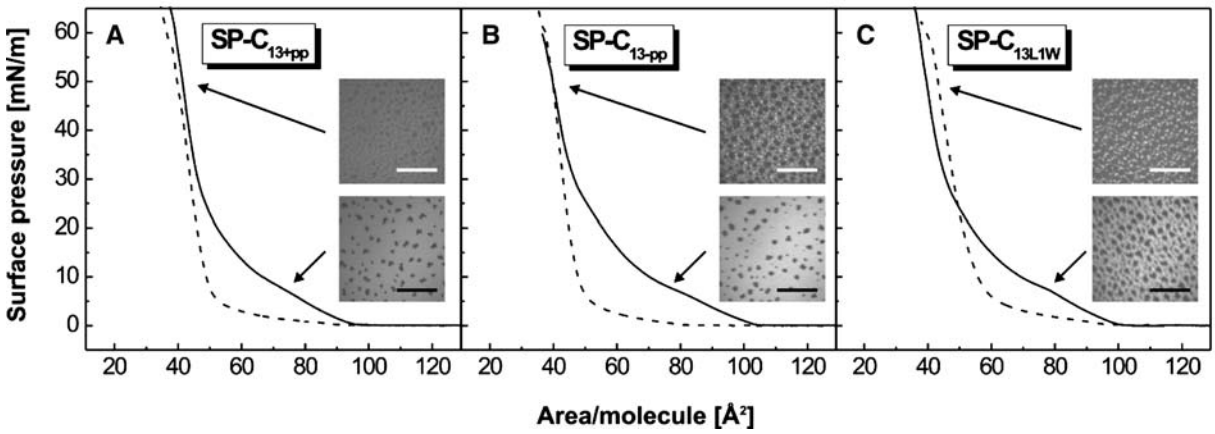


**Fig. 6** Pressure/area isotherms of DPPC/DPPG (80:20, mol/mol) monolayers containing SP-$C_{13+pp}$ (**a**), SP-$C_{13-pp}$ (**b**) and SP-$C_{13L1W}$ (**c**). The peptide concentrations were 0.4 mol% (*dashed line*) and 10 mol% (*solid line*), respectively. The *insets* show fluorescence images of lipid monolayers doped with 0.2 mol% Bodipy-PC and containing 10 mol% of the SP-$C_{13}$ peptides obtained at 5 and 55 mN/m. The *scale bar* is 50 μm. All measurements were performed on pure water at 20°C

probably due to the fact that these extremely truncated peptides do not form extended helical structures anymore. Specific hydrophobic interactions between peptides and lipid acyl chain moieties stabilizing the monolayer are therefore missing.

The observed isotherms displayed similar monolayer properties and were surprisingly shifted to slightly larger molecular areas at low surface pressures than the ones of films containing peptides with 17 amino acids. Reason for this seems to be a highly fluidizing effect of the different SP-$C_{13}$ peptides as can be seen from the fluorescence images obtained at 5 mN/m (Fig. 6a–c). This influence seems to be strongest for SP-$C_{13+pp}$ and SP-$C_{13-pp}$. The peptide SP-$C_{13L1W}$ containing the substituted tryptophan, however, was less effective, probably because of hydrophobic interactions mediated by the aromatic ring which could lead to the formation of peptide aggregates within the protein/lipid monolayer.

## Scanning force microscopy of lipid/peptide mixtures

Scanning force microscopy is a powerful technique to investigate the three dimensional structures formed during compression of surfactant model systems at higher surface pressures. In this study lipid/peptide monolayers compressed to surface pressures of approximately 50 mN/m were transferred onto mica sheets and thus fixated in their momentary physical status. Scanning the surface with a cantilever delivers reliable topographic data of surfactant model systems as was shown by our group and others in the past (Amrein et al. 1997; Bernardino de la Serna et al. 2004; Diemel et al. 2002; Ding et al. 2001; Flanders et al. 2001; Krol et al. 2000; Takamoto et al. 2001; von Nahmen et al. 1997b). The formation of a surfactant confined reservoir is generally assumed to also occur in the physiological lung system and to be pivotal for proper lung function (Schürch et al. 1995, 1998; von Nahmen et al. 1997b).

The multilayer structures for native SP-C have been discussed in the literature in detail (Krol et al. 2000; von Nahmen et al. 1997b). However, for a better interpretation of the topographic images obtained for the modified SP-C peptides SFM pictures of the full length protein are presented here as a reference. Typical for DPPC/DPPG/SP-C monolayers transferred onto mica in the plateau region at around 50 mN/m are large, polygonal patches surrounded by a rim of protrusions which can be attributed to multilayers (Fig. 7a). The protrusions have distinct steps of ~6 nm or multiples of 6 nm (see height profile in Fig. 7a) as has already been reported (Amrein et al. 1997; Galla et al. 1998; von Nahmen et al. 1997b).

Depalmitoylation had a significant effect on the multilayer characteristics (Fig. 7b). Although similar polygonal domains within a distinct SP-$C_{34-pp}$/lipid network were observable the height of the multilayer structures did not exceed a value of 12–14 nm (Fig. 7b). In the case of native SP-C the observed protrusions reached considerable heights up to 50 nm. Since the general phase behavior of SP-$C_{34-pp}$ so far corresponded well to that of native SP-$C_{35+pp}$ we believe that the observed height differences are not due to amino acid sequence differences.

It is generally assumed that palmitoylation enhances protein/membrane interactions (Curstedt et al. 1990) by increasing the hydrophobicity of the protein (Flach et al. 1999). Furthermore, the palmitoyl chains are thought to function as an anchoring device (Johansson et al. 1998). Upon monolayer compression the acyl chains would anchor the peptides to one lipid layer while the helical part would be forced to incorporate into the adjacent double layer performing a hinge like movement. Consequently, the lack of acyl chains in SP-C might not only lead to a reduction in lipid/protein interactions but also to a loss of its ability to connect proximate lipid layers. We therefore conclude from the SFM studies that SP-$C_{34-pp}$ induces the formation of bilayers upon compres-

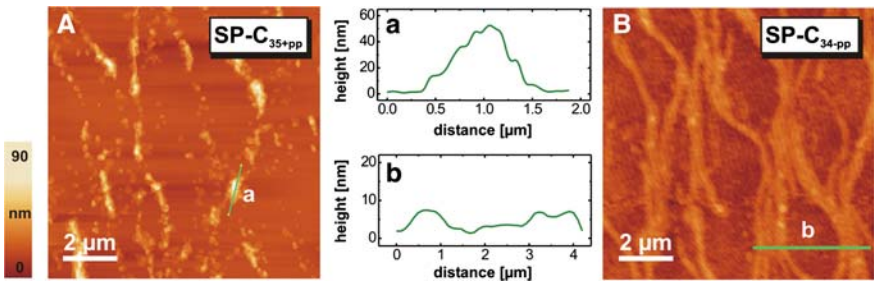


**Fig. 7** SFM topography images of DPPC/DPPG (80:20, mol/mol) monolayers containing 0.4 mol% native SP-$C_{35+pp}$ (**a**) and 5 mol% depalmitoylated SP-$C_{34-pp}$ (**b**). The lipid/peptide films were transferred onto mica from a pure water subphase at 20°C and a surface pressure of 50 mN/m. The height profiles *a* and *b* depict the height differences along the lines shown in **a** and **b**, respectively

sion since its helical part is still long enough to span a lipid double layer. However, as a result of depalmitoylation, it cannot form stacks of bilayers anymore, reason why the observed protrusions only reach a maximum height of 12–14 nm. This assumption would be consistent with a model discussed in the literature proposing that acylation of SP-C may influence the spreading capabilities of a surface-associated surfactant reservoir, but not the structure of either lipids or SP-C in the monolayer at higher surface pressures (Flach et al. 1999).

When truncated SP-C peptides with only 17 amino acid residues where studied with SFM no SP-C characteristic multilayer structures were observed (Fig. 8). These results correspond well with the lack of the typical SP-C plateau in lipid/peptide isotherms and the fluorescence images at higher surface pressures. Only little knobs of 12–16 nm height and a diameter of around 200 nm were observable for palmitoylated as well as non-palmitoylated SP-$C_{17}$. However, one interesting feature concerning the influence of the acyl chains in these shortened peptides becomes apparent from the SFM images. In the case of SP-$C_{17+pp}$ the increase in peptide concentration did not lead to an increase in the amount of knobs (Fig. 8a, b). However, in LB films containing the depalmitoylated peptide increasing the peptide concentration up to 10 mol% lead to a larger amount of protrusions (Fig. 8c, d). These results indicate that SP-$C_{17+pp}$ is able to form protein aggregates whereas its non-palmitoylated analogue distributes homogeneously in the lipid layer. Interestingly, the observed knobs seemed to be located in a delicate network-like structure implying that the truncated peptides are also excluded from the lc phase of lipids during compression and thus are accumulated at the phase boundaries. In the case of the non-aggregating SP-$C_{17-pp}$ this network was visible at higher peptide concentrations.

As is obvious from these SFM measurements SP-$C_{17}$ peptides still possess a certain capability to form surface attached structures although they are not able to span a complete bilayer anymore. From the results obtained so far for these peptides we assume that the observed knobs mainly consist of peptide and perhaps small amounts of lipids forming micellar-like structures at the air/water interface upon compression. The α-helix is obviously too short to adapt to the hydrophobic core of the lipids and to trigger the formation of bilayer structures into which the helix can be incorporated.

When SP-C model peptides with an α-helical length of 13 amino acid residues were studied with SFM a considerable amount of holes and distinct knobs was observed at concentrations of 0.4 mol% (Fig. 9a, c, e). We found such holes as well as knobs in peptide-free monolayers of DPPC and DPPG at surface pressures above 55 mN/m. Increasing the surface pressure at which the LB transfer was performed more holes were identified in the solid supported membrane (data not shown). Such behavior has already been reported in the literature for pure DPPC monolayers at a surface pressure near the film collapse pressure (Schief et al. 2000). The observed holes were attributed to packing defects created where adjacent domain edges with conflicting molecular orientations grow together during the le to lc phase transition. These defects seem to serve as a source of precollapse instabilities, e.g. as regions where material is excluded at surface pressures far below the collapse pressure.

It is interesting to note that the nature of the peptide integrated into the monolayer influences appearance and amount of holes present in the LB film. In the case

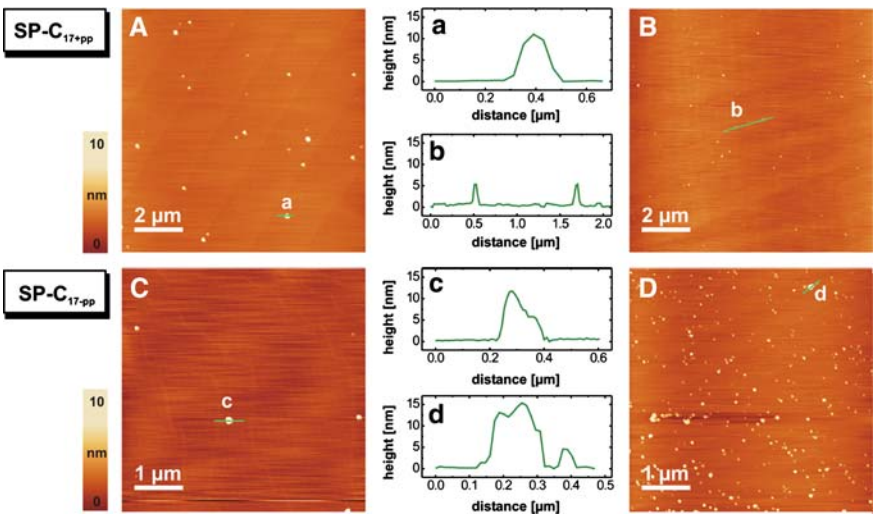


**Fig. 8** SFM topography images of DPPC/DPPG (80:20, mol/mol) monolayers containing SP-$C_{17+pp}$ (**a** 0.4 mol%, **b** 10 mol%) and depalmitoylated SP-$C_{17-pp}$ (**c** 0.4 mol%, **d** 10 mol%). The lipid/peptide films were transferred onto mica from a pure water subphase at 20°C and a surface pressure of 50 mN/m. The height profiles (*a–d*) depict the height differences along the *lines* shown in the topography images (**a–d**)

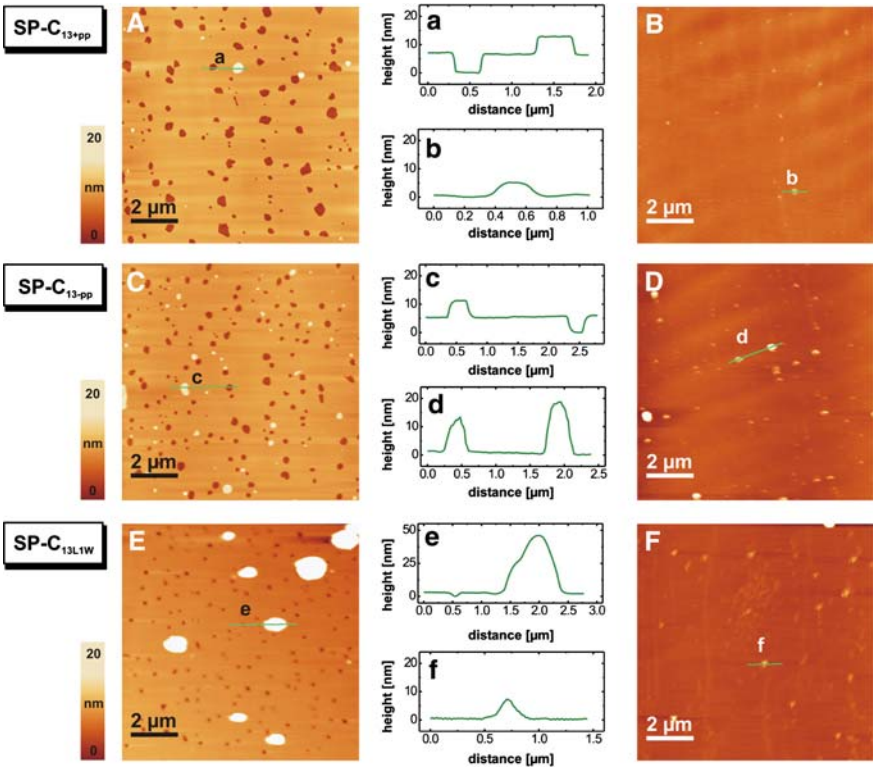


**Fig. 9** SFM topography images of DPPC/DPPG (80:20, mol/mol) monolayers containing SP-$C_{13+pp}$ (**a** 0.4 mol%, **b** 10 mol%), depalmitoylated SP-$C_{13-pp}$ (**c** 0.4 mol%, **d** 10 mol%) and the deacylated derivative SP-$C_{13L1W}$ (**e** 0.4 mol%, **f** 10 mol%). The lipid/peptide films were transferred onto mica from a pure water subphase at 20°C and a surface pressure of 60 mN/m. The height profiles (*a–f*) depict the height differences along the *lines* shown in the topography images (**a–f**)

of depalmitoylated SP-$C_{13-pp}$ (Fig. 9c) the diameter and amount of holes and knobs corresponded to the ones found in the pure lipid layer. These results indicate that the knobs found here probably only consist of lipids and the peptide was irreversibly lost during the transfer. LB films containing the palmitoylated variant SP-$C_{13+pp}$, however, revealed larger holes indicating that more lipids had been extracted from the monolayer. This could be due to the acyl chains of the peptide interacting with the lipids and thus pulling them out of the membrane. However, when the SP-$C_{13L1W}$ mutant was studied clearly smaller holes and significantly larger knobs (diameter: 1 µm, height: 40 nm) were observed (Fig. 9e). We assume that this peptide with its aromatic tryptophan exhibits different intermolecular interactions with lipids leading to excluded protein/lipid aggregates strongly attached to the monolayer.

Increasing the concentration of all three peptides to 10 mol% lead to a disappearance of the holes and to similar topographic characteristics (Fig. 9b, d, f). Only little knobs with heights between 5 and 20 nm were visible and their amount was independent of the peptide used. It is obvious that all peptide molecules have been squeezed-out of the monolayer at the transfer surface pressure of 60 mN/m as was already deduced from film balance measurements. As a result of the LB transfer the excluded material did not stay attached to the monolayer due to a lack of the hydrophobic α-helix and/or missing acyl chains. Since the peptides acted as defects in the monolayer they were excluded from the monolayer instead of the more tightly packed lipids. Therefore an intact monolayer was observable in the SFM images.

## Conclusions

The results presented here reveal that full length SP-C peptides are able to form a surface confined reservoir independent of their palmitoylation state. However, the height and topography of the observed multilayers considerably depends on the existence of the palmitoyl chains since higher structures were detected in the case of palmitoylated SP-$C_{35+pp}$ compared to SP-$C_{35-pp}$. When the α-helical length of the peptides was extremely shortened and only 17 or less amino acids were present no multilayers were detected. Palmitoylation did not influence the topography of the lipid/peptide films but only the aggregation state of the peptide and the stability of the monolayer. The acyl chains, however, seem to act as adhesive moieties modulating interactions between lipids and/or peptides and connecting neighboring layers. Their influence increases with decreasing α-helical length which stresses the importance of hydrophobic interactions mediated by the palmitoyl chains.

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