The Surfactant Peptide KL4 in Lipid Monolayers

PHASE BEHAVIOR, TOPOGRAPHY, AND CHEMICAL DISTRIBUTION

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Studies of different fragments and mutants of SP-B suggest that the function related structural and compositional characteristics in SP-B are its positive charges with intermittent hydrophobic domains. KL4 ([lysine-(leucine)4]4-lysine) is a synthetic peptide based on SP-B structure and is the major constituent of Surfaxin®, a potential therapeutic agent for respiratory distress syndrome in premature infants. There is, however, no clear understanding about the possible lipid–KL4 interactions behind its function, which is an inevitable knowledge to design improved therapeutic agents. To examine the phase behavior, topography, and lipid specificity of KL4/lipid systems, we aimed to study different surfactant model systems containing KL4, neutral dipalmitoylphosphatidylcholine (DPPC) and/or negatively charged lipid in the presence of Ca2+ ions. Surface pressure-area isotherms, fluorescence microscopic images, scanning force microscopy as well as time-of-flight secondary ion mass spectrometry suggest (i) that KL4 is not miscible with DPPC and therefore forms peptide aggregates in DPPC/KL4 mixtures; (ii) that KL4 specifically interacts with DPPG via electrostatic interactions and induces percolation of DPPG-rich phases; (iii) that existing DPPG-Ca2+ interactions are too strong to be overcome by KL4, the reason why the peptide remains excluded from condensed DPPG domains and passively colocalizes with DPPC in a demixed fluid phase; and (iv) that the presence of negatively charged lipid is necessary for the formation of bilayer protrusions. These results indicate that the capability of the peptide to induce the formation of a defined surface-confined reservoir depends on the lipid environment, especially on the presence of anionic lipids.

Pulmonary surfactant, a thin lipid-protein film lining the alveolar/air interface of the vertebrate lung, functions in vivo to lower surface tension, thereby reducing the work of breathing. A significant amount of investigations has been reported dealing with the physiological importance of lung surfactant and the implication of its absence or inability to function in premature neonates and adults (1–3). Over a long period, a lot of biophysical research has been undertaken using some of the major components of lung surfactant to better understand the means by which it is delivered to the air/liquid interface and promotes alveolar stability (4–6). The main phospholipid constituent of pulmonary surfactant is phosphatidylcholine, especially dipalmitoylphosphatidylcholine (DPPC)2 (7). Particular emphasis has been placed on the important role of this major disaturated phospholipid component in reducing surface tension to very low values and thus protecting the alveolus against collapse (8, 9). There also has been a strong interest in other major lipid components, such as anionic phosphatidylglycerol, and phospholipids containing unsaturated acyl chains (10, 11). Besides phospholipids, four proteins, designated as SP-A, SP-B, SP-C, and SP-D, have been found in association with lung surfactant (12, 13), among which SP-A and SP-D are hydrophilic. These two proteins are believed to be related to the storage and transport of lung surfactant as well as to participate in host defense. On the other hand, the hydrophobic proteins SP-B and SP-C are thought to play an important role in promoting the adsorption and spreading of monolayers containing large amounts of DPPC, which by itself only adsorbs slowly and respreads poorly at air/liquid interfaces (8, 14, 15).}

Neonatal respiratory distress syndrome is caused by lung immaturity with a deficiency of surfactant in the alveolar spaces and is a major cause of morbidity and mortality in preterm infants. Studies on replacement therapy on respiratory distress syndrome indicate that SP-B and SP-C are essential constituents of exogenous surfactants (16). Because of microbiological, immunological, economic and purity concerns, many efforts have been made to develop synthetic surfactant replacement formulations, which involve a combination of synthetic lipids with either synthetic or recombinant peptides (17). To understand the role of SP-B in such replacement materials, different spectroscopic techniques have been used to study the interaction between SP-B and phospholipids (18–20). Studies of different fragments and mutants of SP-B suggest that the function-related structural and compositional characteristics in SP-B are its positive charges with intermittent hydrophobic domains (21–24). Based on these structural characteristics a SP-B model peptide with 21 amino acids containing the hydrophobic amino acid leucine (Leu) and cationic lysine (Lys) in the sequence ([lysine-(leucine)4]4-lysine) was synthesized. KL4

2 The abbreviations used are: DPPC, 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine; DPPG, 1,2-dipalmitoyl-sn-glycerol-3-phosphoglycerol; BODIPY-PC, 2-(4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-sn-glycerol-3-phosphocholine; SP-C, surfactant protein C; SP-B, surfactant protein B; SFM, scanning force microscopy; TOF-SIMS, time-of-flight secondary ion mass spectrometry; SI, secondary ion; LB, Langmuir-Blodgett; le, liquid condensed; ie, liquid expanded.

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Interaction of KL4 with Surfactant Lipids

proved to be a potent mimic of SP-B not only in vitro (25) but also in vivo as it improved lung function of premature human infants with respiratory distress syndrome (26).

Although KL4-based replacement surfactants such as Surfaxin® seem to be effective therapeutic agents the molecular mechanism by which the peptide unfolds its effect in lipid mixtures remains unclear. KL4 displays a rather complex behavior when mixed with lipids. In fluid model systems, at low peptide concentrations and/or in the presence of negatively charged lipids KL4 adopts a predominantly α-helical secondary structure. In more rigid membranes, at higher peptide contents and/or in the absence of anionic lipids, KL4 converts to a β-sheet (27). The conformational transition from α-helix to β-sheet was also found to be surface pressure dependent leading to a mainly β-sheet secondary structure in DPPC and DPPC/DPPG monolayers at surface pressures >40 mN/m (28). Only in the presence of pure DPPG did KL4 form an α-helix over the whole pressure range. It is possible that this conformational flexibility is important for the peptides effectiveness in medical treatment, especially because it is assumed that during the breathing process an enrichment of negatively charged lipids in the multilayers formed at higher surface pressures occurs (29). A peptide capable of adapting its conformation to the changing lipid composition during inhalation and exhalation is then thought to represent an effective therapeutic agent.

To obtain a more complete picture of KL4 function and phase behavior in surfactant model systems we performed a systematic monolayer study applying different well established and elaborate techniques such as film balance, fluorescence light microscopy, scanning force microscopy (SFM), and time-offlight secondary ion mass spectrometry (TOF-SIMS). We employed DPPC and DPPG as lipid components for our model system because they represent main constituents of native surfactant (7, 8). The aim of this study was to systematically analyze the phase behavior of KL4 in DPPC, DPPG, as well as DPPC/DPPG (4:1, mol ratio) monolayers in the presence of Ca²⁺ ions. So far, all monolayer studies published on KL4/lipid systems were performed in the absence of Ca²⁺ ions even though this cation is present in the alveolar fluid at a concentration of about ~1.8 mM (30). It was our intention to study the ability of the peptide to induce the formation of multilayers by means of SFM and to identify specific lipid interactions via TOF-SIMS imaging of LB films.

EXPERIMENTAL PROCEDURES

Materials—The lipids used in this study, namely 1,2-dipalmityl-sn-glycero-3-phosphocholine 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 (β-BODIPY® 500/510 C12-HPC, BODIPY-PC) was obtained from Molecular Probes (Eugene, OR). All lipids were used without further purification. Chloroform, methanol, and hexane were high pressure liquid chromatography grade and purchased from Roth (Karlsruhe, Germany). Water was purified and deionized by a multistage system (MilliPore, Billerica, MA) and had a resistivity >18 MΩ·m. HEPES was obtained from Sigma and CaCl2 from Merck (Darmstadt, Germany).

The SP-B model peptide KL4 with the sequence KLLLLKLLLLKKLLKKLLKKLLK was obtained from Richard Mendelsohn, Rutgers University. The concentration of the peptide was estimated by fluorescamine assays. Lipids and peptides were dissolved in chloroform/methanol solution (1:1, v/v).

Surface Pressure-Area Isotherms—All the 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 buffered subphase (25 mM HEPES, 3 mM CaCl2, pH 7, 20 °C). Lipid/peptide monolayers were composed of DPPC, DPPG, or DPPC/DPPG (4:1, mol ratio) and were supplemented with various concentrations of KL4. The lipid/protein mixtures were prepared in a chloroform/methanol solution (1:1, v/v) and spread onto the subphase. After an equilibration time of 10–15 min the monolayers were compressed at a rate of 5.8 cm²/min.

Fluorescence Light Microscopy—All lipid/peptide systems were doped with 0.5 mol % BODIPY-PC. As described in Ref. 31 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 at certain pressures by stopping the barrier. All the measurements were performed on a subphase containing 25 mM HEPES and 3 mM CaCl2, pH 7, at 20 °C.

Preparation of Gold Supports—Preparation of gold supports was done as described in Ref. 32. Glass slides were cleaned by bath sonication at 70 °C, three times alternately in detergent and water. Immediately before evaporation the slides were dried in a nitrogen stream and further treated with argon plasma in a plasma cleaner (PDC 32G-2, Harrick, Ossining, NY) for 3 min. First, 1 nm of chromium was deposited on the surface of the slide, serving as an adhesive layer, onto which 200 nm of gold were sublimed at a rate of 0.01 nm/s. The gold-covered slides were cleaned by rectification for 8 h in a Soxhlet apparatus using n-hexane. They were then dried and used as substrates for the Langmuir-Blodgett transfers.

Langmuir-Blodgett (LB) Transfer—For scanning force microscopy 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 the lipid/peptide mixture was spread from chloroform/methanol (1:1, v/v) solutions onto a buffered subphase of a Wilhelmy film balance (Riegler and Kirstein) 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 1.5 cm²/min until a surface pressure of 50 mN/m was reached. The monolayer was then equilibrated for another 25 min at this surface pressure before transferring the film onto the mica sheet with a velocity of 0.7 mm/min. For TOF-SIMS the samples were transferred onto gold supports using the same procedure as above.
Interaction of KL₄ with Surfactant Lipids

SFM—Scanning force microscopy images of the LB films transferred onto mica sheets 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) operating in contact mode. Silicon nitride tips (Budget Sensors, Sofia, Bulgaria) with a spring constant of 40 N/m. A detailed section height analysis was performed for all the images taken using WSXM/Nanoscope SFM software. The number of protrusions found in a defined height range was plotted as “counts” against the protrusion height. The obtained statistical histograms of section heights were analyzed using Gaussian functions to determine the average protrusion heights.

TOF-SIMS—TOF-SIMS measurements of surface films were transferred onto gold supports obtained on a TOF-SIMS IV (IONTOF, Muenster, Germany) using Bi⁹⁺ as the primary ion at 25 keV. Spectra were taken in bunched mode (focus 3–5 μm) with a mass resolution of 5000–10,000. Cycling time of the instrument was set to 200 μs, allowing the acquisition of spectra up to a mass to charge ratio of 1800. Mass-resolved images were taken at nominal mass resolution (burst alignment mode, focus 300 nm). A surface of 800 μm² was rastered with 256 × 256 pixels (pixel size 312 nm). The primary ion dose did not exceed 8 × 10¹² ions/cm². In line with observations of Biesinger et al. (33), at this primary ion dose no change or inversion of contrast could be detected in any of the measurements performed.

RESULTS

Surface Pressure-Area (π-A) Isotherms—One approach to verify if specific interactions between KL₄ and negatively charged lipids occur in lipid monolayers at an air/liquid interface is to perform film balance measurements. The obtained surface pressure-area (or short π-A isotherms) in combination with fluorescence microscopy images of lipid/peptide films provide fundamental knowledge on phase behavior and mixing properties of KL₄ and thus information on its mode of interaction with individual lipids.

We systematically analyzed mixtures of DPPC, DPPG, and DPPC/DPPG (4:1, mol ratio) with varying KL₄ concentrations by spreading the respective lipid/peptide solutions on a buffered subphase containing Ca²⁺ ions (25 mM HEPES, 3 mM CaCl₂, pH 7, 20 °C) and slowly compressing the monolayer by moving a barrier in a film balance trough. Changes in surface pressure π (with π = γ₀ − γ, where γ₀ is the surface tension of the interface before and γ the surface tension of the interface after monolayer formation) are recorded in dependence of the molecular area A, which is successively reduced upon compression of the film. The isotherms obtained for the studied lipid/peptide mixtures are presented in Fig. 1. They display considerable differences depending on the lipid that can only be attributed to discriminative forces occurring between KL₄ and DPPC or DPPG, respectively.

In the case of DPPC/KL₄ monolayers increasing the peptide concentration leads to a shift of the isotherms to larger molecular areas and a loss of the DPPC-specific plateau at 5 mN/m (Fig. 1a), which is indicative of a liquid-expanded (le) to liquid-condensed (lc) phase transition (34). Such an effect generally occurs when impurities are present in lipid monolayers that suppress the highly cooperative le-lc transition (34). Interestingly, the area shift to larger values is very pronounced at low peptide concentrations and seems to reach a limiting value at higher KL₄ fractions. To evaluate this tendency more accurately we compared the so-called A₂₀ values, which represent the molecular areas of different DPPC/KL₄ mixtures at a surface pressure of 20 mN/m, and plotted them against the peptide concentration (Fig. 1d). This way of presentation of A₂₀ values reveals that the peptide-induced area shift of the isotherms is concentration dependent at KL₄ contents up to 2 mol %. For higher peptide amounts a limiting value of 60 Å² is reached. Aggregation of KL₄ at higher concentrations could account for such a behavior assuming an area requirement of phase-separated molecules that is smaller than that of mixed lipid-KL₄ complexes. Such differences could result from different conformations adopted by the peptide that could allow a tighter packing of KL₄ and DPPC, respectively. Additionally, a slight change in compressibility is observed at peptide amounts larger than 5 mol % leading to a small shoulder in the isotherm. Such a kink could be attributed to either squeeze-out of material or a conformational change of the peptide as was evidenced for SP-B at 40 mN/m (35).

When mixed with DPPG, KL₄ displays a completely different phase behavior than in DPPC/peptide monolayers (Fig. 1b). The most prominent difference is a plateau at higher surface pressures appearing already at very low KL₄ amounts. Plateau height decreases from 42 to 38 mN/m, whereas plateau length increases from 5 to 15 Å² when peptide concentration is increased from 0.4 to 10 mol %. Interestingly, when DPPG/KL₄ films are compressed beyond plateau pressure, molecular areas smaller than those typical for pure DPPG monolayers in the high pressure region are reached. Such a behavior can be attributed to material loss, more precisely to the squeeze-out of peptide together with significant amounts of lipid. This exclusion of material, however, is highly reversible, which was verified by consecutive compression/expansion cycles (data not shown). Another difference is the influence of the peptide on the compressibility of DPPG monolayers in the pressure region beneath 40 mN/m. Minimal increase in KL₄ concentration leads to a significant increase in molecular area as well as compressibility that is a strong indication for a pronounced fluidizing effect. Plotting the A₂₀ values against the peptide amount reveals that the area shift induced by KL₄ almost linearly increases at the higher peptide concentration and does not reach a limiting A₂₀ value (as is indicated by a line drawn through the data points in Fig. 1d). This, too, is in contrast to DPPC/KL₄ monolayers indicating strong interactions, probably of electrostatic nature, between DPPG and KL₄ leading to a more expanded phase.

Because KL₄ seems to behave in totally different ways with neutral DPPC and anionic DPPG, respectively, studying the DPPC/DPPG/KL₄ system should uncover if specific lipid interactions exist in the ternary mixture. As can be seen from the isotherms presented in Fig. 1c DPPG already has an influence on the phase behavior of DPPC. The le-lc plateau vanishes because DPPG, too, acts as an impurity disturbing the highly cooperative phase transition. Adding KL₄ to the DPPC/DPPG mixture only leads to a concentration dependent area shift typical for surface active peptides and otherwise does
not significantly alter the monolayer properties. This is especially surprising because KL₄ affected the phase behavior of DPPG monolayers. We would have expected the appearance of a more pronounced shoulder at $\pi/H_{11011} = 40 \text{ mN/m}$ similar to the one found in DPPG/KL₄ mixtures. The evaluation of $A_{20}$ values, however, revealed a dependence similar to the one found in DPPG/KL₄ monolayers, which is a continuous increase of the $A_{20}$ values with an increasing peptide amount without reaching any limitation (Fig. 1d). It seems that in the ternary mixture a more delicate equilibrium of forces between the individual components exists, which needs further studying.

**Fluorescence Light Microscopy**—We performed fluorescence light microscopy measurements to visualize the morphology of lipid/peptide monolayers at the air/water interface and to further scrutinize lipid-KL₄ interactions. Fluorescence microscopy offers the possibility of studying the le-le phase transition of lipids based on the different partitioning of the fluorescent probe into fluid or condensed regions of the lipid monolayer. The solubility of the fluorescent probe in a given phase depends on lipid packing, and orientation of the lipid acyl chains. Due to the molecular structure of the fluorescent labeled lipid it is not soluble in tightly packed le domains. It is therefore enriched in the surrounding fluid phase and leads to the appearance of dark le domains amid a fluorescent background (33).

In Fig. 2 we present a series of fluorescence microscopic images of DPPC, DPPG and DPPC/DPPG (4:1, mol ratio) monolayers with or without 0.4 mol % KL₄ investigated in a surface pressure range of 0–50 mN/m, where each monolayer is stable over the time scale of the experiments. In the case of DPPC dark slightly kidney-shaped domains embedded in a bright fluorescent phase appear at a surface pressure of 4–5 mN/m corresponding to the plateau observed in the $\pi/A$ isotherms. The observed domains grow in size upon further compression and have a mean diameter of 18–20 $\mu\text{m}$ at a surface pressure of 15 mN/m and of 25 $\mu\text{m}$ at 50 mN/m (Fig. 2a). The peptide obviously induces a phase separation already after spreading of the film. Increasing the surface pressure leads to nucleation and growth of further domains that reach a diameter of 20–25 $\mu\text{m}$ at 50 mN/m.
Pure DPPG monolayers are characterized by the appearance of a large number of small circular domains with diameters well below 5–8 μm (Fig. 2c). Increasing the surface pressure does not result in a significant increase in domain size or additional nucleation of lc domains but only in a continuous loss in contrast because of self-quenching (36). This effect arises from energy transfer among fluorophore molecules when their mole fraction increases and leads to a reduction of emission intensity. In accord with film balance measurements, the addition of cationic KL₄ has a strong fluidizing effect on DPPG monolayers. Fluorescence microscopy images reveal a greater degree of fluorescent lc phase in which large kidney-shaped domains (20–25 μm) already appear at 0 mN/m (Fig. 2d). These domains are larger than the ones found in the DPPG system. Further compression of the monolayer leads to a reduction of the fluorescent fluid phase. The presence of KL₄ in the DPPC/DPPG monolayer leads to a great enhancement of the fluid phase at 0 mN/m and clustering of the observable circular domains (Fig. 2f). At surface pressures exceeding 30 mN/m a pronounced percolation of the condensed domains is discernible. A continuous network of solid phase appears connecting large kidney-shaped domains (30–60 μm), which are evenly distributed in the monolayer. At 50 mN/m, however, the inverse phenomenon is discernible, namely percolation of the fluorescent phase leading to the appearance of branched structures. Larger somewhat distorted kidney-shaped

![Fluorescence images of monolayers consisting of (a) DPPC, (b) DPPC/KL₄ (0.4 mol % peptide), (c) DPPG, (d) DPPG/KL₄ (0.4 mol % peptide), (e) DPPC/DPPG, and (f) DPPC/DPPG/KL₄ (0.4 mol % peptide) measured on 25 mM HEPES, 3 mM, CaCl₂ (pH 7.0) at 20 °C.](image)

"Interaction of KL₄ with Surfactant Lipids"

Pure DPPG monolayers are characterized by the appearance of a large number of small circular domains with diameters well below 5–8 μm (Fig. 2c). Increasing the surface pressure does not result in a significant increase in domain size or additional nucleation of lc domains but only in a continuous loss in contrast because of self-quenching (36). This effect arises from energy transfer among fluorophore molecules when their mole fraction increases and leads to a reduction of emission intensity. In accord with film balance measurements, the addition of cationic KL₄ has a strong fluidizing effect on DPPG monolayers. Fluorescence microscopy images reveal a greater degree of fluorescent lc phase in which large kidney-shaped domains (20–25 μm) already appear at 0 mN/m (Fig. 2d). Interestingly, a very unusual extended network is visible within the fluid fluorescent regions at all analyzed surface pressures. This network seems to consist of bright domains embedded in a less fluorescent dark background and has the appearance of a percolated solid phase. Upon further compression of the film the kidney-shaped dark domains do not significantly increase in size. However, the area of the surrounding fluid or percolated phase first increases and then remains constant.

Because the effect of KL₄ on DPPG monolayers is different from its influence on DPPC films it is of major interest to study the behavior of the peptide in the presence of both lipids. In the peptide-free DPPC/DPPG monolayer large circular domains with a mean diameter of 20–25 μm already appear at 0 mN/m (Fig. 2e). These domains are larger than the ones found in the DPPG system. Further compression of the monolayer leads to a reduction of the fluorescent fluid phase. The presence of KL₄ in the DPPC/DPPG monolayer leads to a great enhancement of the fluid phase at 0 mN/m and clustering of the observable circular domains (Fig. 2f). At surface pressures exceeding 30 mN/m a pronounced percolation of the condensed domains is discernible. A continuous network of solid phase appears connecting large kidney-shaped domains (30–60 μm), which are evenly distributed in the monolayer. At 50 mN/m, however, the inverse phenomenon is discernible, namely percolation of the fluorescent phase leading to the appearance of branched structures. Larger somewhat distorted kidney-shaped domains are still visible at these high pressures and are connected via extended structures with feathery texture. Because percolated phases only appear when DPPG and KL₄ coexist, that is in DPPG/KL₄ monolayers as well as in the ternary system DPPC/DPPG/KL₄, distinct interactions between these two entities are likely to exist.

SFM—Scanning force microscopy is a desirable technique that allows us to explore unique features of biomaterials and to obtain a large variety of surface parameters such as topography, friction, viscoelasticity, and surface potential with high lateral resolution. We report for the first time the influence of KL₄ on monolayer morphology at a surface pressure that in the case of native SP-B facilitates the formation of protrusions. It was our aim to study the influence of KL₄ on the appearance of these three-dimensional structures using SFM and to evidence its
capability of inducing protrusion formation. Although KL$_{4}$ is normally assumed to mimic SP-B quite well there has been no report yet on its ability to induce the formation of a surface-confined reservoir as does native SP-B (35). Fig. 3a shows the topography image ($10 \times 10 \mu m^2$) of a KL$_{4}$ (0.4 mol %) containing LB film composed of DPPC transferred onto mica at a surface pressure of 50 mN/m and scanned under contact mode. Bright regions observable in SFM images correspond to higher structures, whereas dark areas are of lower height, as is indicated by the greyscale bar next to the topographic images. The results obtained for DPPC/KL$_{4}$-LB films suggest the formation of knob-like protrusions with heights of 9–22 nm surrounding rather flat regions (Fig. 3a). These structures are connected to an irregular network that spans the entire area. A detailed statistical histogram analysis of protrusion heights revealed a broad height distribution with its maximum at a value of 9.5 ± 0.4 nm (Table 1).

In monolayers composed of DPPG and 0.4 mol % KL$_{4}$ the formation of stretched patches with an average height of 6.4 ± 0.4 nm was observed (Fig. 3b). Because their height corresponds to the thickness of a lipid bilayer we conclude that in DPPG/KL$_{4}$ formation of bilayer structures occurs. Protrusions of an average height of 9.9 ± 0.5 nm are also visible. These three-dimensional structures are not separated from the stretched bilayer patches but seem to grow from the latter. More precisely, protruding from the three-dimensional structures of bilayer height are smaller knobs with a height difference of 3.5 nm. To verify if these protrusions are possibly already formed in the plateau region at lower surface pressures we performed SFM measurements of LB films transferred at 38 mN/m. The resulting SFM image suggests that protrusion formation already occurs in the plateau pressure region (Fig. 4). Stretched patches similar to the ones found at 50 mN/m were more expanded and possessed an average bilayer height of 4.9 ± 0.3 nm. Because these three-dimensional structures are of lower height than the bilayer protrusions found at 50 mN/m, it can be concluded that protrusion height increases upon further compression of the film under concomitant reduction of the area occupied by the protrusions. Increasing surface pressure most probably also leads to the formation of additional structures protruding from the bilayer patches.

The topography image of DPPC/DPPG/KL$_{4}$ monolayers reveal that KL$_{4}$ also facilitates the formation of multilamellar protrusions in the ternary mixture (Fig. 3c). The appearance of these three-dimensional structures is intermediate to the ones found in the binary mixtures, that is stretched patches (as in DPPG/KL$_{4}$ monolayers) containing a considerable amount of
holes and knobs (as in DPPC/KL₄ mixtures). The height profile reveals structures with average heights of 7.6 ± 0.5 and 12.5 ± 0.3 nm. Apart from this we found non-bilayer protrusions of 3.3 ± 0.2 nm height protruding from the flatter monolayer region. We added this height value to the ones listed in Table 1 although it was not obtained by statistical analysis. The reason for this was that a significant peak in the range of 2.5–3.5 nm was visible in the histogram of Fig. 3c. We therefore felt bound to consider this height in our evaluation and discussion of SFM topography images.

It can be concluded that KL₄ does induce the formation of numerous protrusions independent of lipid head group charge. However, height and appearance of the observed three-dimensional structures strongly depends on lipid composition of the chosen surfactant model system. The presence of both DPPC and DPPG seems to be crucial for the formation of multilamellar protrusions.

**TOF-SIMS—**Laterally resolved TOF-SIMS was used in our study to examine the lateral molecular distribution of the different surfactant model components. We hoped to identify specific interactions between lipids and KL₄ as a result of distinct distribution patterns on the surface. It is known from previous studies that the mass spectra for DPPC and DPPG are very similar because negatively charged lipids do not yield head group-specific secondary ions (SI) and the fragments resulting from the acyl chains are identical to those coming from DPPC (32, 37). To differentiate between the signals arising from these two components and to determine their lateral molecular distribution we used a deuterated DPPG analogue. d62DPPG possesses deuterated acyl chains and was proven to display a very similar phase behavior to the undeuterated analogue (37). Most importantly, d62DPPG shows a significantly different fragmentation pattern than DPPC when it is ionized. For example, a fragment ion with a mass to charge ratio (m/z) of 18 can only be attributed to the secondary ion OD⁻ and can thus be used to identify the localization of d62DPPG on the surface. The distribution of DPPC can be attributed to the specific secondary ions arising from the choline head group (m/z = 104, 184), likewise d62DPPG can be specifically detected by additional fragments of its deuterated acyl chains (m/z = 50, 69, 71). Finally, KL₄ can be identified by SI fragments arising from its amino acid residues (m/z = 30, 82, 84, 86). Mapping the intensities of characteristic secondary ions laterally leads to high resolution mass resolved images that allow us to exactly determine the distribution of the different lipid and peptide components in the different surfactant model mixtures analyzed in this study.

The TOF-SIMS mass resolved images presented in Fig. 5a show the lateral SI distribution of DPPC/KL₄ LB films in which DPPC- and KL₄-specific signals are detected (e.g. m/z = 104, 184 for DPPC and m/z = 82, 30 for KL₄). The bright colored regions are indicative of high ion counting rates of the respective species and are a (however, not quantitative) measure for the concentration of the detected component. As can be seen from the TOF-SIMS images, DPPC and KL₄ form distinct domains that are separated from each other (anti-correlation). This demixing is apparent from the inverse color and SI distribution in DPPC- and KL₄-specific mass resolved images.

Fig. 5b shows the TOF-SIMS images of a d62DPPG/KL₄ surface film with spatially distinct intensities of the component specific secondary ions (e.g. m/z = 71, 69 for DPPG and m/z = 86, 84 for KL₄). The mass resolved images obtained for different SI species indicate that d62DPPG and KL₄, too, form distinct areas enriched in the respective component. The distribution of these domains does not correlate and most interestingly does not show any sign of anticorrelation as was the case in the DPPC/peptide mixture. In comparison to the binary films containing DPPC and KL₄, films consisting of d62DPPG/KL₄ are characterized by smaller and more diffused domains. On closer inspection, however, darker slightly kidney-shaped regions are visible that seem to be depleted in both d62DPPG and KL₄.

Fig. 6 presents the mass resolved images of the DPPC/d62DPPG/KL₄ system showing the distribution of the different signals arising from different fragments. The fragment ions with m/z ratios of 34, 46, 50,

**TABLE 1**

| Monolayer | Transfer pressure (mN/m) | Protrusion height (nm) |
|-----------|--------------------------|------------------------|
| DPPC/KL₄  | 50                       | 9.5 ± 0.4ᵃ             |
| DPPG/KL₄  | 50                       | 6.4 ± 0.4ᵇ             |
| DPPG/KL₄  | 38                       | 9.9 ± 0.5ᵇ             |
| DPPC/DPPG/KL₄ | 50                 | 4.9 ± 0.3ᵇ             |
| DPPG/KL₄  | 38                       | 3.3 ± 0.2ᵇ             |
| DPPG/KL₄  | 38                       | 7.6 ± 0.5ᵇ             |
| DPPG/KL₄  | 38                       | 12.5 ± 0.3ᵇ            |

ᵃ Values obtained from statistical histogram analysis.
ᵇ Average value of heights between 2.5 and 3.5 nm.
Interaction of KL₄ with Surfactant Lipids

FIGURE 5. Mass resolved TOF-SIMS images of (a) DPPC/KL₄ monolayers (0.4 mol % peptide) with positively charged secondary ions resulting from DPPC (m/z = 184, 104) and KL₄ (m/z = 82, 30) and (b) d62DPPG/KL₄ monolayers (0.4 mol % peptide) with positively charged secondary ions resulting from d62DPPG (m/z = 71, 69) and KL₄ (m/z = 84, 86). Bright colored film regions correspond to a high counting rate of the particular fragment ion, whereas darker areas are due to a lower counting rate. LB transfer was performed onto gold-covered glass slides at 50 mN/m from a subphase containing 25 mM HEPES, 3 mM CaCl₂ (pH 7.0) at 20 °C.

DISCUSSION

Synthetic peptides are becoming more and more important in surfactant replacement therapy as they project less biohazardous effects than animal lung extracts, do not contain any immunologically incompatible contaminants, and are easy to produce. A comprehensive knowledge of their interaction with surfactant lipids is critical for an optimal replacement therapy. The physiological significance of the lipid-peptide interaction for an optimal surfactant replacement therapy lies in the fact that they also contribute significantly to the shear viscosities in surfactant preparations. The viscous behavior depends upon both the composition and the interactions between various lipids and proteins that potentially influence their delivery and distribution in the lungs (38). Thus, the knowledge of specific lipid-peptide interactions would enable us to identify physiologically active lipid/peptide compositions and to formulate more efficient surfactant therapeutics.

The major objective of our investigations was to analyze the influence of the synthetic peptide KL₄, which is a potent mimic of native surfactant protein B, on monolayers consisting of the main surfactant lipid components DPPC and DPPG in the presence of Ca²⁺ ions. Our results indicate the existence of differences in the interactions between the peptide and either DPPC or DPPG, which might be of great importance for the understanding of the factors governing lung surfactant function.
The \( \pi - A \) isotherms of different monolayers containing KL4 reveal that the phase behavior of lipid/peptide mixtures depends on the lipid used for the lung surfactant model system. The influence of KL4 on DPPC monolayers seems to be governed by demixing of the individual components and aggregation of the peptide occurring at higher KL4 ratios. Such a phase separation was indeed observed in our fluorescence micrographs (Fig. 2b) and was confirmed by TOF-SIMS measurements as will be discussed later in detail. These conclusions are in accordance with the results obtained from DPPC/KL4 monolayers studied in the absence of Ca\(^{2+}\) ions, which suggested that DPPC and KL4 are immiscible (39).

In contrast to its effect on DPPC, KL4 strongly fluidizes pure DPPG films and even leads to the appearance of a distinct plateau at 40 mN/m. The formation of such a concentration-dependent plateau, which proved to be highly reversible when consecutive compression/expansion cycles were performed with the film balance (data not shown), is not surprising for a peptide that is assumed to mimic SP-B function. Isotherms of SP-B in mixtures with DPPC and/or DPPG generally display kinks at 40 – 45 mN/m, which become more pronounced upon increase in SP-B content (40). The observed plateaus are generally attributed to a protein-induced exclusion of material from the monolayer. What is interesting, however, is that KL4 reveals substantial differences in monolayer behavior when mixed with either DPPC or DPPG, a feature that was not found for SP-B (40). Specific interactions between KL4 and DPPG due to electrostatic attraction could account for this behavior. We therefore conclude that KL4, too, triggers a controlled squeeze-out of material into the subphase, however, only when negatively charged lipids are present in the monolayer.

As the isotherms of the binary mixtures indicated that KL4 strongly interacts with DPPG but not with DPPC, we had hoped to identify these discriminative forces also in the ternary system and, in particular, to observe the specific plateau at 40 mN/m. This was unfortunately not the case. It is possible that the effective concentration of negatively charged lipid in the ternary mixture is too low to observe specific DPPG-KL4 interactions on a macroscopic level with the film balance technique, especially if DPPC and DPPG are not miscible, as is probably the case in the presence of Ca\(^{2+}\) ions (see discussion of fluorescence microscopy results). Under conditions of phase separation the amount of free (diffusible) anionic lipid would be further reduced and thus the probability of phosphatidylglycerol-KL4 interactions decreased. Note that the Ca\(^{2+}\) ions present in the subphase most probably compete with KL4 for DPPG in the monolayer that could further prevent interactions of the peptide with anionic lipids. It therefore seems that a highly

![DPPC](image1.png)

![d62DPPG](image2.png)

![KL4](image3.png)

![Overlay](image4.png)

FIGURE 6. Mass resolved TOF-SIMS images of a DPPC/d62DPPG/KL4 monolayer (4:1:0.4, mol ratio) with positively charged secondary ions resulting from DPPC (m/z = 184), d62DPPG (m/z = 50), and KL4 (m/z = 84). All three components are represented by an image showing the intensity of the respective secondary ion fragment (shown left). Bright colored film regions correspond to a high counting rate of the particular fragment ion, whereas darker areas are due to a lower counting rate. The same image is presented in a different background primary color for correlation analysis (shown right). Additionally, a three-color overlay represents the sum image of the DPPC (blue), d62DPPG (green), and KL4 maps (red). LB transfer was performed onto gold-covered glass slides at 50 mN/m from a subphase containing 25 mM HEPES, 3 mM CaCl\(_2\) (pH 7.0) at 20 °C.
Interaction of KL₄ with Surfactant Lipids

complex balance of attractive and repulsive forces between lipids, peptide, and subphase Ca²⁺ ions exists, which drives the monolayer phase behavior of the ternary mixture.

Effect of KL₄ on Surface Phase Separation—Studying the morphology of lipid/peptide mixtures with fluorescence light microscopy offered the unique possibility to visualize lipid discrimination by KL₄ and to obtain more detailed information on the mode of peptide action in lipid monolayers. Fluorescence light microscopy not only confirmed the conclusion drawn from film balance measurements that the peptide is not miscible with DPPC and strongly interacts with DPPG. We also observed a phenomenon of percolation in the presence of negatively charged lipids.

As was expected for zwitterionic DPPC, in the absence of KL₄ large kidney-shaped domains typical for this lipid (34, 41) appeared at 5 mN/m also in the presence of Ca²⁺ ions (34, 41). When peptide was added to DPPC, extended dark domains were already visible immediately after spreading in the DPPC/KL₄ monolayer, which is a clear indication of demixing of the individual components. Most probably two phases that differ in lipid packing density and/or tilt angle of the fatty acids are formed. Because the typical DPPC plateau indicative of a lipid packing density and/or tilt angle of the fatty acids are individual components. Most probably two phases that differ in lipid packing density and/or tilt angle of the fatty acids are formed. Because the typical DPPC plateau indicative of a le-lc phase transition is still visible in the corresponding isotherms, we conclude that condensed DPPC-rich domains are formed that are embedded in a fluid KL₄-rich matrix. Phase contrast would then result from the fluorescent dye being preferably partitioned in the less dense peptide-rich phase.

When DPPG is studied in the presence of Ca²⁺ ions, a large amount of little dark domains is already visible at 0 mN/m. Such a behavior was to be expected because Ca²⁺ ions have the propensity to reduce the repulsive forces between the negatively charged head groups of DPPG by bridging two molecules and thus inducing a greater degree of condensation at very low surface pressures (42). Binding of Ca²⁺ ions to DPPG is assumed to be accompanied by dehydration of the phosphates in the head group region (42, 43). These Ca²⁺-DPPG complexes probably act as crystallization sites and lead to the separation of tightly packed Ca²⁺-bridged and dehydrated DPPG domains from a fluid le-phase.

Addition of KL₄ to DPPG monolayers leads to a considerable reduction in number and increase in size of condensed Ca²⁺-bridged DPPG domains, that is the reduction of crystallization sites, compared with the KL₄-free system. Electrostatic interactions between DPPG and the peptide could have an influence on the line tension of DPPG domains and would account for the kidney-shaped form of the condensed domains. An unusual feature is the appearance of a network that has a strong resemblance with a percolated solid phase. Percolation is defined as the appearance of a continuous cluster of domains allowing the tracing of a path across the entire phase without leaving it (44). The concept of percolation was initially developed for the problem of fluid movement in porous medium and has recently been applied to biological samples (45). It is comparable with usual temperature- or pressure-induced phase transitions, however, without being temperature or pressure dependent. As in our case, composition of monolayers can determine the appearance of percolation and define the percolation threshold. Also the addition of peptides can induce or at least influence the percolation transition as was mentioned earlier in studies of SP-C containing lung surfactant model systems (46, 47). In our study, KL₄ seems to induce percolation of DPPG-rich domains due to strong electrostatic interactions. Such a behavior could account for the pronounced fluidizing effect exerted on DPPG monolayers if we assume that percolated structures are not as tightly packed as domains originating from a simple le-lc phase transition.

Monolayers consisting of DPPC and DPPG seem to be immiscible in the presence of Ca²⁺ ions because large domains are already visible under highly expanded film conditions. These results are in line with data from the literature where DPPC/DPPG mixtures with more than 10% DPPG displayed a high percentage of condensed lipid at low surface pressures (42). A similar behavior has been described for monolayer mixtures of DPPC/DPPS at 4:1 in the presence of Ca²⁺ ions (41).

When KL₄ is added to the DPPC/DPPG monolayer a considerable clustering of the numerous condensed domains is found at a pressure of 0 mN/m (Fig. 2f). In addition, percolation of condensed domains leading to a continuous network of dark le-phase is visible. Because this phenomenon is only detected when DPPG and KL₄ are simultaneously present in the monolayer, percolation can be attributed to electrostatic interactions between these two components. The appearance of branched structures, however, connecting large kidney-shaped domains is typical only for the ternary mixture. The reason for the formation of these feathery structures is still unclear and remains to be elucidated.

Topography of Lipid/KL₄ Monolayers—SFM is a unique technique to map the topography of biological samples at high resolution and was used in this study to investigate the ability of KL₄ to facilitate the formation of multilamellar protrusions. Such structures could play a pivotal role in the breathing cycle because they possibly present docking sites for surfactant material delivered from the subphase for monolayer refinement and are assumed to also occur in the physiological lung system (48, 49). To date, formation of a surface confined reservoir has been described for surfactant proteins B (35, 50) and SP-C (31, 51) as well as model peptides such as SP-B (1–25) (52). We now report for the first time the influence of KL₄ on the formation of three-dimensional structures in different lipid monolayers. Our results indicate that KL₄ indeed induces protrusion formation at high surface pressures in the presence of Ca²⁺ ions. Three-dimensional structures are formed independent of head group charge and reveal a pattern that is very similar to the one found in SP-C- and SP-B-rich surface films (35, 50). Their height, however, strongly depends on head group charge: protrusions of bilayer height are only detected in the presence of DPPG, whereas DPPC/KL₄ mixtures are characterized by a multitude of structures with varying heights. In the ternary mixture, however, multilayers of bilayer height are formed indicating that both lipid species are necessary for efficient formation of a surface-confined reservoir.

In all lipid mixtures tested in this study SFM images show the appearance of an irregular network of either aligned knobs or stretched patches surrounding flat areas that most likely represent the former solid domains that coalesced upon compres-
regions were different lc domains seem to have collided and therefore are the remains of the former fluid phase enriched with KL4, which was squeezed-out into the subphase. The appearance of these network structures, especially the polygonal shape of most of the individual units, strongly resembles the one found in other surfactant protein/lipid systems (35, 50–52). The fact that these protrusions are formed indicates that KL4 stabilized if not actively induced their formation. Other model peptides of SP-C that consisted of significantly truncated and/or depalmitoylated SP-C analogues and were tested in a previous study (53) did not show any sign of protrusion formation, which underlines the fact that defined structure/function relationships exist governing the effective squeeze-out of material.

As mentioned above, the appearance of three-dimensional structures clearly depended on the kind of lipid present in the surfactant model system. In the case of DPPC/KL4 films the protrusions consist of knob-like structures aligned like a string of pearls along the former lc boundaries (Fig. 3a). The heights are not uniform and range from 4 to 22 nm with a distribution maximum at 9.5 nm. Similar disc-like structures of equal height were observed for SP-B containing DPPC/DPPG films and were explained with the formation of protein protrusions containing only a limited amount of lipids (35). Such a squeeze-out of peptide aggregates would be in line with film balance and fluorescence microscopy results that led to the assumption of phase separation and peptide aggregation. And it would explain the inhomogeneous height distribution found in SFM images. We conclude from these results that, in DPPC films, KL4 seems to display a phase behavior very similar to native SP-B.

In DPPG/KL4 monolayers, however, stretched patches with height differences of 6.4 nm that correspond to the thickness of a lipid bilayer were found. Protruding from these patches are three-dimensional structures of additional 3.5 nm height that could be attributed to a lifting-out of peptide aggregates from these areas. This observation is rather unusual for a structural mimic of native SP-B, especially if one considers that such bilayer protrusions have so far mainly been found for SP-C (35, 54, 55). Then again, it has to be kept in mind that structural differences between KL4 and the C-terminal part of SP-B exist despite their sequence homology (56), which could account for the deviations from SP-B behavior. The surface structure of KL4 is characterized by a continuous distribution of its positive charges over the entire helical circumference, whereas SP-B residues 64–79 form an amphipathic α-helix (57, 58). It is therefore tempting to conclude that, in the presence of anionic lipids, KL4 has a mechanism of action similar to SP-C, as has been proposed by Gustafsson et al. (57), and induces a highly cooperative formation of bilayer structures already in the plateau region. The heights found for the bilayer patches (4.9 nm at 38 mN/m and 6.4 nm at 50 mN/m) also correspond to the ones found for SP-C containing films (6.5 nm) measured in contact mode (35). Pressure-dependent conformational changes of the peptide, as has been described by Cai et al. (28), might be responsible for the height differences found at 38 and 50 mN/m, respectively. However, the α-helix formed by KL4 also differs from the one of SP-C with virtually only non-polar residues (59). These differences could account for formation of peptide protrusions on the bilayer patches, a feature that has not been reported for SP-C containing films yet. The mode of action of the peptide with lipids therefore supposedly differs from both SP-B and SP-C, as has been proposed earlier (58).

In ternary mixtures of DPPC/DPPG/KL4, at least two bilayer steps are discernible in the SFM images. This observation confirms the assumption that negatively charged lipids are necessary for the generation of bilayer protrusions. But it also reveals that DPPC is obviously required for reaching higher protrusion levels. Therefore, a different molecular mechanism for squeeze-out of material than in DPPG/KL4 monolayers has to occur. The found non-bilayer structures of 3.3 nm height protruding from the bottom layer are most likely due to peptide/lipid aggregates, which were lifted out from the monolayer.

We believe that the influence of the lipid environment on lateral distribution and mixing properties of the peptide is mainly responsible for the observed topographic differences in the molecular mechanisms of protrusion formation. However, conformational changes of the peptide due to different lipid environments cannot be ruled out. From literature it is known that the KL4 secondary structure is highly sensitive to lipid composition, peptide amount, monolayer fluidity, and the presence of Ca2+ ions (27, 28). For example, KL4 adopts an α-helical structure when molecular packing density of lipids is low (e.g. in monolayers at low surface pressure or in bilayers containing unsaturated lipids) and charge neutralization due to interaction with negatively charged lipids occurs. Conditions favoring high lipid packing density, such as high surface pressures and reduced negative charge density resulting from addition of Ca2+ ions, seem to trigger the interconversion from α-helix to β-sheet (27, 28). The conditions chosen in our experiments, e.g. saturated lipids and a subphase containing Ca2+ ions, should favor the formation of a β-sheet structure especially at higher surface pressures. The presence of oligomeric KL4 β-sheet assemblies would involve reduction of free energy due to the formation of maximum interstrand hydrogen bonding as has been reported for hydrophobic hexapeptides (60). And it would explain the observed tendency of KL4 to aggregate and form the non-bilayer protrusions visualized with SFM. It would therefore be highly interesting to study the secondary structure of KL4 in monolayers at the experimental conditions chosen in this work and to obtain further insight into the mechanisms behind the protrusion formation process.

Lateral Distribution of Lipids and Peptide—Laterally resolved TOF-SIMS enabled us to study the molecular distribution of surfactant model components and to possibly identify a lipid specificity of KL4 interaction. The observed anti-correlation between DPPC and KL4 distribution supports the assumption that these compounds demix in the binary mixture leading to separate domains (Fig. 5a). Interestingly, phase separation was also observed in d62DPPG/KL4 monolayers (Fig. 5b). However, there was no correlation between lipid and peptide distribution, not even an anticorrelation. We therefore conclude that interactions of KL4 with negatively charged d62DPPG seem to exist that affect the lateral distribution of the molecules. The observed kidney-shaped darker regions visible in both the d62DPPG and the KL4 ion maps are probably the result of matrix effects. It has been described in literature that
the physical state of the lipids and their chemical environment account for different signal intensities of secondary ions (50, 61). We therefore assume that the kidney-shaped domains contain more tightly packed Ca\(^{2+}\)-bridged d62DPPG domains from which the peptide is excluded. Due to their different physical state d62DPPG molecules in these densely packed areas obviously yield lower fragmentation affinities. KL\(_4\) would then colocalize with d62DPPG, which is not bound to Ca\(^{2+}\) ions and form the intermediate phase connecting the d62DPPG-Ca\(^{2+}\) domains. To take up the idea of percolation we suppose that this intermediate phase probably represents the percolated regions in which specific interactions between peptide and negatively charged lipid probably exist.

The investigation of DPPC/d62DPPG/KL\(_4\) monolayers revealed that d62DPPG is strictly separated from DPPC and KL\(_4\) and forms distinct round domains (Fig. 6). It is possible that due to the immiscibility of DPPC and d62DPPG in the presence of Ca\(^{2+}\) ions, KL\(_4\) is excluded from the d62DPPG-rich phase connected by a strong hydrogen bonding network and Ca\(^{2+}\) ions bridging individual anionic lipids. KL\(_4\) would then necessarily be localized in the DPPC-rich bulk but probably would remain immiscible with DPPC in the colocalized region. However, the resolution of TOF-SIMS is not high enough to visualize the demixing of DPPC and KL\(_4\) in the ternary mixture. It is also too low to identify whether the protrusions of bilayer height observed with SFM are actually membranes or only peptide aggregates. It is important to know that TOF-SIMS is a highly surface-sensitive technique. As the information depth of TOF-SIMS is as good as 1–3 molecular monolayers and as 90% of the information is gained from the uppermost monolayer, the distribution of d62DPPG in the lower layers of the protrusions cannot be ruled out. Taking into account that the observed contrasts can be found for all detected fragment ions of the three molecular species artifacts due to matrix effects are unlikely. Thus, the detected distribution of fragment ions represents the distribution of molecules in the uppermost monolayer of the LB sample. Therefore, we strongly believe that KL\(_4\) does seem to colocalize with the DPPC-rich phase (even if it probably forms separate peptide aggregates from DPPC) whether or not DPPG is present in underlying multilayers.

A similar colocalization with DPPC was evidenced for SP-C (32) and SP-B (37), however, in the absence of Ca\(^{2+}\) ions. In these mixtures, too, it was assumed that the immiscibility of DPPC and DPPG attributed to structural differences in the head group region and the presence of a strong hydrogen network also existing in the absence of cations (50) led to the exclusion of the surfactant proteins from the more condensed DPPG-rich phase. Electrostatic interactions between the surfactant proteins and the anionic lipids were assumed to be too weak to overcome the strong intermolecular hydrogen bonding network formed within the DPPG-rich domains. We therefore conclude that as long as experimental conditions favoring formation of separated DPPG domains are chosen it will be difficult to evidence the existence of specific lipid/KL\(_4\) or even lipid-surfactant protein interactions.

**Conclusion**—The present study on phase behavior, topography, and chemical distribution of lipid/KL\(_4\) systems revealed: (i) that KL\(_4\) does not specifically interact with DPPC and forms peptide aggregates in DPPC/KL\(_4\) mixtures; (ii) that electrostatic interactions between KL\(_4\) and DPPG exist, which lead to percolation of the DPPG-rich phase; (iii) that in the presence of Ca\(^{2+}\) ions KL\(_4\) cannot break up the existing DPPG-Ca\(^{2+}\) interactions so it passively colocalizes with DPPC under formation of a demixed fluid phase; and (iv) that protrusions of bilayers or multilayers are only formed in the presence of negatively charged lipid, whereas in DPPC-containing mixtures three-dimensional structures mainly consist of peptide aggregates. It would be of major importance to study in a next step KL\(_4\)-containing surfactant model systems in the absence of Ca\(^{2+}\) ions and investigate whether the peptide does specifically interact with DPPG. Further studies on secondary structural aspects, still a matter of controversy, would also be of major interest for a detailed understanding of the conformational changes that could drive the peptides ability to induce percolation of DPPG-rich domains.

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