

Effects of a Cationic and Hydrophobic Peptide, KL4, on Model Lung Surfactant Lipid Monolayers

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ABSTRACT We report on the surface behavior of a hydrophobic, cationic peptide, [lysine-(leucine)₄]-lysine (KL4), spread at the air/water interface at 25°C and pH 7.2, and its effect at very low molar ratios on the surface properties of the zwitterionic phospholipid 1,2-dipalmitoylphosphatidylcholine (DPPC), and the anionic forms of 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) and palmitic acid (PA), in various combinations. Surface properties were evaluated by measuring equilibrium spreading pressures (π_e) and surface pressure-area isotherms (π - A) with the Wilhelmy plate technique. Surface phase separation was observed with fluorescence microscopy. KL4 itself forms a single-phase monolayer, stable up to a surface pressure π of 30 mN/m, and forms an immiscible monolayer mixture with DPPC. No strong interaction was detected between POPG and KL4 in the low π region, whereas a stable monolayer of the PA/KL4 binary mixture forms, which is attributed to ionic interactions between oppositely charged PA and KL4. KL4 has significant effects on the DPPC/POPG mixture, in that it promotes surface phase separation while also increasing π_e and π_{max} , and these effects are greatly enhanced in the presence of PA. In the model we have proposed, KL4 facilitates the separation of DPPC-rich and POPG/PA-rich phases to achieve surface refinement. It is these two phases that can fulfill the important lung surfactant functions of high surface pressure stability and efficient spreading.

INTRODUCTION

A significant body of literature exists concerning the physiological importance of lung surfactant and the implications of its absence or inability to function in premature neonates and adults (Goerke, 1974; Hills, 1990; Creuwels et al., 1997). Correspondingly, much biophysical research has been carried out using some of the major components of lung surfactant to better understand the means by which it is delivered to the alveolus/air interface and promotes alveolar stability (Fleming and Keough, 1988; Pastrana-Rios et al., 1994; Wang et al., 1995). Particular emphasis has been placed on the important role of a major disaturated phospholipid component, dipalmitoylphosphatidylcholine (DPPC) (Yu et al., 1983; Holm et al., 1996), in reducing surface tension to very low values and thus protecting the alveolus against collapse. There also has been a strong interest in other lipid components, such as anionic phosphatidylglycerol (PG), and phospholipids containing unsaturated acyl chains (Tanaka et al., 1983; Cockshutt et al., 1991). Besides phospholipids, four proteins, designated as SP-A, SP-B, SP-C, and SP-D, have been found in association with lung surfactant (Hawgood and Shiffer, 1991; Johansson and Curstedt, 1997), among which SP-A and SP-D are hydrophilic and believed to be related to the storage and transport of lung surfactant. Meanwhile, the hydrophobic proteins SP-B and SP-C are thought to play an important role in promoting the adsorption and spreading of monolayers con-

taining large amounts of DPPC, which by itself normally does not spread spontaneously at air/water interfaces (Hawgood et al., 1985; Takahashi et al., 1990; Yu and Possmayer, 1990).

In the treatment of respiratory distress syndrome (RDS), which results from the lack of lung surfactant, surfactant replacement materials that are effective, inexpensive, and easy to process are desired (Tanaka et al., 1986; Shapiro, 1989; Jobe, 1993). However, mixtures of synthetic phospholipids alone do not possess surface properties comparable to those of natural lung surfactant. In contrast, materials reconstituted with synthetic phospholipids and the hydrophobic protein SP-B appear to impart full biophysical properties akin to those of lung surfactant (Revak et al., 1991). 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 in bilayer vesicles (Baatz et al., 1990; Oosterlaken-Dijksterhuis et al., 1992; Vincent et al., 1993). 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 (Revak et al., 1988; Waring et al., 1989; Baatz et al., 1991; Longo et al., 1993). To mimic such a characteristic structure, 21 amino acid peptides containing the hydrophobic amino acid leucine (L), with either cationic arginine (R) or lysine (K), in the sequence (RL₄)₄R or (KL₄)₄K, respectively, were synthesized and found to enhance the monolayer properties of DPPC/PG systems significantly, similar to the effect of SP-B (Cochrane and Revak, 1991). An artificial lung surfactant, containing 1,2-dipalmitoylphosphatidylcholine/1-palmitoyl-2-oleoylphosphatidylglycerol/palmitic acid (DPPC/POPG/PA) and KL4, was reported to be very effective in the clinical treatment of animal and

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human RDS (Cochrane et al., 1996; Revak et al., 1996). Therefore, it appears that such a peptide behaves very similarly to SP-B and provides a means of substituting for SP-B in reconstituted surfactant systems. The study of the surface properties of this peptide alone and in the presence of the various relevant lipids should broaden our understanding of the role of the peptide in this system and, in turn, provide a better mechanistic model for lung surfactant structure and function in general.

In most *in vitro* lung surfactant studies involving monolayers formed at the air/water interface, either a bubble surfactometer or a Langmuir monolayer apparatus is used to measure dynamic surface tension when the available surface area is rapidly expanded and compressed. In these dynamic measurements it is often difficult to capture a physical picture of molecular events, because the exact composition of different components at the surface under a given set of conditions is unknown (Prokop and Neumann, 1997). It appeared important to us, therefore, to step back and to examine the more static properties of monolayers containing one or more typical molecules used in such model studies. Recognizing that static properties do not exactly mimic the dynamic situation, in this approach we are in a position to better establish any relationships between exact composition and surface pressure. Thus we report the equilibrium spreading pressure π_e , π - A isotherms (surface pressure π versus area per molecule A), and the surface phase behavior of KL4, DPPC, POPG, and PA monolayers individually and as mixtures under the same conditions. Besides the major component DPPC, we have used POPG, because it represents a major unsaturated anionic component in lung surfactant, and PA, because it has been used in earlier studies dealing with the possible role of SP-B (Longo et al., 1993) and because it has been included in artificial lung surfactant replacement systems containing DPPC, POPG, and KL4 (Cochrane and Revak, 1994).

MATERIALS AND METHODS

Materials

The lipids used in this study were 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-*rac*-glycerol (POPG), palmitic acid (PA), and 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-glycero-3-phosphocholine (NBD-PC). They were obtained from Avanti Polar Lipids (Alabaster, AL), with stated purities of 99+%, and were used as received. The POPG was supplied as its sodium salt, [Lysine-(leucine)₄]-lysine, KL4, synthesized as described previously (Revak et al., 1991), was a gift from the R. W. Johnson Research Institute. Throughout this study the amounts of the various ingredients were maintained at a constant molar ratio of DPPC:POPG:PA:KL4 equal to 84:27:48:1 or a weight ratio of 26:8:5:1. This ratio reflects the levels of each component used in earlier clinical studies (Cochrane and Revak, 1994).

Chloroform (99.9% high-performance liquid chromatography (HPLC) grade; Aldrich):methanol (99.9% HPLC grade; Fisher Scientific) (70:30 v/v) was used as the spreading solvent for all mixtures, and each solvent was used as received. The purity of chloroform and methanol was checked by spreading the solvents on water and monitoring surface tension as the surface was compressed. No decrease in surface tension was observed upon

compressing the total area by 70%, so it was assumed that no significant amounts of any surface-active impurities were present in the solvent. All solutions were stored at -20°C .

The water used throughout these studies was triply distilled. The house-distilled water was passed through a Barnstead PCS filtration system and then distilled two additional times, once from an alkaline potassium permanganate solution and once from a dilute sulfuric acid solution. The subphase pH was controlled with a 0.02 M Tris(hydroxymethyl)aminomethane (Tris) (99.9%, Sigma) buffer that was adjusted to pH 7.2 with 1 M acetic acid, HAc (A.C.S. reagent grade; Aldrich). All monolayers were studied on the Tris-HAc buffer subphase with 0.13 M sodium chloride (99+%; Aldrich).

Methods

Equilibrium spreading pressure π_e

Various mixtures of lipids and KL4 were dissolved in the 70:30 mixture of chloroform and methanol at room temperature and then dried under vacuum in a liquid nitrogen bath for at least 12 h. Solid material was sprinkled onto the clean aqueous surface, and the surface tension was monitored using a sand-blasted Wilhelmy plate attached to an Ohaus GA 200-D electronic balance (Ohaus Corp, Florham Park, NJ) equipped with an RS232 port and interfaced to a Macintosh Plus computer. The monolayer was formed on a subphase contained in a Teflon trough designed to be maintained at constant temperature by circulating thermostatted water through a glass coil at the bottom of the trough. This set-up was contained in a larger plexiglass box to reduce drafts. The relative humidity in the box was maintained at 80% or more with open glass containers of water and wet filter paper. It was critical to be sure that the recorded surface tension represented an equilibrium between two coexisting phases, i.e., bulk material and the spread monolayer. Therefore final values of π_e were chosen when there was no further significant change in surface tension with time (less than 0.2 mN/m change per hour), when the value was independent of the amount of excess bulk material on the surface and of the total surface area upon which the monolayer was spread.

Static surface pressure-area isotherms

Surface pressure was measured as described above, except for those experiments in which the Wilhelmy plate was attached to a Cahn 2000 electrobalance (Cahn Co., Paramount, CA) with a chart recorder (Kipp and Zoen model BD40). The chloroform:methanol (70:30 v/v) lipid solutions were spread with a Hamilton microsyringe (Hamilton Co., Reno, NV) and allowed to equilibrate for anywhere between 30 min and 24 h, depending on the mixture identity and state of the monolayer. A mixed spreading technique, in which all components were dissolved into a single spreading solvent, was adopted in all the experiments. The static surface tension was determined when the change in mass of the plate was less than 1 mg (or less than 0.2 mN/m) in 1 h. The area per molecule (A) was varied by three methods: 1) continuous addition of a spreading solution (after the attainment of equilibrium) to a surface of constant area, 2) stepwise reduction of surface area with a movable barrier with a fixed amount of spread material (compression method), and 3) repeated cleaning and spreading of the monolayer film to a given surface concentration (single-shot method). Surface pressure versus area isotherms for all monolayers were constructed by each of the three methods. Good agreement among the three methods was confirmed with both expanded and condensed regions of a monolayer.

The software package, Insight II (v. 2.3.0), developed by Biosym Technologies (San Diego, CA), was used to model the structures of peptide KL4. Three-dimensional structures of KL4 in different conformations were generated and displayed on a SGI computer.

Fluorescence microscopy

The fluorescence microscope used in this study was the same as the one mentioned in a previous study (Koppenol et al., 1997). It was constructed

from a commercially available microscope (Micromaster, Model E; Fisher Scientific). The excitation light from a tungsten-halogen lamp (Fiber-Lite, Dolan-Jenner Industries) was passed through a filter (Zeiss) and reflected down to the water surface via a dichroic mirror (Zeiss). The fluorescence from the surface was collected by the objective lens (40 \times) and passed through the dichroic mirror and an additional barrier filter (Zeiss). The microscope was connected to a video camera (Optronics Engineering) via a video relay lens (10 \times) (Fisher Scientific), and the image was viewed on a monitor (Apple monitor III) and stored on a Videotape (Mitsubishi HS-U65).

The monolayer mixture, which contained 0.5–1% NBD-PC, was spread and allowed to equilibrate before any images were recorded. Surface pressure was measured with a Nima pressure transducer (Nima, Coventry, England). Preliminary studies revealed a negligible effect of the dye on the π -A diagram of the peptide and each phospholipid.

RESULTS

Equilibrium spreading pressure

The equilibrium spreading pressure (π_e) values of each component studied, plus selected mixtures, are presented in Table 1. The value reported for PA was obtained not by spreading, but by adsorption upon saturating the underlying substrate solution with palmitic acid. Because of the significant solubility of the palmitate ion at pH 7.2, it was not possible to spread PA on pH 7.2 water. We emphasize that a stable π_e value was not obtained without saturating the subphase. Consistent with earlier observations (Koppenol, 1996), DPPC showed essentially no tendency to spread at 25°C over a 24-h period. However, at 45°C, \sim 4°C above the critical temperature T_c at which DPPC bilayers undergo a gel-to-liquid-crystalline phase transition, π_e was found to reach 45 mN/m within 1 min. This indicates the very poor spreading characteristics of DPPC at temperatures below 41°C. On the other hand, the much more fluid POPG ($T_c \approx 0^\circ\text{C}$) spread within seconds to a value of π_e equal to 46 mN/m. The peptide KL4 showed a tendency to spread to

\sim 30 mN/m at 25°C, but it took 20 h to attain this equilibrium value.

In Table 1, the data for various binary mixtures of KL4 with DPPC, POPG, or PA are also presented. Note that a mixture of DPPC/KL4 spread with a π_e value very close to that of KL4 itself, whereas that for POPG/KL4 is just slightly greater than that of POPG alone. In the case of PA, where a stable π_e spreading on a pH 7.2 subphase could not be determined because of dissolution, the presence of KL4 produced a stable monolayer with π_e comparable to the value for PA on a saturated subphase. In Table 1 we also report values of π_e for mixtures of DPPC/POPG and DPPC/POPG/PA in the molar ratios described above, in the absence and presence of KL4. The DPPC/POPG binary mixture spreads to an equilibrium value of 17 mN/m, less than half that of POPG alone. This is unlike the case of either DPPC/KL4 or POPG/KL4 mixtures, where the π_e value is very similar to that of the component with the higher π_e in the mixture. For the three-component mixture of DPPC/POPG/PA, π_e is very similar to that of DPPC/POPG, i.e., much lower than that of POPG alone. It is interesting to note the significant increase in π_e when the small amount of KL4 is added to either mixture.

Static surface pressure-area (π -A) isotherms

Fig. 1, *a–d*, contains various π -A isotherms at 25°C for KL4, DPPC, POPG, and PA individually, and with corresponding data for each of the lipids in the presence of KL4. In Table 1 are listed values of π_{max} , the maximum surface pressure attainable under static conditions. As mentioned previously in Materials and Methods, at collapse for each system. π_{max} is not reported for PA, because of its significant dissolution under the conditions of this experiment. Note that, in Table 1, with the exception of DPPC, the values of π_e and π_{max} for each sample system are about the same, indicating that π_{max} represents the surface pressure at equilibrium between the monolayer and bulk phases. For DPPC, we note the very high π_{max} relative to a π_e being 0, which reflects its unique ability to maintain itself in a highly overcompressed state despite its poor initial spreading characteristics.

The π -A curve for KL4 alone appears to be very stable up to a π_{max} of \sim 30 mN/m. If we assume at “lift-off,” where the surface pressure begins to increase significantly above zero, that the entire surface is covered with peptide molecules, it is possible to estimate the area per KL4 molecule to be \sim 3.5 nm². The possible meaning of this value in the context of computer modeling results will be presented in the Discussion.

As presented in Fig. 1, *b* and *c*, the π -A isotherms for DPPC and POPG are in excellent agreement with earlier reports (Koppenol, 1996). The lack of a stable surface pressure for PA alone at pH 7.2 illustrates the strong tendency of PA, in the form of palmitate ion, to dissolve into the underlying subphase. Fig. 1, *b–d*, also depicts the π -A

TABLE 1 Equilibrium spreading pressure, π_e , and collapse pressure, π_{max} , at 25°C on pH 7.2 Tris buffer for various components and their mixtures*

System	π_e (mN/m)	π_{max} (mN/m)
DPPC	0	42
POPG	42	44
PA [#]	26	—
KL4	32	32
DPPC/KL4	32	32
DPPC/KL4	32	41
POPG/KL4	46	43
PA/KL4	27	24
DPPC/POPG	17	21
DPPC/POPG/PA	23	21
DPPC/POPG/KL4	41	42
DPPC/POPG/PA/KL4	42	40

*Compositions of all mixtures are in the molar ratio of 87:27:48:1 (DPPC:POPG:PA:KL4). See Materials section for discussion.

[#]The equilibrium spreading pressure of PA at pH 7.2 was measured on a subphase saturated with PA. On an unsaturated subphase, stable values of π_e and π_{max} could not be obtained.

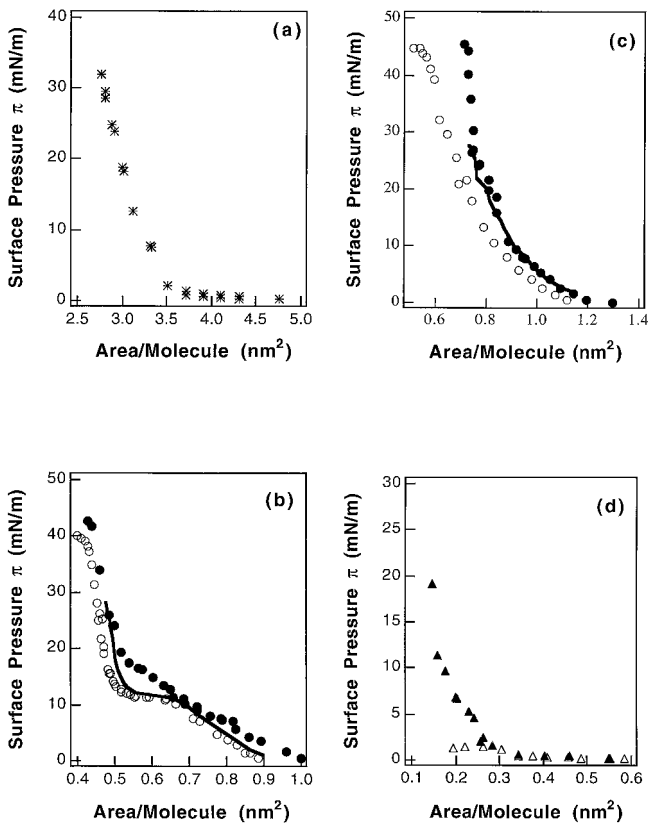


FIGURE 1 (a) Surface pressure (π) versus area per molecule (A) isotherm at 25°C for KL4 (*) monolayer spread on 0.02 M Tris buffer, pH 7.2, 0.13 M NaCl. (b) π - A isotherm at 25°C for DPPC (○) and binary mixture of 84:1 DPPC/KL4 (●) monolayers spread on the same Tris buffer as in *a*. The solid curve (—) represents the calculated π - A isotherm according to the proportion of each component in the mixture. (c) The π - A isotherm at 25°C for POPG (○) and a binary mixture of 27:1 POPG/KL4 (●) monolayers spread on the same Tris buffer as in *a*. The solid curve (—) represents the calculated π - A isotherm according to the proportion of each component in the mixture. (d) π - A isotherm at 25°C for PA (△) and a binary mixture of 48:1 PA/KL4 (▲) monolayers spread on the same Tris buffer as in *a*.

isotherms at 25°C in the presence of KL4 for DPPC, POPG, and PA, respectively. The area per molecule for a mixture at a particular π was calculated as the total surface area of the trough divided by the total number of molecules on the available surface. As seen in the various figures, KL4 has a small but significant effect in shifting the π - A isotherms of DPPC and POPG to the right, producing a greater π for a given surface area than observed with the phospholipids alone. As shown in Fig. 1 *d*, the PA/KL4 mixture forms a stable monolayer on the surface. According to our calculations, this surface pressure cannot result merely from the small amount of surface active KL4 present in the mixture (1:48), and thus KL4 has a significant effect in stabilizing the PA in monolayer, i.e., producing a π_{\max} equal to that of π_e (Table 1). This stabilization would appear to be due to the electrostatic interaction of KL4 with PA to form a more water-insoluble species, with perhaps some dissolution of any excess ionized PA. At a molar ratio of 1:48, if all five

lysines on each KL4 molecule were involved in an interaction with PA, the charge ratio would be roughly 5:48, and there still would be excess PA ions that could desorb.

In Fig. 2, *a* and *b*, are given the π - A isotherms of DPPC/POPG versus DPPC/POPG/PA and DPPC/POPG versus DPPC/POPG/KL4 versus DPPC/POPG/PA/KL4 in the usual proportions. Again, in Table 1 the values of π_{\max} are presented, and they are shown to agree very well with the corresponding values of π_e . In the case of DPPC/POPG/PA, the area used to construct the π - A isotherm in Fig. 2 *a* was calculated by assuming that no PA was present, because of complete desorption from the surface. Indeed, agreement between this isotherm and that for DPPC/POPG is excellent when compared in this manner. Also note in Table 1 that both π_{\max} and π_e are the same for DPPC/POPG and DPPC/POPG/PA, again indicating that the complete desorption of PA most likely has occurred. The fact that π_{\max} for DPPC/POPG binary mixture agrees with its π_e , and only reaches 20 mN/m, a value about half of the π_{\max} for DPPC and POPG, appears to be important to our understanding of this system and will be discussed later. In this regard it is also interesting to note that KL4 added to both DPPC/POPG and

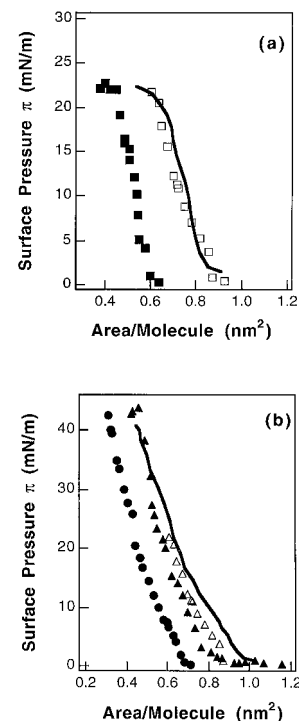


FIGURE 2 (a) The π - A isotherm at 25°C for a DPPC/POPG (84:27) mixture (□) and a ternary mixture (84:27:48) of DPPC/POPG/PA (■) monolayers spread on the same Tris buffer as in Fig. 1 *a*. The solid curve (—) represents the calculated π - A isotherm for a DPPC/POPG/PA mixture, assuming complete desorption of PA from the surface. (b) The π - A isotherm at 25°C for a DPPC/POPG (84:27) mixture (△), a DPPC/POPG/KL4 (84:27:1) mixture (▲), and a DPPC/POPG/PA/KL4 (84:27:48:1) four-component system (●) of monolayers spread on the same Tris buffer as in Fig. 1 *a*. The solid curve (—) represents the calculated π - A isotherm for DPPC/POPG/PA/KL4, assuming complete desorption of PA from the surface.

DPPC/POPG/PA (Fig. 2 *b*) produces more condensed monolayers with π_e and π_{\max} values that are now increased from 20 mN/m to ~ 40 – 42 mN/m.

Fluorescence microscopy

In the fluorescence images, a homogeneous light field represents a liquid-expanded (LE) monolayer, whereas the appearance of dark spots reflects a liquid-condensed phase that cannot be penetrated by the fluorescence probe. An increase in the fraction of the dark regions therefore reflects greater surface phase separation. Although images are not shown here, tracer amounts of NBD-PC dye mixed with either KL4 or POPG show that the individual components form homogeneous liquid-expanded monolayers over the whole π range. In Fig. 3 we present a series of fluorescence microscopic images of four monolayer systems over roughly the same surface pressure range, ~ 3 – 25 mN/m, where each monolayer is stable over the time scale of the experiments. Consistent with previous reports (McConnell, 1991), DPPC itself undergoes an LE to LC phase transition

at a π of 10 mN/m, corresponding to the plateau observed in the π -*A* isotherm. The DPPC and POPG mixed monolayer without KL4 exhibits surface phase separation at a π of ~ 8 mN/m, but at higher π less condensed phases are formed compared to that of DPPC alone at the same surface pressure. The addition of KL4 to the DPPC/POPG or DPPC/POPG/PA systems greatly increases surface phase separation at very low values of π , whereas at higher π , more LC phase is formed. We presume that the darker condensed regions are predominantly DPPC-rich, whereas the expanded phase is predominantly POPG-rich and POPG/PA-rich. It is assumed that this effect on surface phase separation by KL4 is most likely linked in part to its ability to electrostatically interact with POPG and PA.

DISCUSSION

Surface properties of the peptide KL4

The synthetic peptide KL4, when present in a relatively small amount, exerts a significant effect on the monolayer

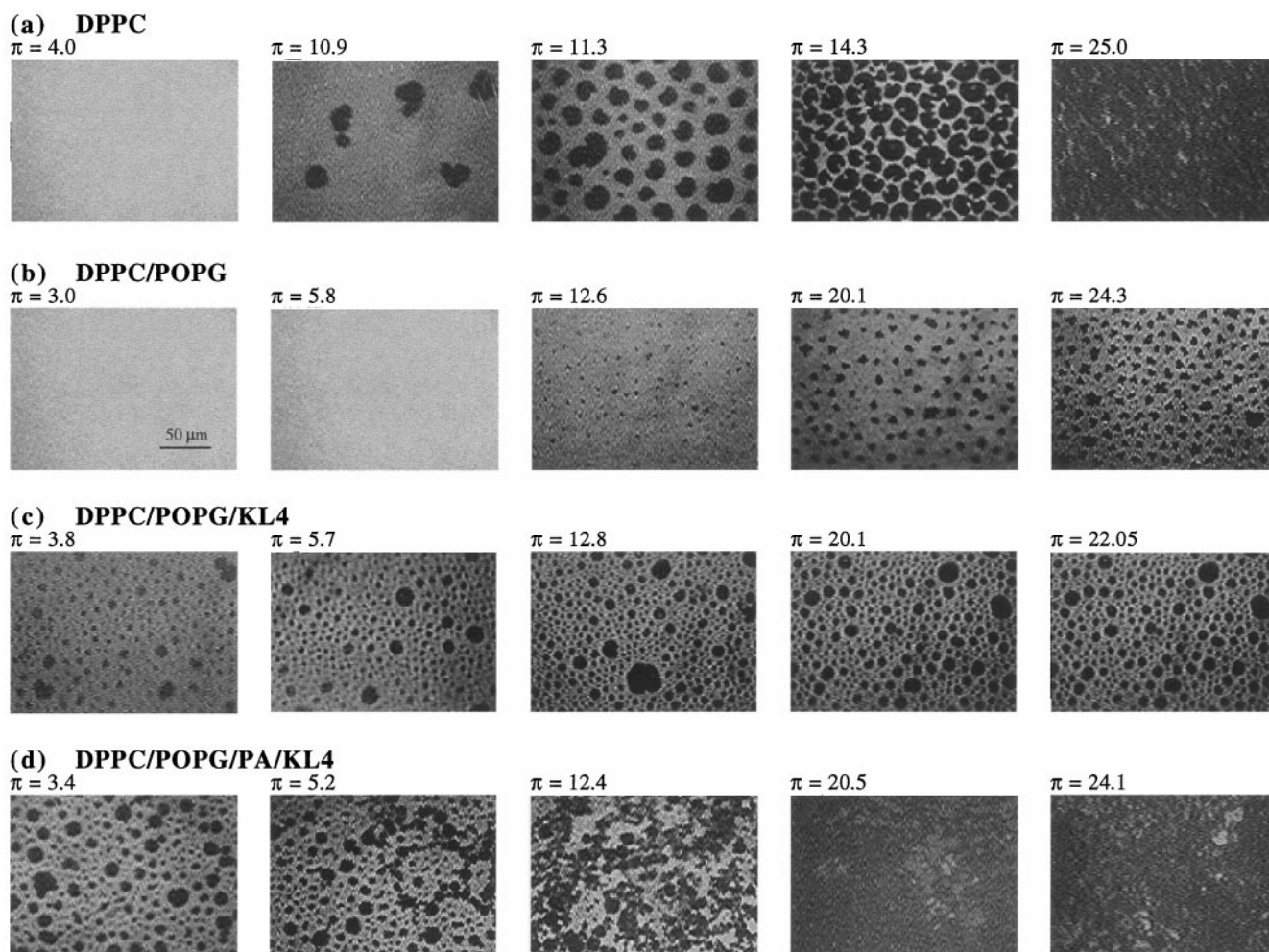


FIGURE 3 Fluorescence images of various monolayers, spread at 25°C on 0.02 M Tris, pH 7.2, 0.13 M NaCl. (a) DPPC; (b) DPPC/POPG (84:27); (c) DPPC/POPG/KL4 (84:27:1); (d) DPPC/POPG/PA/KL4 (84:27:48:1).

behavior of the lipid combination used in this study and as a clinical substitute for natural lung surfactant. Further investigation of its conformation at the interface, therefore, would be useful. We have shown above that KL4 alone forms very stable monolayers up to a π of 30 mN/m, and assuming closest packing at lift-off, an area per KL4 molecule of 3.5 nm² was estimated. This translates into ~ 0.17 nm²/amino acid, which is consistent with the literature values of ~ 0.2 nm²/amino acid for amphiphilic proteins on the surface, in general, and for SP-B (Taneva and Keough, 1994). Using the Insight program, and assuming that a conformation with its backbone oriented parallel to the interface is adopted by the peptide, and that the area per molecule at closest packing is a circle swept out by the backbone end-to-end distance, we calculated an area per molecule of 18 nm² and 48 nm², respectively, for the α -helix and β -sheet. Clearly, the value obtained from the π -A isotherm corresponds more closely to that of the α -helix, but it is still significantly different. From a three-dimensional view of the α -helix peptide, it can be observed that the five charged lysine groups are evenly distributed around the axis of the helix. We would expect, however, that the charged groups would orient preferentially at the air/water interface toward the water, resulting in a twisted α -helix. We have estimated the dimensions of such a distorted α -helix and found the area per molecule to be 4.5 nm², in much better agreement with the experimental value of 3.5 nm²/molecule. It is interesting to note that SP-B is an amphiphilic protein (Takahashi et al., 1990) with a predominant secondary structure in monolayers and in lipid bilayers, reported to exist as strands of α -helix and helix in a distorted form (Perez-Gil et al., 1993; Kang et al., 1996).

Lipid/lipid and lipid/peptide interactions in monolayers of binary mixtures

It is generally acknowledged that the ability of natural and artificial lung surfactants to attain a low surface tension is due to the presence of a high proportion of DPPC. Furthermore, the presence of other components, such as PG, unsaturated phospholipids, and the hydrophobic proteins SP-B and SP-C, promote the normally poor spreading characteristics of DPPC, below its gel-to-liquid-crystalline transition temperature of 41°C. It is interesting to note from this study that the mixture of DPPC and POPG, and that of DPPC, POPG, and PA cannot achieve as high a static surface pressure upon compression as do DPPC and POPG alone. Indeed, for both mixtures, the values of π_e and π_{max} are reduced by more than half for the compositions used in this study. The fact that a simple mixture of DPPC and POPG is not enough to provide proper properties is also demonstrated in the very slow rates of adsorption from DPPC/POPG liposomes previously reported (Yu and Possmayer, 1992; Wang et al., 1995). From a previous study of bulk phase behavior using thermal analysis (unpublished results from this laboratory) and NMR (Wiedmann et al., 1993),

there appears to be a high level of miscibility of these two components in the bilayer. The low π_e and π_{max} of ~ 20 mN/m for these mixtures (Table 1) likewise points to significant miscibility between DPPC/POPG, which reduces the ability of POPG to spread, and for both components to attain a high collapse pressure. In the absence of KL4 at pH 7.2, it is shown that PA does not contribute to the overall surface activity, because of desorption of palmitate ions to the underlying aqueous solution. From the fluorescence microscopic studies it can be seen that a LE/LC phase transition occurs in this mixture, although no plateau is observed in the π -A isotherm (Fig. 2 *a*). This is a further indication that DPPC and POPG exhibit some level of miscibility in the monolayer. Quantification of the dark domains (LC phase) in a DPPC/POPG (1:1) mixture indicated less than the expected 50% LC phase, if DPPC was completely immiscible with POPG under conditions similar to those of this study (Koppenol et al., 1997). Thus, although we cannot quantify the area fraction of dark domains in the 84:27 mixture of DPPC/POPG because of very irregular domain shapes, all of our results suggest that not all of the DPPC "precipitates" as a separated LC surface phase upon compression. We conclude that the LC phase is highly enriched in DPPC, that the LE phase consists of the remaining DPPC "dissolved" in POPG, and that it is this partial miscibility of DPPC and POPG that gives rise to reduced values of π_e and π_{max} . In this context, therefore, we hypothesize that any factor tending to promote the phase separation of DPPC from POPG should improve the ability of the mixture to attain high surface pressures and to respread.

To evaluate this hypothesis further, we can analyze the nature of the various experimental π -A isotherms in relation to those calculated from the individual component isotherms, assuming individual contributions to π -A in proportion to composition at any π . If the calculated isotherm superimposes on the experimental isotherm, we have either "ideal" mixing or complete surface immiscibility. If the experimental isotherm is shifted to the left of the calculated one (more condensed), we can assume miscibility and a negative deviation from ideality due to strong intercomponent interactions. A shift to the right (more expanded) leads to miscibility with a positive deviation from ideality, suggests stronger interactions between like components than between unlike, and indicates a tendency to demix (Gaines, 1966). However, care must be taken in relating surface free energy to interactions, because one must also consider the entropic contribution to the surface free energy related to packing and steric factors.

As shown in Fig. 1 *b*, for the DPPC/KL4 mixture the experimental π -A curve is more expanded than the calculated one, indicating that the interaction between KL4 and DPPC may be slightly repulsive. This, together with the fact that π_e for DPPC/KL4 is the same as that of KL4 alone, suggests that DPPC and KL4 likely form a highly immiscible mixture. For the POPG/KL4 mixture, experimental and calculated π -A curves at $\pi < 25$ mN/m agree quite

well, and thus there is no hint of any strong specific interaction between the two oppositely charged components. It is impossible to compare calculated and measured π - A curves beyond the KL4 collapse pressure of 32 mN/m. However, there are some features of the POPG/KL4 isotherm at high surface pressure regions (>30 mN/m) that are worth pointing out. First, there is an increased deviation of POPG/KL4 isotherm from that of POPG alone, which is also observed as a steeper POPG/KL4 π - A curve with greater slope. Second, no discontinuity or kink point is observed at the KL4 collapse pressure of 32 mN/m. These features strongly indicate that KL4 is retained in the monolayer, rather than being squeezed out, even at $\pi > 32$ mN/m. We attribute the retention of KL4 above its π_{\max} to interaction between POPG and KL4 by electrostatic attraction. On the other hand, electrostatic attraction alone cannot account for the observed steeper slope of the isotherm at this high π region. Therefore, packing or steric effects may play a role in the monolayer of POPG/KL4.

Surface phase separation induced by KL4 in monolayers of three- or four-component systems

As KL4 is added to the DPPC/POPG mixture, the monolayer becomes more condensed than that of DPPC/POPG alone, indicating some level of attractive interaction. This condensation effect, together with the increased π_e and π_{\max} for the ternary system relative to DPPC/POPG alone, was also observed when Ca^{2+} was injected into the subphase of a DPPC/POPG monolayer, only to a less significant extent (Koppenol et al., 1997). Ca^{2+} also was shown to affect the surface phase behavior of DPPC/POPG in a manner similar, but less significant than that of KL4, i.e., promoting LC phase formation (increased number and size of domains) at comparable π , and it was suggested earlier that the solubility of POPG in DPPC at the air/water interface is reduced by the presence of Ca^{2+} (Koppenol et al., 1997). Studies of mixtures of other zwitterionic and anionic phospholipids also showed phase separation to be induced or enhanced by the addition of Ca^{2+} , or by positively charged proteins (Gilmanshin et al., 1994; Nag et al., 1994; Williams et al., 1995). Such lateral phase separation recently has been observed with monolayers of extracted calf pulmonary sample (Discher et al., 1996). In a monolayer of the DPPC/POPG/KL4 mixture, the same type of selective electrostatic interaction between KL4 and POPG occurs, leading to surface phase separation, although it occurs within the surface instead of binding from beneath the surface in the subphase. The increased immiscibility between DPPC and POPG in the presence of small amounts of KL4 within the monolayer therefore gives rise to the increased π_e , π_{\max} and to the better overall spreadability of the ternary mixture.

The use of PA in the system and the amount used were primarily motivated by its inclusion in the artificial lung surfactant system developed with the use of KL4 (Cochrane

and Revak, 1994). However, other studies involving SP-B have placed great emphasis on the stabilizing effects of SP-B on PA monolayers through electrostatic interaction, and the potential significance of these effects in the functioning of lung surfactant (Longo et al., 1993; Lipp et al., 1996, 1997). Whatever the ultimate importance of SP-B and PA interactions in actual lung surfactant, from this study it has been shown that KL4, a possible model for SP-B, is essential in maintaining a stable monolayer containing PA in the ionized state at physiological pH values around 7.0. With PA maintained in a monolayer of DPPC and POPG by the presence of KL4, the same effect of raising π_e and π_{\max} occurred, as with DPPC/POPG. Thus in this sense PA is not actually required to see an effect of KL4 on π_e and π_{\max} . However, from the π - A isotherms (Fig. 2 *b*) and fluorescence micrographs, we clearly see some significant differences between DPPC/POPG/KL4 and DPPC/POPG/PA/KL4. First of all, as PA is added to the DPPC/POPG/KL4 mixture, the isotherm of the four-component system, plotted assuming the absence of PA (*solid line*), is significantly more expanded than the actual DPPC/POPG/KL4 curve, in contrast to DPPC/POPG versus DPPC/POPG/PA, where PA appears to be absent from the monolayer because of desorption. Thus PA is retained in the four-component system because of KL4. More striking are the corresponding fluorescence images, which show that surface separation is greatly enhanced when PA is added to the DPPC/POPG/KL4 system. Thus PA may indeed, through introduction of KL4, provide an even greater tendency toward the phase separation of DPPC from other components, which is required for enhanced lung surfactant activity.

These conclusions appear to be quite consistent with earlier spectroscopic studies of SP-B and KL4 with POPG alone, and with a mixture of DPPC/DOPG formed as bilayers (Baatz et al., 1991; Vincent et al., 1993). Here it was shown that although no dramatic perturbation of the inner structure of the POPG bilayer occurred, the DPPC/DOPG lipid bilayers were markedly ordered by SP-B or KL4. Moreover, the very broad phase transition temperature range observed indicated far less cooperativity of the mixture in the transition region, which implies a phase separation of lipids induced by SP-B or KL4.

CONCLUSION

In summary, we report that through electrostatic interactions with both POPG and PA in a monolayer, KL4 exerts its effects by promoting phase separation of these lipids from DPPC, resulting in exclusion of other components from the DPPC phase, but not from the surface. It is a widely held belief that pulmonary surfactant must undergo substantial refinement of its composition before it can achieve a high surface pressure. We propose here that this refinement proceeds in part at least via a mechanism of surface phase separation, which is induced by a cationic peptide such as KL4. It is the formation of these phase-

separated domains that may reconcile the apparent contradictory attributes of lung surfactant in artificial and natural systems, i.e., efficient spreading of the anionic lipid LE phase and the ability to sustain a high surface pressure caused by the DPPC-rich phase.

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