

Penetration Depth of Surfactant Peptide KL₄ into Membranes Is Determined by Fatty Acid Saturation

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ABSTRACT KL₄ is a 21-residue functional peptide mimic of lung surfactant protein B, an essential protein for lowering surface tension in the alveoli. Its ability to modify lipid properties and restore lung compliance was investigated with circular dichroism, differential scanning calorimetry, and solid-state NMR spectroscopy. KL₄ binds fluid lamellar phase PC/PG lipid membranes and forms an amphipathic helix that alters lipid organization and acyl chain dynamics. The binding and helicity of KL₄ is dependent on the level of monounsaturations in the fatty acid chains. At physiologic temperatures, KL₄ is more peripheral and dynamic in fluid phase POPC/POPG MLVs but is deeply inserted into fluid phase DPPC/POPG vesicles, resulting in immobilization of the peptide. Substantial increases in the acyl chain order are observed in DPPC/POPG lipid vesicles with increasing levels of KL₄, and POPC/POPG lipid vesicles show small decreases in the acyl chain order parameters on addition of KL₄. Additionally, a clear effect of KL₄ on the orientation of the fluid phase PG headgroups is observed, with similar changes in both lipid environments. Near the phase transition temperature of the DPPC/POPG lipid mixtures, which is just below the physiologic temperature of lung surfactant, KL₄ causes phase separation with the DPPC remaining in a gel phase and the POPG partitioned between gel and fluid phases. The ability of KL₄ to differentially partition into lipid lamellae containing varying levels of monounsaturations and subsequent changes in curvature strain suggest a mechanism for peptide-mediated lipid organization and trafficking within the dynamic lung environment.

INTRODUCTION

Lung surfactant is a lipid-rich substance containing key proteins that minimizes surface tension in the alveoli and provides a barrier against disease (1,2). Lung surfactant undergoes a cycle of lipid adsorption and resorption at the alveolar air-fluid interface. Lipid polymorphisms that change the geometry and arrangement of lipid headgroups have been postulated to be critical for this process not only in the lung, but also in other membrane-membrane mediated events (3,4). The lipid constitution in mammalian lung surfactant is heterogeneous but is dominated by zwitterionic PC (70–80%) and 10–20% anionic PG and PI (5); ~50% of the PC lipids and almost all of the anionic lipids in lung surfactant

are monounsaturated. Levels of the lipids are relatively conserved in the lungs of vertebrates (6), with the preponderance of DPPC being particularly striking. The stability of DPPC-enriched monolayers at air-water interfaces is of particular relevance in the lung environment. However, the lipid composition of lung surfactant is not sufficient to explain its unique properties. Critical to its function are the lung surfactant proteins, which are present at relatively low levels (7). In particular, SP-B, which accounts for 0.7–1.0% of the dry weight of lung surfactant, or <0.2 mol % relative to the lipids, is required for proper lung function (8). Disruption of the SP-B gene in mice confers embryonic lethality (9) and mutations cause respiratory failure (10,11). Inadequate protein levels are a leading cause of RDS in premature infants (12).

A multitude of roles for SP-B in surface tension minimization, intracellular surfactant trafficking, and respiratory dynamics in general have been proposed and experimentally established (13). Current therapies for RDS primarily rely on administration of lung surfactant isolates from animal sources to replenish SP-B (14,15). The native form of SP-B is a highly hydrophobic, 78–81 residue protein that functions as a homodimer and contains seven disulfide bridges (16–18). The hydrophobicity and disulfide bridges within SP-B make purification or heterologous expression of the protein in large quantities untenable. Structurally, SP-B has been postulated to be primarily helical based on CD and FTIR studies of the isolated protein in a phospholipid environment (19,20). However, more detailed structural studies of SP-B

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Abbreviations used: PC, phosphatidylcholines; CD, circular dichroism; PG, phosphatidylglycerol; PI, phosphatidylinositol; CPMAS, cross-polarization with magic angle spinning; CSA, chemical shift anisotropy; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPC-d₆₂, 1,2-d₆₂-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; H_{II}, inverted hexagonal phase; LUV, large unilamellar vesicle; L_{αα}, fluid lamellar phase; L_β, gel lamellar phase; MLV, multilamellar vesicle; NMR, nuclear magnetic resonance; P/L, peptide/lipid molar ratio; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPC-d₃₁, 1-d₃₁-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPG 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol; POPG-d₃₁, 1-d₃₁-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol; RDS, respiratory distress syndrome; SP-B, surfactant protein B; ssNMR, solid-state nuclear magnetic resonance; TFE, trifluoroethanol; TM, transmembrane.

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have been precluded by its hydrophobicity and consequent problematic isolation and purification (21–23). Synthetic, peptide-based lung surfactant replacements designed to replicate the properties of SP-B have received noticeable attention (24) as the use of synthetic analogs would remove the immunologic risks associated with animal-derived surfactant and allow for greater restorative consistency. Peptide mimics that rely on the presumption of amphipathic helicity in SP-B have been pursued both clinically and as model systems for understanding the structure and dynamics of the protein and lipids that impart the unique physical properties of lung surfactant. Understanding how peptide analogs of SP-B alter lipid properties could lead to the design of SP-B mimetics with enhanced biophysical properties and therapeutic lifetimes. For example, oligo N-substituted glycines (phenylethyl, 2-butyl, or 4-aminobutyl substituents attached to the nitrogen of glycine), or peptoids, have been designed to mimic the helicity of SP-B and have shown potential (25).

KL₄, or sinapultide, was designed based on amino acid residues 59–80 of SP-B and has particularly strong surface tension reducing properties (26). The primary amino acid sequence of KL₄ is a repeating pattern of four hydrophobic leucines separated by hydrophilic lysines: KLLLLKLLLLK LLLLLKLLLLK. KL₄ has shown promise as a replacement for therapies that rely on exogenous SP-B (27–31). KL₄-based surfactant therapy relies on a formulation of the peptide with DPPC, POPG, and a spreading agent, palmitic acid. Molecular-level information on how synthetic peptides can modulate surface tension in alveolar compartments is limited; however, lucinactant, the clinical name for KL₄ in a lipid formulation, has been approved as an agent for treatment of RDS because of its efficacy. In vitro assays as well as animal studies have shown the ability of KL₄ to lower surface tension in different lipid systems (26,32). In clinical studies, administration of lucinactant to very premature infants was noticeably more effective in treating RDS than other commercially available formulations (33). KL₄ improves respiratory status and was found to prevent the appearance of diffuse, granular radiopacity seen in chest radiographs typical of RDS and meconium aspiration syndrome (27,28). A recent antimicrobial function of KL₄ was also suggested, in line with recent reports concerning the antibacterial properties of SP-B (34,35). However, the biophysical properties of KL₄ and their relation to alleviating RDS remain the focal point of current investigations, particularly in regard to its structure and organization in lipid environments.

Artificial lung surfactant formulations simplify the problem of studying the underlying biophysical aspects of lung surfactant function against the complicated, heterogeneous background that exists in vivo. Of particular interest is that KL₄ retains many of the macroscopic properties of SP-B even though it bears little resemblance to the biologically produced protein other than a similarity in charge distribution to the C-terminus.

With an understanding of the properties of KL₄, nonnatural peptide analogs of SP-B that exploit the protein's helical and amphipathic qualities could be made to extend or enhance artificial lung surfactant therapies. Thus, understanding how KL₄ affects the molecular and biophysical properties of lipids is of particular relevance for the treatment of various forms of RDS. The use of calorimetry and CD in combination with ssNMR can provide unique insight into KL₄-induced changes in lipid phase properties. In this study, static ²H and ³¹P NMR, MAS ¹³C NMR, CD, and DSC were employed to investigate the properties of mixtures of KL₄ with 4:1 DPPC/POPG and 3:1 POPC/POPG lipid vesicles. The former lipid composition was selected to mirror the lipid composition of several lung surfactant studies and lucinactant. The latter composition is similar to formulations used in numerous studies of amphipathic membrane-active peptides (36–38) and allows direct comparison of the physical properties of KL₄ with other biologically active peptides. Lipid phases of these compositions can also be found in localized areas of the alveoli during the surfactant cycle.

MATERIALS AND METHODS

Synthesis of KL₄

KL₄ (KLLLLKLLLLKLLLLKLLLLK) was synthesized via automated solid-phase peptide synthesis on a Wang resin (ABI 430, ICBR, UF). The peptide was cleaved from the resin with 90% TFA/5% triisopropylsilane/5% water and ether precipitated. The crude product was purified by reverse-phase high-performance liquid chromatography using an acetonitrile/water gradient, and purity was verified by mass spectrometry. Dried peptide was weighed and dissolved in methanol to a stock concentration of ~1 mM, and aliquots were analyzed by amino acid analysis (Molecular Structure Facility, University of California, Davis, CA) for a more accurate determination of concentration.

Preparation of peptide/lipid samples

POPC, DPPC, POPG, POPC-d₃₁, DPPC-d₆₂, and POPG-d₃₁ were purchased as chloroform solutions (Avanti Polar Lipids, Alabaster, AL) and concentrations were verified by phosphate analysis (39) (Bioassay Systems, Hayward, CA). The lipids were mixed at a molar ratio of 4:1 DPPC/POPG and 3:1 POPC/POPG in chloroform and aliquoted. For samples containing peptide, a methanol solution of KL₄ was added to lipid solutions with final protein/lipid (P/L) molar ratios ranging from <1:1000 to >1:50. The samples were dried under a stream of nitrogen with the sample temperature maintained at 42–50°C in a water bath. The resulting films were suspended in cyclohexane, flash-frozen, and lyophilized overnight to remove residual solvent.

CD experiments

For CD experiments, 3 mg of peptide-lipid powder were solubilized in 1 mL 10 mM HEPES buffer, pH 7.4, with 140 mM NaCl to achieve a concentration of 40 μM KL₄ with 4 mM lipids. Samples were placed in a 50°C water bath to facilitate solubilization accompanied by three to five freeze-thaw cycles with vortexing to achieve equilibration. Peptide-lipid MLVs were extruded through 100 nm filters (Avanti Polar Lipids, Alabaster, AL) 15–25 times above the *T_m* of the lipids to form LUVs just before CD analysis. CD experiments were performed on an Aviv model 215 (Aviv, Lakewood, NJ) at 45°C using a 195–260 nm wavelength range, a 1 nm step size,

and averaging of 10–25 scans. A control spectrum of 40 μ M KL₄ in TFE was also collected. Background contributions from the buffer and LUVs were removed by subtracting appropriate controls.

DSC experiments

For DSC experiments, 2 mg of peptide-lipid powder was solubilized in 1 mL of 5 mM HEPES buffer, pH 7.4, with 140 mM NaCl, 1 mM EDTA, and, in some cases, 5 mM CaCl₂ to achieve a 3 mM lipid concentration. Samples were placed in a 50°C water bath to facilitate solubilization accompanied by three to five freeze-thaw cycles with vortexing to achieve equilibration. Peptide-lipid MLVs were extruded through 100 nm filters (Avanti Polar Lipids, Alabaster, AL) 15–25 times at 45°C to form LUVs and degassed just before DSC. DSC experiments (MicroCal, LLC, Northampton, MA) were conducted over a range of 10–70°C at a scan rate of 1°C/min and run in triplicate.

³¹P and ²H NMR experiments

For each static ssNMR sample, 20–30 mg of peptide-lipid powder was placed in a 5 mm diameter NMR tube with 200–300 μ L of buffer containing 5 mM HEPES pH 7.4, 140 mM NaCl, and 1 mM EDTA in ²H depleted water (Cambridge Isotopes, Andover, MA). Samples were made using 4:1 DPPC-d₆₂/POPG, 4:1 DPPC/POPG-d₃₁, 3:1 POPC-d₃₁/POPG, 3:1 POPC/POPG-d₃₁, DPPC-d₆₂, POPC-d₃₁, or POPG-d₃₁ lipid preparations. Some NMR samples contained 5 mM CaCl₂; however, we saw no significant difference in NMR line shapes between samples with or without Ca²⁺. NMR samples were subjected to three to five freeze-thaw cycles with gentle vortexing to form MLVs before data collection. ³¹P NMR data were collected on a 600 MHz Avance system (Bruker, Billerica, MA) using a standard 5 mm broadband probe. Proton decoupling (25 kHz) was employed during acquisition to remove dipolar couplings. Spectra were acquired at 34°C, 39°C, and 44°C with 1024–2048 scans and a 5 s recycle delay between scans to minimize radiofrequency sample heating. ²H NMR data were collected on a 500 MHz Bruker Avance system using a standard 5 mm broadband probe and quad echo sequence (90°- τ -90°- τ -acq with τ = 30 μ s) with a B₁ field of 40 kHz. Spectra were acquired at 34°C, 39°C, and 44°C for all samples with 1024 or 2048 scans and a 0.5 s recycle delay between scans. To monitor the phase transitions of the DPPC/POPG mixtures, for some samples spectra were also collected over a range of 30–44°C in 2° increments.

DePaking analysis of NMR data was accomplished using previously published algorithms that simultaneously dePake and determine macroscopic ordering for partially aligned lipid spectra using Tikhonov regularization (40). ³¹P NMR spectra were referenced to phosphate buffer before dePaking, and dePaked spectra were quantitated by fitting the resulting two peaks with Lorentzian line shapes. Assignments of ²H resonances were made based on published values (41,42).

MAS NMR analysis

For each sample, 40 mg of lipid containing KL₄ ¹³C'-enriched at positions L9 and L10 at a P/L ratio of 1:33 or 1:100 were hydrated in 1 mL of buffer containing 10 mM HEPES pH 7.4, 140 mM NaCl, and 1 mM EDTA. Samples were subjected to 3–5 freeze-thaw cycles with gentle vortexing to form MLVs before pelleting the lipids by centrifugation. The pellets were packed directly into MAS rotors and NMR data were collected at temperatures of ~-5°C, 25°C, and 40°C. NMR samples contained ~20 mg of the lipid-peptide mixture.

MAS experiments were performed on a 500 MHz DRX system (Bruker, Billerica, MA) using a doubly tuned 4 mm magic angle spinning probe (Doty Scientific, Columbia, SC). CPMAS experiments employed a ¹H 90° pulse width of 3.2 μ s followed by a contact time of 2 ms and 100 kHz ¹H CW decoupling. A 25% ramp was applied on the ¹H channel during cross polarization. Spectra were acquired at a spinning speed of 5 kHz with a total of 512–2048 scans for each spectrum. The rotor speed was regulated to \pm 3 Hz for all MAS experiments, and each transient was collected with

a sweep width of 50 kHz and 20 ms of acquisition (1024 points) with a recycle delay of 3 s between transients.

For the ¹H spin diffusion experiments (43), residual ¹³C magnetization was abolished by four 90° pulses before excitation of ¹H magnetization. After a 90° pulse on the ¹H channel, a T₂ filter was applied for 10 rotor periods (2 ms) to dephase ¹H magnetization in any rigid molecules before the t₁ period for evolution of the ¹H magnetization. After the evolution period, the magnetization was stored along the z axis for 100–200 ms to allow ¹H spin diffusion before the magnetization was flipped back to the x-y plane with a 90° pulse. The ¹H magnetization was then transferred to neighboring ¹³C spins by a short (0.5 ms) cross-polarization period followed by detection of the ¹³C signal with 100 kHz ¹H CW decoupling. The 2D spectra were collected with 512 transients per t₁ point and 96 spectra were collected over the course of 19.2 ms in t₁ (a dwell time of one rotor period) using States-Haberkm-Ruben phase-sensitive detection.

RESULTS

Secondary structure of KL₄ interacting with lipid bilayers

CD spectroscopy was utilized to investigate the conformation of KL₄ in the presence of unilamellar lipid vesicles. The effects of two lipid systems—a 4:1 DPPC/POPG lipid mixture and a 3:1 POPC/POPG lipid mixture—on peptide secondary structure were investigated. Fig. 1 shows the CD spectra obtained for KL₄ in these two environments. Spectra were collected at 45°C to ensure that both lipid systems were in the liquid lamellar phase. As a control, the CD spectrum of KL₄ in 50% TFE is shown for comparison. The CD spectra have features characteristic of helical secondary structure, as evidenced by minima at 206–208 and 222 nm. The spectra differ for the two lipid environments, and the spectrum for KL₄ interacting with 4:1 DPPC/POPG shows greater helicity than the spectrum for KL₄ interacting with 3:1 POPC/POPG lipids. Of interest, the samples containing DPPC/POPG LUVs have similar ellipticity to the peptide in TFE, with the minimum at

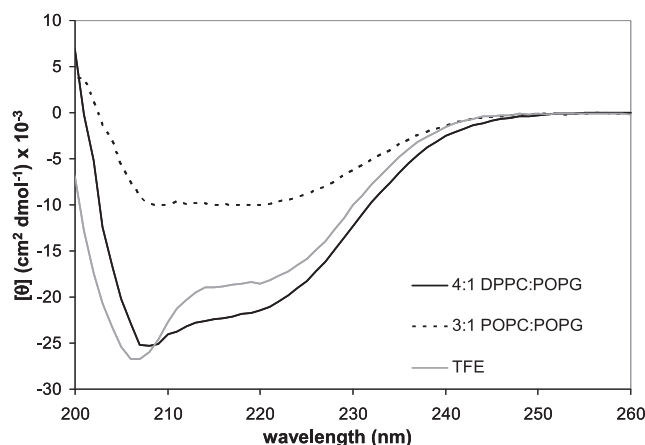


FIGURE 1 CD spectra at 45°C of KL₄ at a P/L molar ratio of 1:100 in 4:1 DPPC/POPG (solid dark curve), at a P/L molar ratio of 1:100 in 3:1 POPC/POPG (dashed curve) and in 50% TFE (solid light curve). The final peptide concentration was 40 μ M in all three samples.

206–208 significantly lower than the minimum at 222 nm. Similar spectra have been observed for peptides that were constrained to form π -helices in buffer (44). In a first attempt to understand the origins of the differences seen in the CD spectra, the data were fit with standard deconvolution software (45), resulting in secondary structure estimates of 40–45% α -helix, 40–45% random coil, and 10–20% β -sheet for KL₄ in POPC/POPG LUVs and >85% α -helix, <15% random coil, with negligible β -sheet for KL₄ in DPPC/POPG LUVs. However, the quality of the fits was poor, particularly for the sample containing DPPC/POPG LUVs, due to the nonstandard shape of the CD spectra relative to canonical α -helices, which have minima of similar depths at 222 and 208 nm, and to the lack of data below 200 nm, where light scattering from the lipids prevents signal measurement. Given that KL₄ does not form a typical amphipathic α -helix when projected on a helical wheel (Fig. 2 A), it is highly probable that the peptide forms helices that might vary in helical pitch or even hydrogen-bonding patterns. More amphipathic helical wheel projections for KL₄, which represent structures that KL₄ might adopt in lipid bilayers, along with their hydrophobic moment values, are shown for comparison (Fig. 2, B and C).

DSC suggests KL₄ decreases DPPC/POPG lipid miscibility

Shown in Fig. 3 are DSC thermograms for 4:1 DPPC/POPG LUVs containing varying levels of KL₄. Adding POPG to DPPC in a 1:4 ratio results in a lipid transition temperature below 37°C, >4° lower than the T_m for pure DPPC, due to the monounsaturated fatty acid group in POPG. Of note is the gradual shift of T_m to a higher temperature as KL₄ levels are increased. At 0.2 mol % peptide, a higher temperature shoulder appears in the phase transition. This shoulder grows with increasing levels of peptide until two peaks with T_m values of 37°C and 39°C are clearly seen at 1 mol % peptide. At 2 and 3 mol % peptide the higher temperature transition becomes dominant. This bifurcation in the phase transition suggests domain separation of the lipids, possibly due to electrostatic interactions between the cationic peptide and anionic POPG. This phase separation is striking given the low percentages of peptide, and is similar to behavior noted

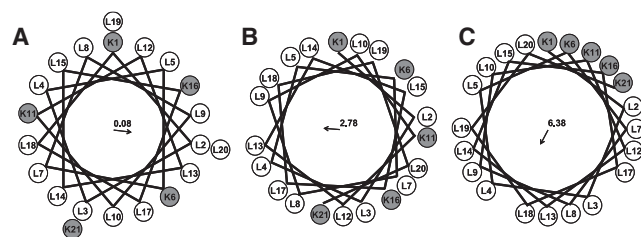


FIGURE 2 Helical wheel representations of KL₄ assuming (A) a canonical α -helix, (B) a helix with a pitch intermediate between α -helix and π -helix, and (C) a π -helix.

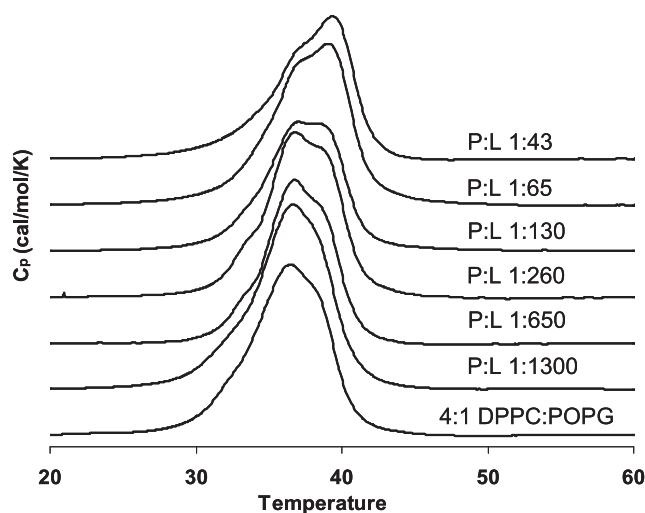


FIGURE 3 DSC scans for 4:1 DPPC/POPG LUVs with KL₄ at the indicated P/L ratios. The onset of phase separation is apparent at a P/L ratio of 1:260 and continues with increasing amounts of peptide.

previously for 3:1 DPPC/POPG MLVs (46). It also agrees with a previous observation of domain formation via Langmuir trough studies of KL₄ interacting with fluorescently labeled DPPC in a DPPC/POPG/PA system (47). This behavior may be important for the function of the peptide in vivo during the lipid resorption/adsorption cycle (13). However, from the DSC data it is not possible to determine whether the separate transitions are due to the formation of separate POPG-enriched and POPG-depleted DPPC lipid domains or to another physical phenomenon. In particular, leucine-rich TM peptides have been demonstrated to cause phase separation when interacting with DPPC. The two transitions observed in DSC traces are attributed to melting of bulk lipids and peptide-associated lipids (48). Given the high percentage of leucines in KL₄, close association of the peptide with lipid fatty acyl chains is expected. Other than a small increase at low concentrations of KL₄, no significant change in ΔH_{cal} was seen. DSC samples with 5 mM CaCl₂ showed a similar behavior, indicating that the effects of KL₄ on the L_{β} -to- L_{α} phase transition in this lipid system is not calcium-dependent.

Effect of KL₄ on lipid headgroup interactions

ssNMR was used to obtain a molecular-level view of how KL₄ interacts with phospholipids in the two bilayer systems. ³¹P NMR spectra at 44°C for 3:1 POPC/POPG and 4:1 DPPC/POPG MLVs containing varying concentrations of KL₄ were collected and dePaked, allowing a clear determination of the individual, time-averaged CSAs of the PC and PG headgroups (Fig. 4). Because data were collected on a 600 MHz NMR spectrometer, macroscopic alignment of the vesicles occurred, as evidenced by the downfield features in the lamellar line shapes being lower in intensity than is typically seen for phospholipid dispersions at lower

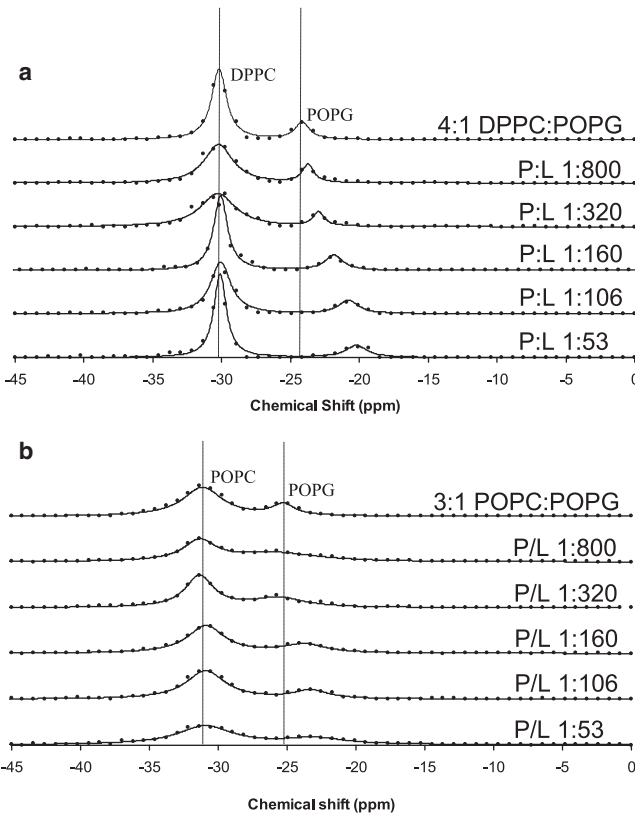


FIGURE 4 DePaked phosphorous NMR spectra of (top) 4:1 DPPC/POPG and (bottom) 3:1 POPC/POPG MLVs with KL₄ at the indicated P/L ratios with fits assuming Lorentzian line shapes.

magnetic fields (Supporting Material). As such, standard fitting of the chemical shift powder patterns was not possible. Instead, a dePaking algorithm that includes a Tikhonov regularization procedure for correcting for the macroscopic alignment was utilized (40). From this analysis it is possible to determine the spans of the CSA patterns and hence deduce the effects of KL₄ on the phospholipid headgroups. Differences in PG and PC headgroup orientations relative to the membrane normal lead to differences in their CSA values (49). With increasing levels of KL₄, the PC CSA was invariant, but the PG CSA lessened with increasing peptide levels. The decrease in the POPG CSA on addition of KL₄ was consistently seen in all lipid systems studied, regardless of which lipid was deuterated. To assess the underlying cause of the changes in POPG CSAs, ³¹P NMR spectra were collected on individual lipids alone and with 1.5 mol % KL₄; the ³¹P NMR spectra and their corresponding dePaked spectra are provided in the Supporting Material. Only small changes in individual lipid CSAs were seen on addition of KL₄. However, the CSA for POPG alone is significantly smaller than that observed for POPG in 4:1 DPPC/POPG or 3:1 POPC/POPG mixtures before the addition of KL₄. For the binary lipid mixtures containing higher concentrations of KL₄, the ³¹P CSAs are more comparable to

those of the neat lipids. Thus, KL₄ clearly affects the interactions of the PC and PG lipids, and possibly their miscibility. Table 1 shows CSA values for lipid mixtures and comparisons with individual lipids. These results indicate that when POPG interacts with PC headgroups, electrostatic interactions cause the PG headgroups to reorient, leading to a subsequent increase in their averaged CSA values. Addition of KL₄ disrupts this interaction, causing the PG CSAs to move toward values seen for POPG alone.

At low P/L ratios, lipid alignment along the static magnetic field is seen in spectra of lipid systems, as evidenced by disproportionately high perpendicular edges for the static powder patterns. Lipid alignment in high magnetic fields was observed previously (40,50,51) and is caused by the negative diamagnetic susceptibility inherent to phospholipids. In lipid bilayers, the cooperative alignment of the magnetic moments of individual lipid molecules leads to a large bulk magnetic susceptibility resulting in an overall macroscopic ordering of the sample within the magnetic field. Increasing amounts of KL₄ disrupt this alignment in a concentration-dependent manner, as evidenced by the gradual increase in the parallel edges of the ³¹P NMR spectra. The reduction in alignment of the lipid lamellae on addition of KL₄ suggests that the peptide disrupts lipid-lipid interactions. The ability of peptides to affect lipid magnetic field alignment, particularly for amphipathic peptides, was previously noted (52–56). However, reports on the influence of amphipathic peptides on lipid alignment have been primarily anecdotal, and the diversity in lipid composition, peptide concentration, and experimental conditions used to date prohibit a systematic evaluation of how peptide sequences and lengths affect macroscopic lipid organization. Finally, for our systems, although the POPG CSA values decrease on addition of KL₄, the lipids clearly remain in a lamellar phase above the phase transition temperature.

TABLE 1 ³¹P CSA values (in ppm) for 3:1 POPC/POPG and 4:1 DPPC/POPG with varying concentrations of KL₄ (peaks in the dePaked spectra had full widths at half-height of 1–5 ppm)

mol % KL ₄	4:1 DPPC/POPG		3:1 POPC/POPG	
	DPPC	POPG	POPC	POPG
0	44.8	38.2	45.8	38.6
.06	46.0	37.6	46.0	38.5
.13	45.4	37.8	46.2	38.7
.2	44.8	37.4	44.0	38.4
.31	45.2	37.1	45.1	38.6
.63	45.8	36.2	45.3	37.3
.94	46.1	35.2	46.4	36.6
1.0	45.0	35.2	44.7	36.7
1.89	45.5	34.9	45.8	36.9
3.0	45.0	32.7	44.5	35.3
Individual lipids				
	DPPC	POPG	POPC	
0	48.0	34.4	46.2	
1.5	44.6	34.7	47.3	

²H NMR studies of DPPC/POPG lipid miscibility on addition of KL₄

To determine whether the domain formation observed in the DSC experiments was due to phase separation of the DPPC and POPG lipids, ²H NMR spectra were collected over the temperature range of the phase transitions for samples that contained either deuterated DPPC or deuterated POPG (Fig. 5). A first-moment analysis of these data to determine the phase transition temperatures is provided in the [Supporting Material](#). From these spectra, it is clear that in the 4:1 DPPC/POPG samples the lipids are fully miscible, with the DPPC and POPG melting at similar temperatures. The phase transition seen for deuterated DPPC is at a slightly lower temperature (midpoint of 30.7°C) than for deuterated POPG (31.1°C). This is partly because a larger percentage of the fatty acyl chains is deuterated (80% vs. 10%) (57), but it may also reflect slight differences in POPG content between the two samples since they were made from different lipid stock solutions. For the samples that do not contain KL₄, the spectra at intermediate melting temperatures are a superposition of gel phase and liquid phase spectra. Addition of KL₄ affects the phase transition temperature of both lipids in a similar manner, based on the first-moment analysis. However, in the POPG-d₃₁ spectra a larger percentage of the dynamic lipid phase is evident, and in the DPPC-d₆₂ spectra the dynamic phase is absent at lower temperatures, as can be seen by comparing spectra at 30°C and 32°C. The peptide also leads to an increase in temperature at which the lipids are completely melted (36°C vs. 40°C for deuterated DPPC; 38°C vs. 40°C for deuterated POPG), consistent with the DSC data. Attempts to determine the fractions of liquid phase and gel phase by spectral subtractions with samples containing differing percentages of KL₄ were unsuccessful due to variations in the lipid alignment and acyl chain order parameters. Thus, addition of KL₄ leads to some phase separation of the lipids at lower temperatures, with a significant fraction of POPG remaining in the gel phase. Above the phase transition temperature of the domain with the higher *T_m*, the peaks in the ²H spectra coalesce to a single resonance for each position in the acyl chain, suggesting the absence of separate

domains in the fluid phase or exchange between domains is fast on the NMR timescale.

Effects of KL₄ on lipid acyl chain dynamics

Further analysis of ²H NMR spectra above the lipid phase transition temperatures allows determination of the effect of KL₄ on lipid acyl chain dynamics in the fluid phase, and provides insight into its partitioning depth into the bilayers. ²H NMR spectra for 3:1 POPC-d₃₁/POPG and 4:1 DPPC-d₆₂/POPG MLVs at 44°C with varying levels of KL₄ were collected and dePaked (Fig. 6). For 3:1 POPC-d₃₁/POPG MLVs, only small decreases are seen in the ordering of the POPC *sn*-1 acyl chains on addition of KL₄. In contrast, addition of KL₄ to 4:1 DPPC-d₆₂/POPG MLVs increases the ordering of the DPPC acyl chains, particularly toward the middle of the bilayer. This suggests that either 1), KL₄ inserts deeply into the DPPC/POPG bilayers, restricting the motional freedom of the acyl chains; or 2), an electrostatic interaction of the peptide with the lipid headgroups occurs, resulting in a change in the lipid packing and headgroup conformation. However, the ³¹P experiments described above establish that the phosphate moieties in the PC headgroups are unaffected by KL₄, ruling out the latter possibility.

Lipid acyl chain order parameters were determined by measuring the quadrupolar splitting ($\Delta\nu_Q$) for deuterium atoms at various positions along the acyl chain and determining their order parameters, S_{CD} (Eq. 1). The order parameters provide a measure of the average angular fluctuations around the bilayer normal at each acyl chain segment (58):

$$\Delta\nu_Q = \frac{3}{4} \frac{e^2 q Q}{h} (3\cos^2\theta - 1) S_{CD}. \quad (1)$$

For saturated C-D bonds, the quadrupole coupling, $\frac{3}{4} \frac{e^2 q Q}{h}$, is 167 kHz in the static limit (59). If the bilayers adopt random orientations with respect to the magnetic field, the resulting spectra of perdeuterated acyl chains are comprised of a superposition of axially symmetric powder patterns arising from each deuterated position. The intensities of these powder patterns follow the well-established distribution function $p(\theta) \propto \sin(\theta)$, where θ is the angle between the

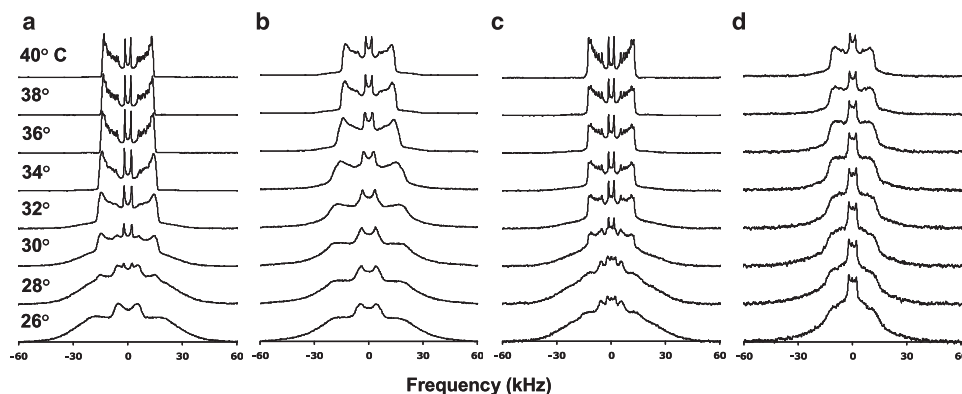


FIGURE 5 Deuterium NMR spectra as a function of temperature for (a) 4:1 DPPC-d₆₂/POPG MLVs, (b) 4:1 DPPC-d₆₂/POPG MLVs with KL₄ at a P/L molar ratio of 1:53, (c) 4:1 DPPC/POPG-d₃₁ MLVs, and (d) 4:1 DPPC/POPG-d₃₁ MLVs with KL₄ at a P/L molar ratio of 1:33.

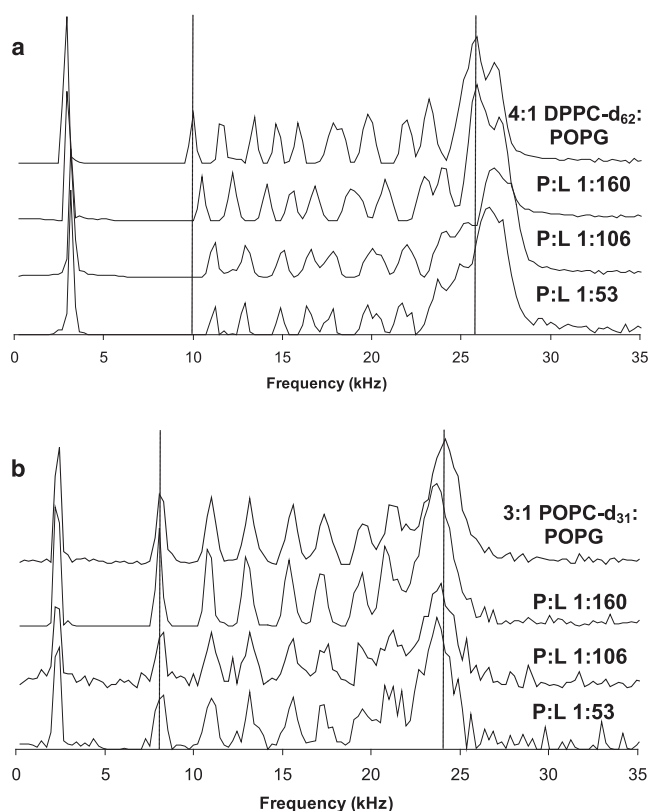


FIGURE 6 DePaked deuterium NMR spectra of (top) 4:1 DPPC-d₆₂/POPG and (bottom) 3:1 POPC-d₃₁/POPG MLVs with KL₄ at the indicated P/L ratios. Spectra were taken at 44°C.

bilayer normal and the magnetic field. The overlaid powder patterns can be deconvoluted using a standard inversion (dePaking) procedure (60). For a sample in which the lipid bilayers show a tendency to align in the magnetic field, the probability distribution function must be adjusted. Assuming that the magnetic field leads to an ellipsoidal deformation of the MLVs, the probability distribution becomes (40):

$$p_{(E)}(\theta) \propto \sin(\theta) [1 - (1 - \kappa_E) \cos^2 \theta]^{-2}. \quad (2)$$

Here κ_E refers to the square of the ratio of the long to short axes of the ellipsoids. Using an iterative procedure, which simultaneously determines κ_E and dePakes the spectrum, the order parameters, S_{CD} , can be determined with high accuracy in a partially aligned sample along with the degree of alignment. Order parameters calculated using deconvoluted spectra (Fig. 6) are tabulated in the [Supporting Material](#); the resulting order parameter profiles are graphed in Fig. 7. The order parameter profiles clearly indicate that KL₄ has different effects on POPC versus DPPC lipids, with a small decrease in order for the POPC lipids and a larger increase in order for the DPPC lipids. The profiles also yield a more detailed picture of how KL₄ affects the lipid dynamics, with carbon positions 2–8 being the most affected in the POPC lipid acyl chains and positions 9–16 the most affected

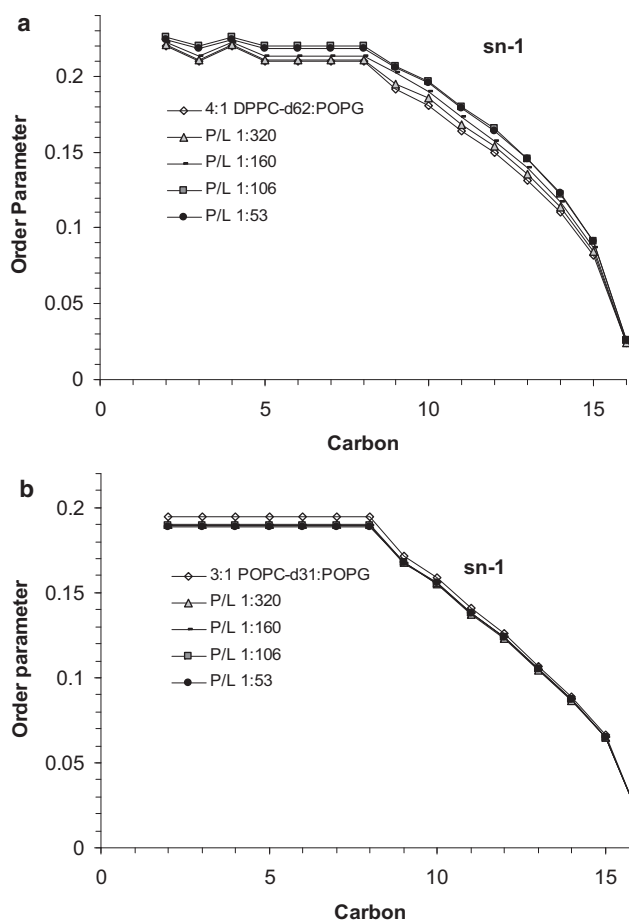


FIGURE 7 Order parameter profiles for the *sn*-1 chain of (top) DPPC-d₆₂ in 4:1 DPPC-d₆₂/POPG and (bottom) POPC-d₃₁ in 3:1 POPC-d₃₁/POPG MLVs with KL₄ at the indicated P/L ratios.

in the DPPC lipid acyl chains. The effects of KL₄ on the PG lipids were similarly monitored by collecting ²H NMR spectra for 4:1 DPPC/POPG-d₃₁ and 3:1 POPC/POPG-d₃₁ MLVs and calculating the order parameter profiles for the *sn*-1 chain on POPG in these lipid mixtures ([Supporting Material](#)). In these experiments an overall decrease in ordering of the POPG acyl chains, particularly in the plateau region, is seen on addition of KL₄. Decreases in the order parameters are seen at all the acyl positions in POPG in 3:1 POPC/POPG MLVs and are greater than the changes for POPC in the plateau region. The POPG acyl chain order parameters in 4:1 DPPC/POPG MLVs do not appear to be as affected by KL₄; however, a comparison of the results further down the acyl chain for POPG-d₃₁ when mixed with DPPC versus POPC again demonstrates that the peptide interacts with these lipid systems in a manner that depends on the degree of saturation of the fatty acid chains.

To clarify the effects of KL₄ on the individual lipids in the binary lipid mixtures, ²H NMR spectra of neat lipids, DPPC-d₆₂, POPC-d₃₁, and POPG-d₃₁, with and without 1.5 mol % KL₄ were also collected, dePaked, and assigned ([Supporting](#)

Material). A comparison of these order parameter profiles with those for the lipids in binary mixtures shows that the DPPC order parameters for 4:1 DPPC/POPG MLVs with P/L ratios $> 1:200$ are higher than would be expected for DPPC alone, and the POPG order parameters are also higher than expected for POPG alone. Within the resolution of the ^2H NMR experiment, no phase separation is seen. Thus, KL_4 is most likely interacting with both lipid populations in the fluid phase rather than segregating the lipids and interacting with a single phase above the L_β -to- L_α phase transition.

The observed changes in order parameters are seen more clearly when the data are viewed as the change in order parameter at each position along the acyl chain on addition of peptide relative to the order parameters of the lipids without the peptide. Shown in Fig. 8 are the changes in order parameters at particular methylene positions in the *sn*-1 palmitoyl acyl chains for the individual lipids in 3:1 POPC/POPG and 4:1 DPPC/POPG MLVs, respectively, on addition of peptide at a P/L molar ratio on the order of 1:100. From these graphs, the behavior of the PG lipids relative to the PC lipids is clearly offset, but the trends with respect to acyl chain position are similar. The offset of the profiles for the PG lipids is consistent with the ^{31}P NMR findings described above, showing that association of KL_4 with the lipids leads to a change in the orientation PG headgroups and an overall decrease in the ^{31}P CSAs for the POPG lipids. Thus, the offset in the PG profiles in Fig. 8 relative to the PC profiles does not reflect significantly less internal order in the PG lipid methylene chains per se, but instead reflects a change in the average orientation of the PG lipid director relative to the membrane normal.

The larger decrease in order parameters for POPG versus POPC in 3:1 POPC/POPG MLVs reflect the reorientation of the PG headgroup, and the lipid acyl chains are most likely equally affected in terms of the internal dynamics of the methylene chains. From their profiles, one can see that for this lipid system the methylenes in the plateau region are more affected than those further down the acyl chain. This behavior is somewhat in contrast to changes observed for antimicrobial peptides, which typically cause larger changes in order further down the methylene chain (37,61,62), and suggests that the interaction of KL_4 with monounsaturated lipids is unlike antimicrobial amphipathic helices, which have a much higher percentage of charged residues and are known to disrupt membrane bilayers. Changing the order of amino acids in the primary sequence of KL_4 to mimic a standard amphipathic α -helix abolishes its effectiveness as a surfactant (63). However, its behavior is similar to that of the C-terminus of SP-B, SP-B_{59–80} (64), and saposin C, a protein that is important for lipid enzymology and is closely related to SP-B (65,66).

Of particular interest regarding the role of KL_4 in lung surfactant formulations is its effects on 4:1 DPPC/POPG MLVs. From the order parameter profiles, one can see that KL_4 clearly increases in order along the DPPC acyl chains,

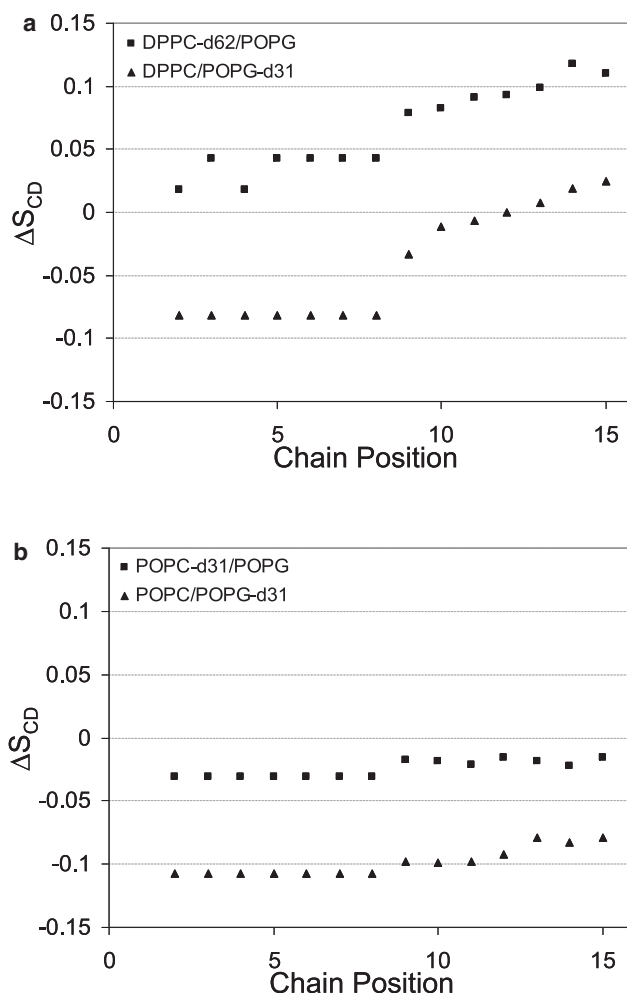


FIGURE 8 Changes in *sn*-1 d_{31} -palmitoyl acid chain order parameters for PC lipids (squares), and POPG (triangles) in 4:1 DPPC/POPG MLVs (top) and 3:1 POPC/POPG (bottom).

with the greatest effect at the center of the bilayers. The profile for POPG also shows an increase toward the center of the bilayers but a decrease in the plateau region. However, as discussed above, the decrease reflects a change in the average orientation of the POPG headgroup, and if this change in orientation is taken into account, the internal order at individual methylene positions in the POPG acyl chain is increasing over the entire length of the acyl chain. We previously observed these same trends for SP-B_{59–80} (64). Similar behavior has also been observed on addition of the human antimicrobial peptide LL-37 to DMPC bilayers (61). Increases in lipid acyl chain ordering have been observed in response to polyelectrolyte binding to lipid headgroups (67,68), as well as in response to the addition of small molecules, such as cholesterol (69,70), or TM peptide helices that partition into the acyl chain region of the lipids (71,72). In the case of polyelectrolyte binding, larger changes are seen for the plateau region of the lipids, reflecting a change in overall orientation of the lipid packing and headgroups.

Smaller changes are also seen near the center of the bilayers and are interpreted as resulting from closer packing of the lipids on binding of the electrolyte. It is unlikely that KL₄ is affecting the lipid bilayers in this manner, since the ³¹P results above show little change in the dynamics of the PC headgroups. For TM helices similar in length to KL₄, increases in order parameters that are similar in magnitude are seen for both the methylene positions in the plateau region and toward the center of the bilayers; these trends are observed in both POPC and DPPC bilayers. This is expected because insertion of a TM helical peptide primarily affects the thickness of the bilayer, and it would interact with both the lipid acyl chains and headgroups across the entire span of the bilayers. In contrast, for cholesterol, larger changes in order parameters are observed toward the center of the bilayer (69) because cholesterol partitions to the bilayer interior and does not strongly interact with the lipid head groups. The effects of KL₄ and SP-B_{59–80} on the order parameters in DPPC/POPG bilayers are most similar to those of cholesterol. This suggests that the bulk of the peptides are partitioning deep within the lipid bilayers. The higher degree of ordering toward the center of the bilayer could be due to the leucines in KL₄ penetrating deeply into the bilayer and decreasing the mobility of the acyl chains. However, the smaller changes seen in the plateau region (carbons 3–8) suggest that the peptide does not adopt a TM orientation. These changes in order parameters indicate that KL₄ lodges into the hydrophobic region of the bilayer while maintaining its helix axis perpendicular to the bilayer normal. This type of interaction would lead to a negative curvature strain within the lipid bilayers, a phenomenon that has been hypothesized to be important for lung surfactant function (4). Of interest, ²H spectra of 3:1 POPC/POPG lipid mixtures collected at 10°C (data not shown) show that KL₄ increases acyl chain order in a manner similar to that seen for 4:1 DPPC/POPG mixtures at 44°C. Thus, the contrast in the effects of KL₄ on lipid dynamics in the two lipid systems at physiologic temperatures is related to the difference between the temperature of observation and the phase transition temperatures of the individual lipid systems.

Another interesting outcome is seen when one compares the effect of KL₄ on DPPC and POPC acyl chains in mixed DPPC/POPG and POPC/POPG bilayers relative to the PC lipids alone (Fig. 9). Given the highly conserved lipid composition found in mammalian lung surfactants, POPG is most likely a critically important factor in the physical properties of surfactant. For both lipid systems, the incorporation of POPG leads to a greater change in the order parameter profiles of the PC lipids on addition of KL₄. Although the anionic POPG interacts with the peptide through electrostatic interactions with the lysine side chains, this interaction seems to increase the miscibility of the peptide with the PC lipids in the fluid phase rather than leading to phase separation, as evidenced by its increased effects on the PC lipid order parameters with addition of POPG.

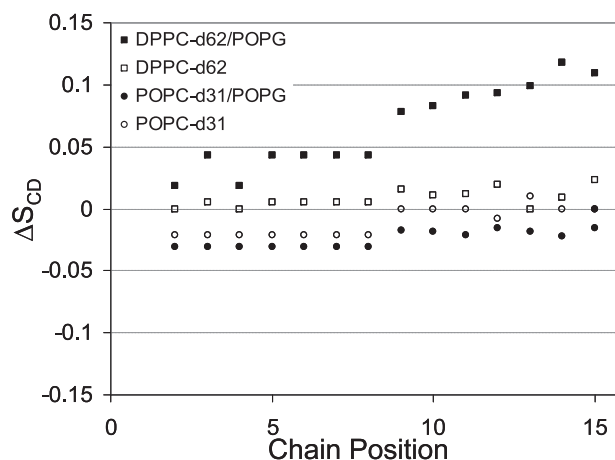


FIGURE 9 Changes in *sn*-1 d₃₁-palmitoyl acid chain order parameters for DPPC (squares) and POPC (circles) MLVs with (closed symbols) and without (open symbols) POPG on addition of KL₄ at P/L molar ratios of ~1:100.

Binding and insertion depth of KL₄ into lipid bilayers

To more directly gauge the insertion depth of KL₄ into the DPPC/POPG and POPC/POPG bilayers, samples were prepared for ¹H spin diffusion experiments. For these experiments, the peptide was ¹³C' enriched at positions L9 and L10, and initial CPMAS spectra were collected to assess the dynamics and cross-polarization efficiency of the peptide (Fig. 10 a). The sample containing DPPC affords high-quality CPMAS spectra with an easily discernible ¹³C' resonance at 172 ppm. However, ¹³C' resonances are not as clearly seen in the CPMAS spectra for the sample containing POPC at 25°C and 40°C, and cross polarization is less efficient. Cooling the samples down to nearer the phase transition temperature of the lipids leads to the appearance of peptide ¹³C' signal at 174 ppm. Thus the peptide is clearly bound and immobilized in the fluid-phase DPPC/POPG bilayers on the NMR timescale, but when interacting with the POPC/POPG bilayers at physiologic temperatures it is only transiently bound or highly mobile. This contrasts with previous observations of the binding of KL₄ to POPC/POPG LUVs in 5 mM HEPES buffer alone (73). Similar variations in peptide dynamics for a peptide ion channel mixed with lipids in organic solvents or bound to lipid LUVs from buffer have been observed (74). Of interest, these differences in dynamics do not necessarily correlate to global changes in secondary structure, as the isotropic chemical shifts for enriched ¹³C' positions in KL₄ in the two POPC/POPG sample preparations are indistinguishable when the samples are flash-frozen (at ~172 ppm) and the two preparations yield similar CD spectra. However, we do see a slight change in the isotropic chemical shifts between the two POPC/POPG preparations when the lipids are in the fluid phase: the more dynamic preparation, resulting

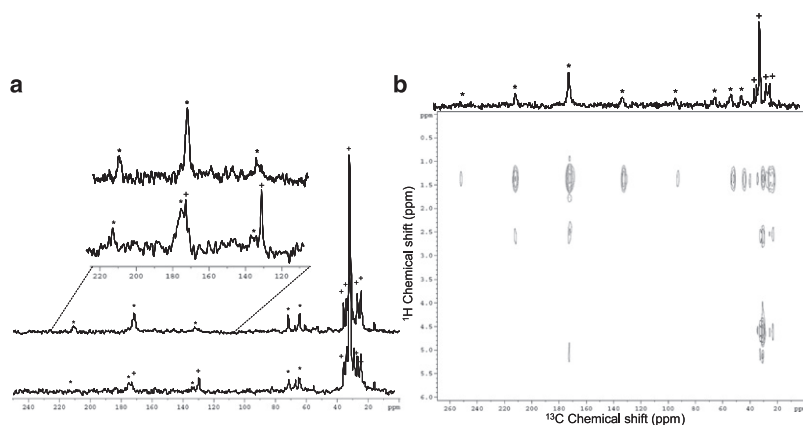


FIGURE 10 (Left) CPMAS spectra of KL₄ in 4:1 DPPC:POPG vesicles (top), collected at 40°C, and 3:1 POPC:POPG vesicles (bottom), collected near 0°C, at a P/L molar ratio of 1:33. The peptide is ¹³C'-enriched at positions L9 and L10. Peptide resonances (*) and lipid resonances (+) are indicated. In the expansion of the region for the ¹³C' isotropic and first spinning sideband resonances, the attenuation of peptide signal in 3:1 POPC/POPG vesicles can be seen and the lipid signals are more prevalent. (Right) 2D ¹³C-detected ¹H spin-diffusion spectrum for 1:33 KL₄ in 4:1 DPPC/POPG with the peptide (*) and lipid (+) ¹³C resonances indicated in the *t*₁ = 0 experiment (top).

from mixing KL₄ with lipids in an organic phase, has an isotropic chemical shift near 174 ppm (Fig. 10 *a*), whereas the less dynamic preparation has an isotropic chemical shift of 172 ppm (73). Carbonyl ¹³C' signals are particularly susceptible to slight variations in hydration and/or hydrogen bonding (75), which would explain these observations. The transient nature of the KL₄ interactions with the POPC/POPG bilayers would also explain why its effects on acyl chain order are attenuated relative to its effects on order in DPPC/POPG bilayers.

The interaction of KL₄ with the DPPC/POPG bilayers in the fluid phase was characterized by means of ¹H spin diffusion experiments. Fig. 10 *b* shows a representative ¹³C-detected ¹H spin diffusion spectrum at a mixing time of 100 ms, in which an interaction of the peptide ¹³C' resonances at ~172 ppm with the lipid acyl chains at a ¹H chemical shift of 1.3 ppm is clearly seen. Although the lipids are also exchanging polarization with water, as evidenced by a cross-peak at a ¹H chemical shift of 4.7 ppm and a ¹³C chemical shift of 30 ppm, no appreciable signal corresponding to spin diffusion between the peptide and water is seen. This observation is consistent with the peptide penetrating deeply into the lipid bilayers (43).

DISCUSSION

The dynamic air-fluid interface in the lung requires lung surfactant to possess specific attributes to lower surface tension and allow rapid respreading of lipids. Lung surfactant relies on relatively low levels of lung surfactant proteins B and C (<0.2 mol %) to alter the structure, dynamics, and phase properties of the lipids. The peptide KL₄ similarly affects the macroscopic properties of the lipids and has been pursued as a clinical replacement for SP-B in therapeutic formulations (30,33). However, the molecular mechanisms behind its properties have not been fully elucidated. FTIR measurements of SP-B interacting with lipid bilayers indicate that it is a helical protein with the helices oriented perpendicular to the membrane normal (19,20). FTIR studies of KL₄ in which the amide I' band was monitored led to con-

flicting models of the structure and orientation of the peptide. In early work using oriented bilayers at limited hydration, investigators concluded that KL₄ was helical in 7:3 DPPC/DPPG but spanned the bilayers (76). Later work with DPPC and DPPG mixed monolayers at an air-water interface indicated that KL₄ lies along the surface of the lipids as a mixture of helix and sheet (77). However, in both of these studies it was assumed that KL₄ forms either a classic α -helical or β -sheet secondary structure with standard backbone conformations and hydrogen-bonding distances. Additionally, the amide I' band is sensitive to the solvation environment of the peptide being monitored, and thus variable partitioning into the lipid environments could explain the observed changes in stretching frequencies. More recently, an *in vitro* transcription-translation system, which utilizes the *Escherichia coli* inner membrane protein leader peptidase (Lep) to report on the integration of TM helices into ER membranes, was used to assay whether KL₄ or SP-B_{59–80} would partition as TM helices in the context of a Lep model protein (78). In that study, KL₄ was found to incorporate into the protein as a TM helix whereas SP-B_{59–80} did not. However, the accuracy of this host-guest system for predicting the partitioning of positively charged amphipathic sequences has been called into question given the complexity of the translocon protein assembly and mechanisms of glycosylation (79–81). The possible secondary structures of KL₄, as well as its interactions with specific lipids found in lung surfactant, need more thorough examination. In an attempt to gain a molecular-level understanding of how KL₄ alters lipid properties, we undertook an ssNMR examination of how KL₄ interacts with two lipid formulations: 4:1 DPPC/POPG and 3:1 POPC/POPG.

Our CD measurements, as well as previously published work (46), show KL₄ to be helical in both POPC/POPG and DPPC/POPG lipid mixtures. However, in all probability, KL₄ does not form a classic α -helix if the helix axis is perpendicular to the membrane normal, since this would place the lysines evenly around the helix (Fig. 2 *A*). Lysine- and leucine-rich peptides have been shown to adopt different amphipathic secondary structures at the air-water interface

and on polymer surfaces based on the amino acid pattern of the residues (82,83). Similar driving forces would exist at the lipid bilayer surface. A possible explanation for how KL₄ partitions as a helix into the bilayers is that it may form a structure in which the lysines lie on one side of a helix. The length and flexibility of the lysine side chains would allow either an amphipathic helical conformation of KL₄, which preferentially binds at the lipid interface (Fig. 2 B), or a π -helix conformation in which the peptide deeply penetrates into the bilayer while retaining electrostatic interactions between the lysine side chains and the lipid phosphate groups by “snorkeling” (Fig. 2 C). We recently determined the structure of KL₄ in 3:1 POPC/POPG MLVs via ssNMR dipolar recoupling experiments (73). The average backbone torsion angles found were (-105° , -30°), which allow KL₄ to form an amphipathic helix in which the lysines lie on one half of the peptide helix as in Fig. 2 B. This would allow the peptide to preferentially interact with the lipids at the bilayer interface, but would make deeper penetration into the hydrophobic region of the lipids unfavorable. Measurements of the structure of KL₄ in DPPC/POPG MLVs suggest i to $i + 5$ hydrogen bonding (A. K. Mehta and J. R. Long, unpublished data). Of interest, the CD spectrum for KL₄ interacting with DPPC/POPG LUVs is very similar to that observed for recently published peptides that are constrained by covalent bonds to form π -helices (44).

Another consideration is whether the helical form of KL₄ might span the bilayers in a TM conformation. The snorkeling of lysine residues was previously postulated to stabilize the TM WALP and KALP peptides (84). However, the length of the lysine side chain limits its placement relative to the peptide termini for a TM orientation to be observed. Of specific interest regarding the orientation of KL₄ are the 22 amino acid peptides KKLLKLLLLLLLLLKKLLKK and KLLLLLKKLLLLLLLLLKK, which have been characterized in POPC membranes. The former peptide adopts a TM orientation, whereas the latter peptide exchanges slowly between different orientations in the bilayers (85). These peptides bear a striking homology to KL₄ in both amino acid content and hydrophobic to hydrophilic ratio, but only the sequence in which the lysines are distributed more toward the termini is able to achieve a stable TM configuration. The thermodynamic penalty imposed by placing KL₄ in a TM orientation would be prohibitive since it would result in charged amino acids partitioned into the hydrophobic core. Thus KL₄ most likely binds lipid membranes in an amphipathic helical conformation with the helix axis parallel to the plane of the bilayers. This orientation is supported by comparison of the effects of KL₄ and SP-B_{59–80} on lipid dynamics (86). Both peptides yield strikingly similar results when the changes in the dynamics of fully hydrated POPC/POPG and DPPC/POPG mixtures are examined, and their effects are somewhat similar to those of the antimicrobial peptide LL-37 at low peptide/lipid ratios (61). It has been established that both LL-37 and SP-B_{59–80} form helical structures at lipid interfaces rather than spanning

lipid bilayers. In all likelihood, the studies that found KL₄ to adopt a TM orientation suffered from the conditions of the experiments; however, it is likely that deep partitioning of the peptide into the membrane is energetically more favorable, and this may be a key feature in its function within lung surfactant.

The variation in the amphipathic helical structure of KL₄ sets it apart from classic amphipathic α -helices. Cationic, leucine-rich amphipathic helical peptides have been pursued as antimicrobial agents because their positive charge leads to preferential targeting of the anionic-rich membranes typical of prokaryotes (87). These peptides are specifically intended to form amphipathic α -helices based on naturally occurring antimicrobial peptides, and disrupt membranes on binding through the carpet mechanism or the formation of toroidal or barrel stave pores (87–89). However, these peptides have a significantly higher percentage of charged residues ($>50\%$ vs. $<25\%$ for KL₄), and the periodicity of the hydrophobic and hydrophilic residues is designed to favor classic amphipathic α -helices. We find no indication of membrane disruption by KL₄, consistent with the notion that its mechanism of interaction with lipids differs from that of antimicrobial peptides. The higher hydrophobicity of the amphipathic KL₄ helices modeled in Fig. 2 would favor more interaction with the lipid acyl chains relative to the lipid headgroups and thus would exert less positive curvature strain on the lipid bilayers.

Our data on lipid dynamics support our conclusions regarding the helical structure and orientation of KL₄. In POPC/POPG bilayers, a decrease in acyl chain order on addition of peptide suggests partitioning of the peptide into the lipid headgroup region. The acyl chain ordering in the DPPC/POPG lipids is more marked at positions 9–15 than in the plateau region, suggesting deep penetration of the peptide into the lipid bilayer with less perturbation of the headgroup region than is seen for leucine-rich TM helices. A π -helical conformation with snorkeling of the lysines would allow KL₄ to penetrate more deeply into saturated lipid bilayers while remaining perpendicular to the bilayer normal.

The proposed snorkeling of KL₄ within DPPC/POPG lipid multilamellar vesicles would have important consequences for membrane structure and function. This represents a novel peptide-lipid interaction that is unusual in comparison to what has been seen for membrane active amphipathic α -helices. Although the binding of KL₄ to POPC/POPG vesicles suggests that it could bind to these lipids in a manner similar to amphipathic α -helices, its ability to snorkel in DPPC/POPG lipids would have a different effect on lipid biophysics, particularly in the alveoli. Some of the thermodynamic costs of peptide penetration can be relieved by the formation of alternative nonlamellar structures, such as hexagonal phases (90,91). The formation of alternative lipid geometries, specifically the inverted H_{II} phase, has been found to be important for lung surfactant function (4). It has also been hypothesized that SP-B causes negative curvature strain in lung surfactant

and may even favor the formation of a hexagonal phase. Although we did not observe a hexagonal phase in this study, the partitioning of KL₄ deep into the DPPC/POPG bilayers would significantly increase negative curvature strain. The addition of small levels of cholesterol or POPE could further affect the curvature strain in the lipid bilayers and cause the formation of a hexagonal phase. Studies of the effect of KL₄ on other model lung surfactant compositions are ongoing. Preliminary work with DPPE/POPG lipids indicates that KL₄ promotes the formation of the H_{II} phase at lower temperatures. Alternatively, lipid geometry could be critical during fusion of lipids to the air-surfactant interface, and KL₄ snorkeling could be an essential feature for the proper shuttling of lipids. Our studies also highlight that the properties of KL₄ are lipid-dependent, and thus KL₄ could play a role in differential lipid partitioning to the air-water interface.

CONCLUSIONS

The unique interplay among lipid saturation, peptide penetration, and peptide structure we observed could explain the unique properties of KL₄ in the dynamic lung environment. DSC, ³¹P, ²H, ¹³C CPMAS, and ¹H spin diffusion data all indicate that KL₄ binds peripherally to 3:1 POPC/POPG lipids through electrostatic interaction of the lipid phosphates with the positively charged lysines. A different interaction is seen with 4:1 DPPC/POPG lipids, suggesting that the peptide penetrates to a far greater depth in the bilayer. CD measurements indicate that the peptide is helical in both lipid environments, although the amount and character of the helix are lipid-dependent; alterations in helix pitch would allow KL₄ to adapt its structure to match the environment for different insertion depths. Thus, both peptide structural plasticity and acyl chain saturation play an important role in determining the insertion level of KL₄. The variations in peptide penetration would directly affect the stability and composition of lipid structures in lung surfactant by increasing positive curvature strain in POPC-enriched domains and increasing negative curvature strain in DPPC-enriched domains. These changes in curvature strain would provide a mechanism for lipid trafficking from lamellar bodies and tubular myelin to the air-water interface in a manner selective for DPPC. The enrichment of DPPC at the air-water interface has been postulated to be one of the major roles of SP-B. The plasticity of KL₄ in its structure and insertion depth could be a consequence of the spacing of hydrophilic amino acids every five residues; the paucity of aromatic residues, which favor partitioning to the lipid interface; the high percentage of leucine residues, which favors interactions with lipid acyl chains; the choice of lysines for the charged amino acids, which allows for side-chain snorkeling; or a combination of these factors. The importance of each of these characteristics to the partitioning, insertion, and structure of amphipathic helices relevant to lung surfactant is the subject of ongoing studies. With

the findings presented here, a more thorough molecular model can be established to explain how this small peptide modulates lipid properties and drive the development of future SP-B mimetics.

SUPPORTING MATERIAL

Eight figures and five tables are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(09\)00667-5](http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)00667-5).

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