

Comparison of Bilayer and Monolayer Properties of Phospholipid Systems Containing Dipalmitoylphosphatidylglycerol and Dipalmitoylphosphatidylinositol

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Bilayer and monolayer phase behavior at the air–water interface of dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylinositol (DPPI), and their binary mixtures with dipalmitoylphosphatidylcholine (DPPC), in the context of their possible roles in lung surfactant function, have been systematically compared. Surface properties, as a function of temperature and composition, were evaluated and analyzed by measuring equilibrium surface pressure–area isotherms (π – A), equilibrium monolayer collapse pressure (π_c), and minimum area per molecule at the monolayer collapse pressure (A_c) and by direct observation using fluorescence microscopy. Bilayer properties were evaluated by measuring the main bilayer phase transition temperature (T_m) and the bilayer equilibrium spreading pressure (π_e) as a function of temperature and composition. Through thermodynamic analysis, it was found that DPPC/DPPG mix ideally in both the monolayer and bilayer states, whereas DPPC/DPPI (further supported with DPPC/Soy PI) are phase-separated with partial miscibility in bilayers and miscible with very significant positive deviations from ideality in monolayers. This behavior is attributed to the distinct differences between PG and PI headgroup properties as reflected in their physical size, state of hydration, and possible conformational flexibility, despite identical net negative charge and identical acyl chain headgroup properties. PC and PG exhibit very similar headgroup properties, which allow the dipalmitoyl chain properties to dominate over headgroup effects in bilayers and monolayers. In contrast, the unique PI headgroup properties dominate over the dipalmitoyl chain effects giving rise to very different surface and bilayer phase behavior.

Introduction

It appears from a large body of evidence that a complex mixture of phospholipids and proteins, making up lung surfactant, is required to provide normal lung function during breathing, as well as certain protective effects against infection and inflammation.¹ Although lung surfactant, packaged in the form of lamellar bodies, which are essentially bilayered structures, is made up of a variety of phospholipids and proteins, a number of interesting aspects of these systems are worthy of more simplified physical chemical analyses using model systems. In this study, we have attempted to carry out some physical chemical studies primarily on two synthetic phospholipids that have the same nonpolar portion but differ in polar headgroup: anionic dipalmitoylphosphatidylglycerol (DPPG) and anionic dipalmitoylphosphatidylinositol (DPPI). Such a comparison is possible for the first time, since until now, DPPI had not been available for such study.

Dipalmitoylphosphatidylcholine (DPPC) is the primary phospholipid component in lung surfactant, making up 55–60% of the zwitterionic phosphatidylcholine (PC) lipid.¹ Interestingly, in normal lung surfactant, phosphatidylglycerols (PG) also are present at a rather high

level relative to other lipids (compared to other mammalian tissues) at about 10% of total phospholipid, whereas phosphatidylinositols only exist at a level of about 2–5%.^{1,2} Before normal birth at term, in prematurely born infants suffering from a lack of lung surfactant from respiratory distress syndrome (RDS), or in other pulmonary disease states, such as cystic fibrosis, the PG content can be very low or even absent while the level of PI can be as high as the normal post-term level of PG at about 10%.^{1,2} This unique physiological event makes a physicochemical comparison of PG and PI particularly interesting for further study.

Recognizing that this entire physiological process is quite complex, involving phospholipids with different nonpolar acyl chains and polar headgroups and various pulmonary proteins, it still appears important to try to systematically learn more about the bilayer and monolayer properties of a model PG (e.g., DPPG) and PI (e.g., DPPI) and their relation to DPPC, the major component of lung surfactant. It is also important to systematically characterize their ability to mix with DPPC, so as to influence bilayer and monolayer phase behavior and the ability of bilayer spreading on an aqueous surface to occur from such binary mixtures.

In this study, we evaluate and systematically compare the following: (1) The bilayer and monolayer phase behavior of DPPC, DPPG, and DPPI individually as a function of temperature by measuring T_m , the gel to liquid crystalline bilayer phase transition temperature; the equilibrium spreading pressure, π_e , from bulk phospholipid

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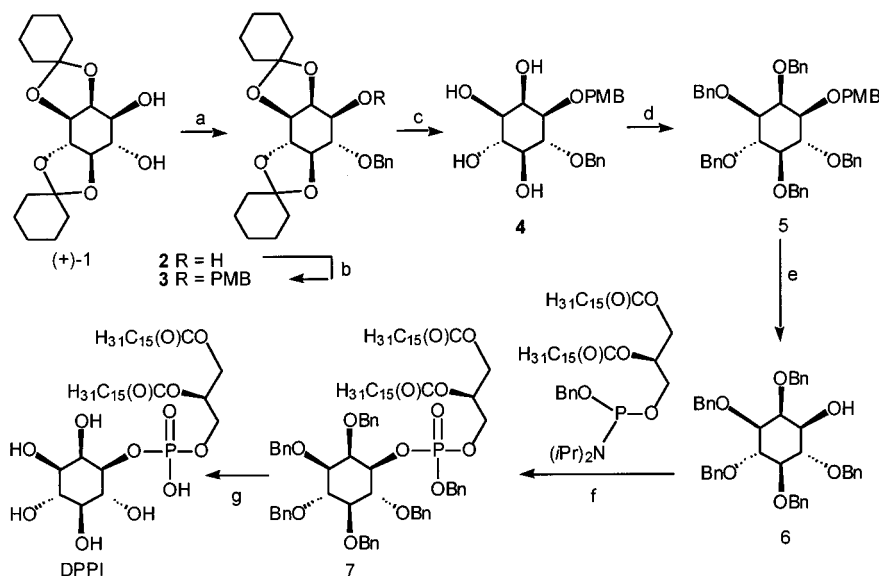
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Scheme 1



bilayer phases; and the surface pressure–area (π – A) isotherms, coupled with fluorescence microscopy, as a function of temperature. (2) Comparable studies were conducted by combining DPPC with DPPG and with DPPI in binary mixtures, to closely assess any critical and characteristic differences, such as physical size, state of hydration, and possible conformation, despite identical net negative charge and identical acyl chains due to the PG versus PI headgroup. To our knowledge, the only monolayer study on PI mixed with a PC phospholipid previously carried out was on bovine liver PI/DSPC monolayers which showed almost complete phase separation up to 20 mol % PI.³

Experimental Section: Materials and Methods

Materials. Phospholipids. The following phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), with a stated purity of >99% and were used as received: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-glycerol sodium salt (DPPG), 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC), and soybean L- α -phosphatidylinositol sodium salt (Soy PI). As specified by Avanti Polar Lipids, Inc., the fatty acid distribution in Soy PI is (33.2% 16:0, 7.1% 18:0, 5.5% 18:1, 46.8% 18:2, 7.1% 18:3, and other 0.3%).

Optically active 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-phosphoryl)-*D*-myo-inositol (DPPPI) was synthesized, for the first time to our knowledge, from (+)-1,2,5,6-di-*O*-cyclohexylidene-myo-inositol (optical purity greater than 99% enantiomeric excess) according to the procedure⁴ described in Scheme 1. The synthetic DPPPI (protonated form, molecular weight 810 g/mol) was characterized by ^1H and ^{32}P NMR and mass spectrometry, in which no appreciable impurity could be detected.

Solvents for Bilayer and Monolayer Surface Chemical Studies. The solvent used in all DPPC and Soy PI studies was chloroform (99.9% ACS high-performance liquid chromatography (HPLC) grade; Sigma Aldrich Co., Inc., Milwaukee, WI). DPPG and DPPI were dissolved in a heated cosolvent of chloroform/methanol (99.93% ACS HPLC grade; Sigma Aldrich) at a 99:1 volumetric ratio. The presence of any surface-active impurities in chloroform and methanol was checked by spreading them on water, and the surface tension was then monitored as the surface was compressed. No decrease in surface tension was observed upon

compression. The house-distilled water was passed through a five-cartridge Barnstead PCS filtration system with a resistivity of 18 M Ω cm and then distilled two additional times, once from an alkaline potassium permanganate solution and once from a dilute sulfuric acid solution, and collected in a glass container. The calcium content of the triply distilled water was analyzed by atomic emission spectroscopy and was found to be less than 9.5 ppb (2×10^{-7} M). The presence of 1 mM EDTA (ethylenediamine tetraacetic acid, 99.999%, Sigma Aldrich) in the subphase had a negligible effect on these selected anionic phospholipid monolayers, as also reported by others.⁵

All experiments were carried out using pH 7.40 buffer containing 10 mM Tris Ultrapure (hydroxymethyl-aminomethane, 99.9%, United States Biochemical Co.) and 150 mM NaCl (99.999%, Sigma Aldrich) adjusted to pH 7.40 with 1 N HCl volumetric standard (ACS reagent grade, Sigma Aldrich). These conditions were chosen since it has been shown in the literature that bilayer characteristics such as the bilayer phase transition temperature, T_m , are independent of pH and ionic strength at the selected pH and ionic strength.⁶

Methods. Thermal Analysis Studies. Phospholipid-containing chloroform solutions were placed in 15 μL Seiko aluminum differential scanning calorimetry (DSC) pans (Seiko Instruments Inc., Horsham, PA) designed to be hermetically sealed. These were dried for at least 24 h to remove all of the organic solvent in a vacuum oven (Precision Vacuum Oven, Precision Scientific, Winchester, VA). The appropriate amounts of pH 7.40, 150 mM NaCl/10 mM Tris buffer were added to the phospholipid samples in the aluminum DSC pans in order to make the lipid concentration 15% on a weight/weight (w/w) basis. This lipid concentration was chosen since it has been shown in our laboratory and in others that T_m is independent of the hydration level (fully hydrated) at a lipid concentration of 20% w/w or less.^{6–10} The Seiko DSC pans were then hermetically sealed with a Seiko hermetic sealer (Seiko I, Seiko Instruments Inc., Horsham, PA) to retain the level of hydration and vortexed (Super-Mixer, Cole-Parmer, Chicago, IL) at 50–60 $^\circ\text{C}$ for 3–6 h to ensure complete hydration of the phospholipid molecules.

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The gel to liquid crystalline phase transition temperatures, T_m , of the fully hydrated samples were measured under a dry nitrogen purge in a Seiko SSC 220C DSC (Seiko Instruments) fitted with an automated liquid nitrogen cooling accessory. Dry nitrogen gas was used as the purge and liquid nitrogen as the coolant. The measured DSC data were analyzed using a coupled Seiko DSC 5200 Data Station that consists of a Hewlett-Packard computer (model 712/60) using a UNIX platform. Samples were heated and cooled at 1 °C/min and found to give results in agreement with those obtained at scanning rates as low as 0.5 °C/min. The DSC was calibrated for melting points and heats of fusion using pure indium, gallium, and cyclohexane. At least four melting and four cooling scans were carried out to ensure T_m reproducibility. No hysteresis was observed on melting; therefore, all reported T_m values were taken from endotherms, as determined from the intersection of the endothermic baseline and the tangent of the onset of the melting peak.

One chromatographic spot on silica gel 60 F₂₅₄ precoated plastic sheets (EM Science, Cherry Hill, NJ) was observed using thin-layer chromatography (TLC) which confirmed phospholipid stability after thermal analysis. Two solvent mixtures were used for the TLC analyses: (1) 76% chloroform, 20% methanol, 2% acetic acid, and 2% water and (2) 69% chloroform, 27% methanol, and 0.04% water.

Surface Chemical Studies. For all surface chemical studies, the subphase was contained in a Teflon trough designed to maintain a constant temperature by circulating thermostated water through a glass coil at the bottom of the trough for surface pressure–area (π - A) monolayer experiments or in a jacketed glass beaker for bilayer spreading studies. Subphase and air temperatures were maintained at 25, 37, and 45 °C by a circulating water bath and temperature feedback system, respectively. All experiments were carried out in an aluminum box to prevent surface contamination and air drafts and to maintain a relative humidity of 80% or more with open evaporating dishes containing triply distilled water and wet filter paper.

Bilayer equilibrium spreading pressure, π_e , measurements were performed with the Wilhelmy plate technique using a platinum plate and a Cahn model 2000 Electrobalance (Cahn Co., Nicolet Instruments, Madison, WI). There are two ways to determine π_e of a bilayer: (1) deposit on or inject below the surface an aqueous lipid suspension and (2) sprinkle dry lipid powder directly from the manufacturer's bottle. We have used both ways, in selected cases, to show that both methods of determining π_e give the same results, although the kinetics of attaining equilibrium can be different.¹¹ In all reported bilayer spreading experiments, excess solid material was sprinkled onto the clean aqueous surface and the surface pressure at equilibrium due to bilayer spreading creating a monolayer was measured as described above. Final π_e values were determined when the change in the surface pressure of the monolayer was less than 0.2 mN/m per hour, independent of the amount of excess bulk lipid material on the surface and independent of the total surface area upon which the monolayer was spread.

Monolayer surface pressure–area, π - A , measurements of single-component and binary phospholipid mixtures were made by dissolving phospholipids into a single spreading solution that was deposited onto an aqueous surface using a Hamilton microsyringe (Hamilton, Reno, NV). Twenty minutes was allowed for the organic solvent to evaporate before measurements were made. All monolayer experiments were carried out under equilibrium (time-independent) conditions as determined by no more than a change of 1 mg per hour in meniscus mass which is equal to a change of 0.2 mN/m per hour. Monolayer equilibration times, at any given area per molecule, ranged from 1 to 24 h. The area per molecule, A_m , was determined under different conditions by three methods: (1) addition: stepwise addition of phospholipid molecules (after attainment of equilibrium) to a surface of constant area; (2) compression: stepwise reduction of surface area (after equilibrium is reached) with a movable barrier and a fixed amount of deposited material on the subphase; (3) single-shot: repeated cleaning and spreading of the monolayer film of

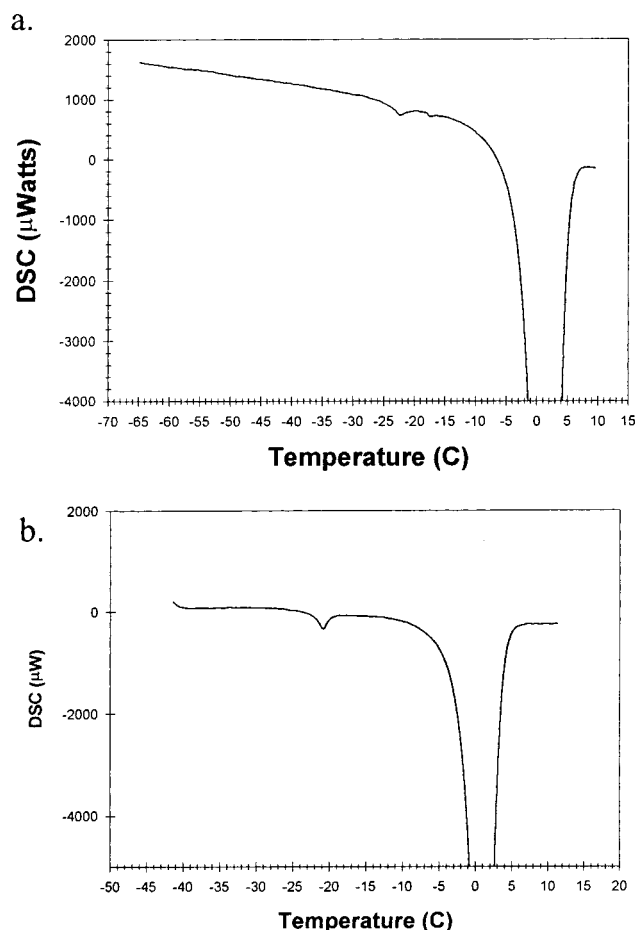


Figure 1. Thermal analysis of various phospholipid bilayers (15% w/w) at a heating/cooling scanning rate of 1 °C/min: (a) DPPI and (b) Soy PI.

a given surface concentration. To avoid any barrier leakage at higher surface pressures and to have relatively shorter equilibration times, methods 1 and 3 were primarily used. To further ensure that no possible overcompression occurred, each isotherm, in its entirety, was also created and verified by method 3. Good agreement among the three methods was confirmed with both expanded and condensed regions of selected monolayers. A_c is determined to be the point on the time-independent isotherm at which the monolayer just reaches the equilibrated collapse surface pressure.

Epifluorescence Microscopy of Monolayers. The equipment used to measure the epifluorescence of monolayers has been described in detail elsewhere^{12,13} and essentially consisted of a commercially available microscope (Micromaster, model E, Fisher Scientific, Pittsburgh, PA) that is connected to a high sensitivity Olympus DEI-750D CCD video camera system (Olympus America, Inc., Melville, NY) that is interfaced to a Mitsubishi HS-U795 SVHS video recorder (Mitsubishi Digital Electronics America, Inc., Irvine, CA) connected to a Sony Trinitron monitor. The phospholipid fluorophore, NBD-PC, was always present in the monolayer at a dye-to-lipid ratio of 1 mol %, since preliminary studies at this level of dye concentration revealed no effect of the dye on the π - A isotherm.¹²

Results

Bilayer Phase Transitions. *Single-Component Systems.* Seen in Figure 1 are the DSC thermograms for DPPI and Soy PI used to determine T_m (± 0.1 °C), the gel to liquid crystalline bilayer phase transition temperature.

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Table 1. Gel–Liquid Crystalline Phase Bilayer Transition Temperature for Binary Mixtures of DPPC/DPPI (15% w/w) Bilayers at a Heating/Cooling Scanning Rate of 1 °C/min

DPPC/DPPI	T_m PI-rich (°C)	T_m PC-rich (°C)
0:100	−24.9	
50:50	−24.4	9.0
70:30	−20.0	37.0
90:10	−8.9	39.5
100:0		41.2

Table 2. Gel–Liquid Crystalline Phase Bilayer Transition Temperature for Binary Mixtures of DPPC/Soy PI (15% w/w) Bilayers at a Heating/Cooling Scanning Rate of 1 °C/min

DPPC/ SOY PI	T_m PI-rich (°C)	T_m PC-rich (°C)
0:100	−22.4	
10:90	−21.7	
30:70	−21.7	
50:50	−22.1	21.7
70:30	−21.4	33.4
90:10	−21.9	36.9
100:0		41.2

As observed previously^{6,14} and confirmed in our laboratory, DPPC and DPPG (data not shown) give very similar T_m values of 41.2 and 40.3 °C, respectively, following the formation of the ripple phase (which exhibits gel short-range order) at T_p , whereas DPPI exhibits a significant decrease in T_m to −24.9 °C (with no ripple phase formation) quite comparable to that observed for Soy PI, with a more variable fatty acid composition, as described in the materials section. Of most significance in the DPPI bilayer state is the extraordinary depression of T_m into a region of about −20 °C (wherein other dipalmitoyl phospholipids have elevated T_m), a value generally associated with highly unsaturated phospholipids, such as dioleoyl PC, as shown in our laboratory and others.⁸

Binary Mixtures. For DPPC/DPPG (data not shown), the T_m values (and T_p) gradually change between the very close values obtained for each pure component. Notably important are the ripple phase (exhibiting gel short-range order) and a sharply single main phase transition at all compositions. Since the T_m values of DPPC and DPPG are so close, if this mixture were immiscible, it would not be expected that two separate distinct endothermic peaks would be detected. Comparing the ideal and experimental phase transition data, however, indicates complete miscibility, as reported by others¹⁵ and confirmed under our conditions.

For all compositions of 50:50 molar ratio and higher of DPPC/DPPI and DPPC/Soy PI, two T_m values (Tables 1 and 2, respectively) were obtained from two major DSC peaks (parts a and b of Figure 2, respectively), excluding that expected for ice. These are interpreted to represent a DPPC-rich phase and a DPPI- or Soy PI-rich phase. Thus, whereas DPPC and DPPG form a miscible bilayer system at all compositions, the introduction of PI with the same two palmitoyl chains appears to cause phase separation with partial miscibility in the bilayer. The significance of the PI headgroup in causing such effects is confirmed by similar behavior with Soy PI. Below 50:50 DPPC/Soy PI (data not shown), it appears that only the Soy PI-rich peak is present effectively solubilizing all of the DPPC molecules among PI molecules. Also notably important is that the broad DPPC-rich peak with a depressed T_m (presumably due to the solubilization of DPPI

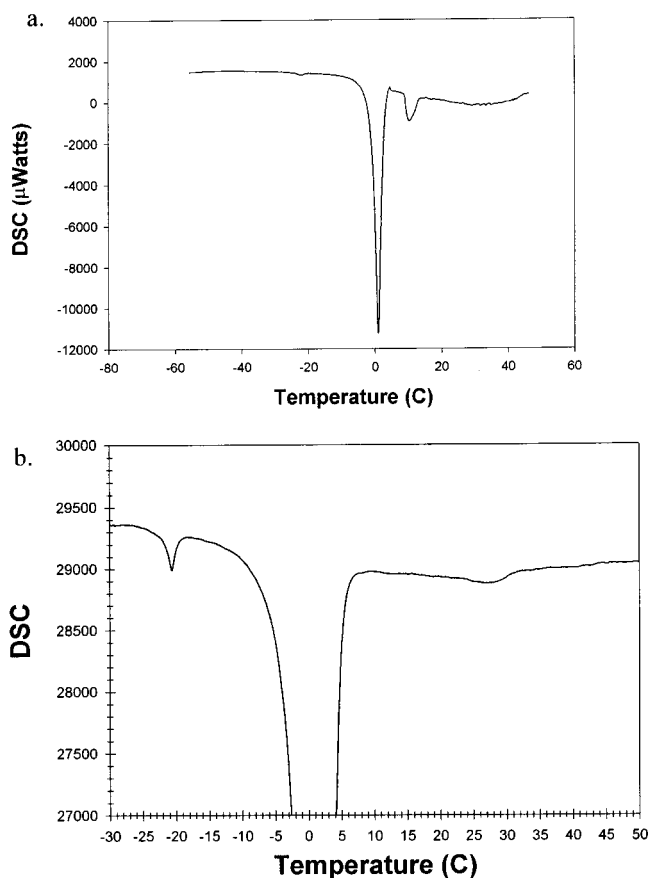


Figure 2. Thermal analysis of binary mixtures of phospholipid bilayers (15% w/w) at a heating/cooling scanning rate of 1 °C/min: (a) DPPC/DPPI 50:50 (15% w/w) and (b) DPPC/Soy PI 50:50.

in DPPC), with no ripple phase formation (indicative of the fluidizing properties of the PI headgroup), is not seen until as much as 50% DPPC is mixed with PI. Moreover, as much as 90 mol % DPPC must be present to significantly elevate the T_m values of both the DPPI-rich and DPPC-rich phases, as seen in Table 1.

Bilayer Spreading. Single-Component Systems. For DPPC and DPPG at 25 °C (gel phase) and 37 °C (ripple phase), below their respective T_m , π_e is observed to be approximately 0 mN/m (defined as any $\pi_e < 0.2$ mN/m), whereas at 45 °C, above the individual T_m values of DPPC and DPPG, each liquid crystalline material spontaneously spreads to $\pi_e = 45 \pm 0.3$ mN/m. Clearly, whereas most dipalmitoyl phospholipid derivatives, such as DPPC and DPPG in their fully hydrated state, have rather high T_m values relative to, for example, 37 °C and therefore remain in the gel or ripple phase under such conditions, DPPI is in its liquid crystalline phase at these temperatures. With such a low value of T_m , it is not surprising that a DPPI bilayer spreads spontaneously to π_e of 45 mN/m on an aqueous interface at 25 °C and higher. This occurs at 25 and 37 °C, in contrast to DPPC and DPPG, presumably, since it is in its highly disordered liquid crystalline phase at these temperatures. The same behavior is also noted for Soy PI because of its similarly reduced T_m .

Binary Mixtures. Spreading of binary mixtures of DPPC/DPPG, DPPC/DPPI, and DPPC/Soy PI produces a similar pattern dependent on the relative values of the mixture T_m and whether the operating temperature is above or below T_m of the mixture. For all DPPC/DPPG compositions at 25 °C (miscible gel phase) and 37 °C (miscible ripple phase), the bilayer $\pi_e \sim 0$ mN/m, whereas at 45 °C (miscible

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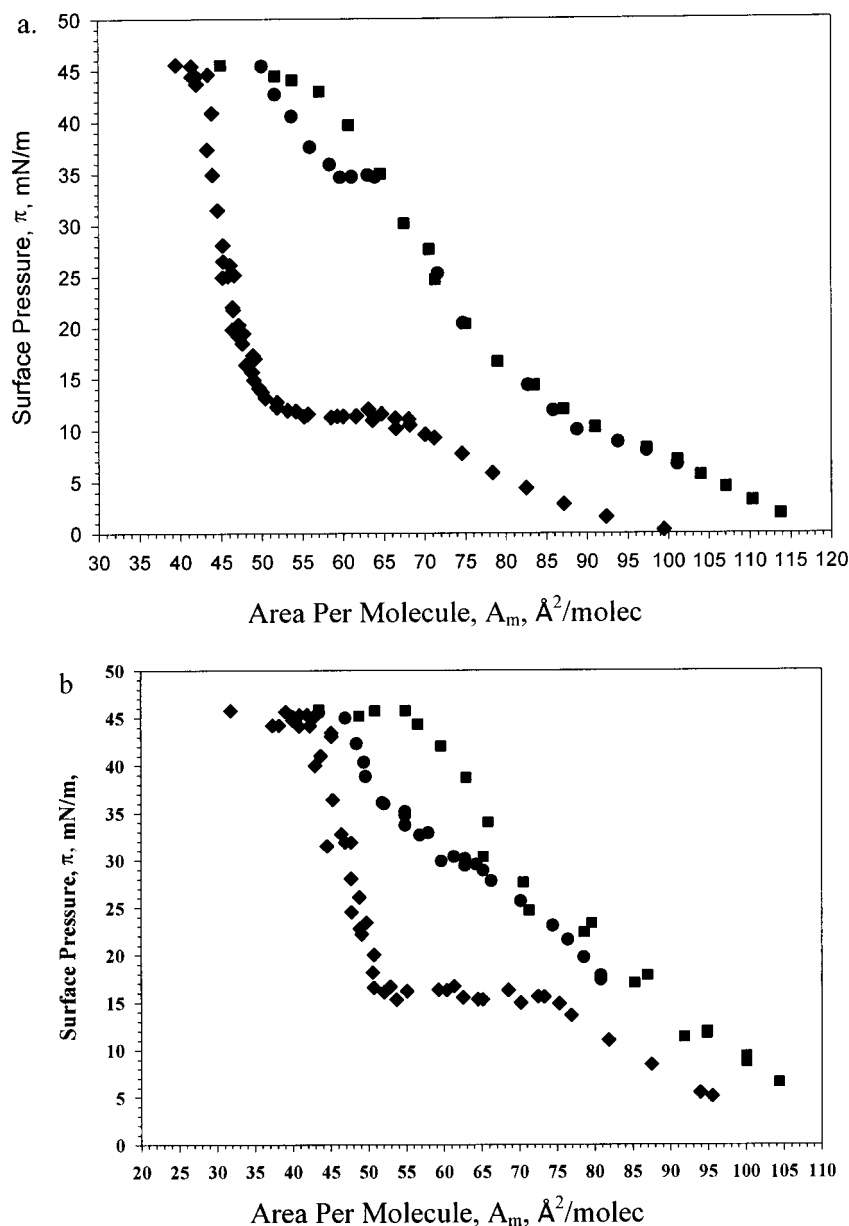


Figure 3. Surface pressure (π) vs area per molecule (A_m) for DPPC and DPPG single-component monolayers at the air–water interface spread on pH 7.40 Tris buffer with 150 mM NaCl: (a) DPPC at 25 °C (♦), 37 °C (●), and 45 °C (■); (b) DPPG at 25 °C (♦), 37 °C (●), and 45 °C (■).

liquid crystalline phase), $\pi_e \sim 45$ mN/m occurs. It is important to note, however, that for DPPC/DPPI and DPPC/Soy PI bilayer binary mixtures at all temperatures and compositions, the bilayer spontaneously spreads to $\pi_e \sim 45$ mN/m, presumably due to demixing into PI-rich and PC-rich phases, thereby allowing the liquid crystalline PI-rich phase to spread at all temperatures. Clearly, bilayer spreading of DPPI relative to DPPG indicates, as does T_m , the significant difference in headgroup effects due to different properties exhibited between the PG and PI polar headgroups, despite their similar net anionic charge. Specifically, the significant decrease (uncharacteristic of dipalmitoyl derivatives where an elevation in T_m is characteristically seen) in T_m of DPPI (below that of ice) due to the PI headgroup is indicative of its very fluid nature in the bilayer state which is consequently reflected in its spontaneous spreading behavior. This fluid nature of a dipalmitoyl phospholipid (DPPI) is a direct reflection of a lower bilayer packing density, greater intermolecular distance between adjacent DPPI molecules

(and Soy PI), and more disorder present in the gel state, despite the presence of dipalmitoyl chains and their characteristic bilayer condensing effect. The unique properties of the PI headgroup of larger headgroup size, greater tendency to imbibe water due to its unique chemical structure giving it the ability to undergo exceptionally strong hydrogen bonding with water, and the conformational flexibility of PI structure (to achieve maximal hydration) give rise to greater separation between adjacent DPPI molecules, so much so that the dipalmitoyl chains cannot condense themselves to any significant degree.

Monolayer Studies. Single-Component Systems. Parts a and b of Figure 3 present π – A isotherms for DPPC and DPPG, respectively, at 25, 37, and 45 °C. For DPPC and DPPG at 25 °C, below their respective bilayer T_m values, these monolayers as observed earlier^{12,16} exhibit a well-defined first-order liquid expanded (LE)/liquid condensed

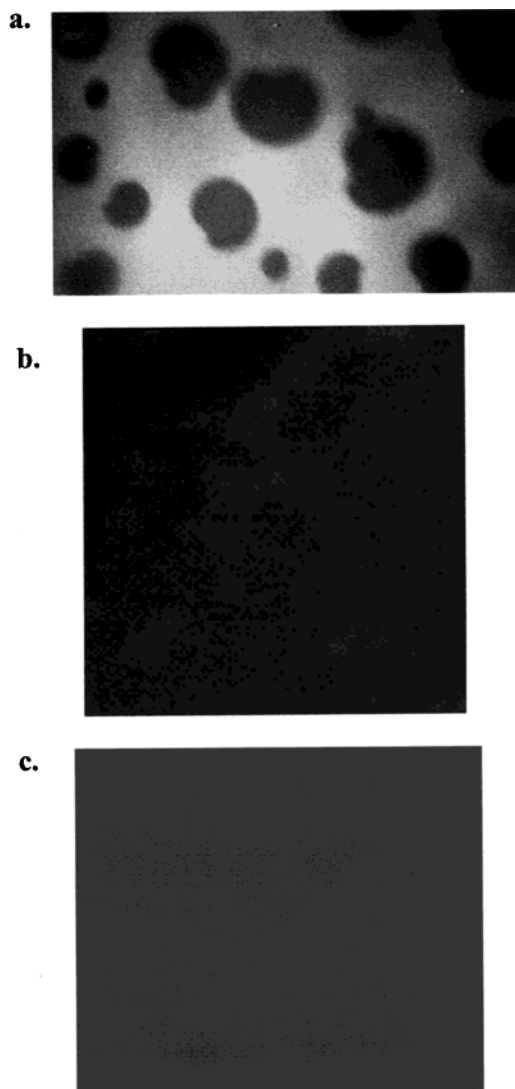


Figure 4. Phase behavior using fluorescence microscopy of DPPC monolayers at the air–water interface spread on pH 7.40 Tris buffer with 150 mM NaCl at various temperatures: (a) 12 mN/m at 25 °C, (b) 35 mN/m at 37 °C, and (c) 40 mN/m at 45 °C.

(LC) surface phase transition of invariant surface pressure, π_t , over a wide range of A_m . At 25 °C, this is at 11.3 mN/m over the A_m range of 53–65 Å²/molecule for DPPC; for DPPG, π_t occurs at 15.3 mN/m over the A_m range of 57–68 Å²/molecule. For DPPC and DPPG at 37 °C (Figure 3) under equilibrium conditions, each monolayer exhibits a well-defined first-order LE/LC phase transition occurring at a higher surface pressure and over a narrower range of A_m than that observed at 25 °C. At 37 °C, π_t occurs at 34.8 mN/m over an A_m range of 58–64 Å²/molecule for DPPC; for DPPG, π_t occurs at 30.8 mN/m over an A_m range of 59–64 Å²/molecule. As seen in Figure 4, at 25 and 37 °C, the DPPC monolayers, as confirmed by fluorescence microscopy (FM), are homogeneous in both the LE phase and the LC phase and phase separated in the region of LE/LC coexistence at π_t , the surface pressure for the LE/LC surface phase transition. Similar behavior was observed for DPPG (data not shown). In Figure 4, at 45 °C, above the T_m of DPPC, fluorescence microscopy shows that the DPPC monolayer is completely homogeneous up to and just below collapse, and the π – A isotherms (Figure 3) are nearly identical and continuous (liquid expanded) right up to the monolayer collapse point. The same behavior was noted for DPPG (data not shown). Upon

very close visual examination of Figure 3, there appears to be a discontinuity in the DPPC isotherms at 37 and at 45 °C in the region of 10–15 mN/m. FM results indicate no region of coexistence by lack of any phase separation. Since there is no evidence for distinct phase separation by FM or characteristic first-order invariance of surface pressure over a finite range of area per molecule on the 37 and 45 °C isotherms in the region of 10–15 mN/m, this discontinuity is not interpreted to be a first-order transition. It may represent a higher-order transition, but we have no other evidence to suggest this. Such a discontinuity was not observed with DPPG at these temperatures.

In Figure 5, it is observed that at 25 and 37 °C, both DPPI and Soy PI isotherms exhibit no phase transitions from the LE to LC regions right up to the monolayer collapse pressure, $\pi_c \sim 45$ mN/m, as confirmed by visual observation using FM by a lack of any domains (data not shown). The Soy PI monolayer isotherm is shifted to the right (slightly more expanded) of the DPPI monolayer isotherm; however, the shapes of the curves appear to be very similar.

The π_c values for DPPI and Soy PI are essentially identical to π_c (± 0.4 mN/m), the collapse pressure, at all temperatures studied (all above T_m), whereas for DPPC and DPPG, with π_c essentially 0 mN/m at temperatures below T_m , both spread monolayers collapse at about 45 mN/m at all temperatures studied, that is, above and below T_m (to be discussed).

Observation of the π – A isotherms in Figures 3 and 5 further reveals interesting headgroup effects. Note that at 25 °C, as expected, DPPG with its anionic charge creates a more expanded LE phase than DPPC, whereas at surface pressures above the LE/LC surface phase transition region, both monolayers become very condensed, in a similar fashion. On the other hand, DPPI (further supported by Soy PI) remains expanded all the way up to collapse.

The minimum area per molecule at the collapse pressure, A_c , for the various systems is of interest, since presumably this is most likely related to the point when the bilayer phase is formed and therefore it might reflect, under equilibrium time-independent conditions, the effective physical size of the polar headgroup in the bilayer phase. At 25 °C, A_c (± 0.3 Å²/molecule) is 40.0 and 41.3 Å²/molecule for DPPC and DPPG, respectively. This trend for DPPG to collapse at a slightly higher A_c than that of DPPC remains at 37 and 45 °C, whereas the liquid expanded regions of the isotherms become more similar. These values of A_c obtained from the equilibrium isotherms, below and above T_c , are in excellent agreement with the size of the PC and PG headgroups in the bilayer state reported in the literature (to be discussed).

It can be seen, by comparing Figures 3 and 5, that at 25 °C, the DPPI isotherm is significantly shifted to the right (much more expanded giving the same surface pressure at a higher area per molecule) of DPPC and DPPG at all surface pressures with a collapse area per molecule, A_c , at 25 °C of 47.5 Å²/molecule, significantly greater than those for DPPC and DPPG, which again agrees with the PI headgroup having the largest headgroup size (to be discussed). The general pattern of such expansion and collapse to a larger area per molecule is also observed with Soy PI. Shifted slightly to the right of the DPPI isotherm, the Soy PI monolayer is more expanded due to the presence of a mixture of saturated acyl chain lengths and multiple unsaturated acyl chains. At 25 °C, in the LE phase, the negatively charged DPPG and DPPI monolayers appear to exhibit almost identical π – A isotherms that are much more expanded than that for the zwitterionic

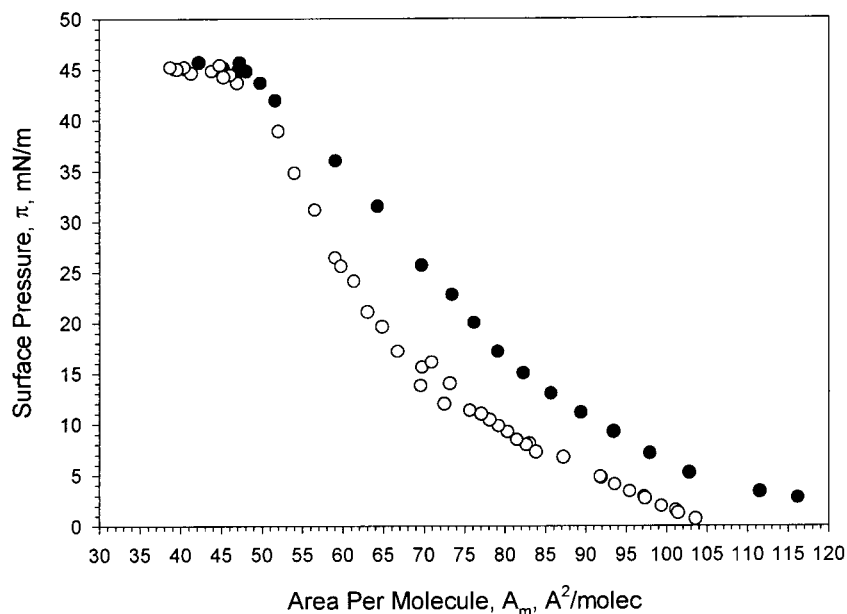


Figure 5. Comparison of the surface pressure (π) vs area per molecule (A_m) isotherms for DPPI (○) and Soy PI (●) monolayers at the air–water interface spread on pH 7.40 Tris buffer with 150 mM NaCl at 25 °C.

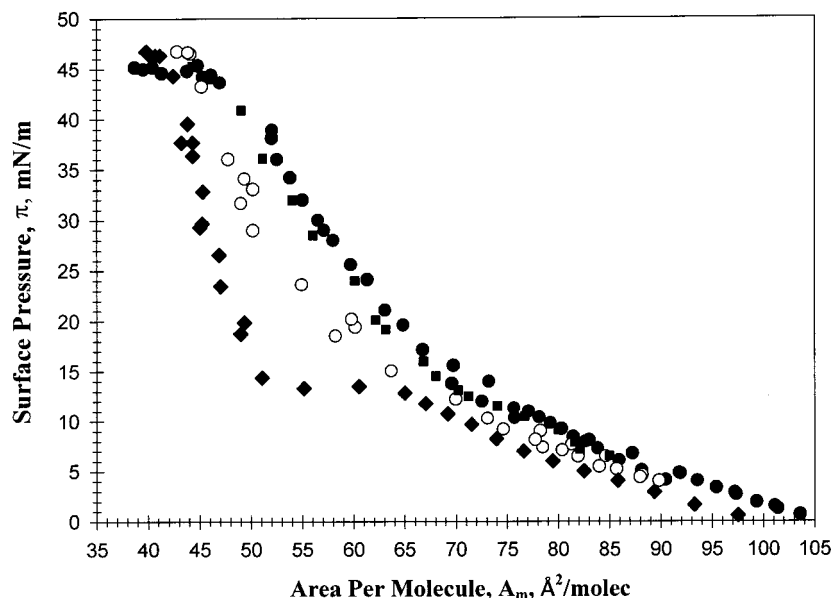


Figure 6. Surface pressure (π) vs area per molecule (A_m) for DPPI (●), DPPC/DPPC 50:50 (■), DPPC/DPPC 70:30 (○), and DPPC/DPPC 90:10 (◆) monolayers at the air–water interface spread on pH 7.40 Tris buffer with 150 mM NaCl at 25 °C.

DPPC, presumably due to a negative charge repulsion. Yet, at higher π values, DPPC behaves more similarly to the DPPC monolayer, while DPPI remains more expanded in the LE phase at all values of π – A . Thus, with the same acyl chains, the PI polar headgroup consistently shows significantly different patterns of surface behavior from those of PG that must be accounted for by headgroup properties other than only net anionic charge (to be discussed).

Binary Mixtures. Isotherms of various monolayers of DPPC/DPPI at 25 °C are depicted in Figure 6 to illustrate certain general points about mixing tendencies caused by the mixtures of the PG versus PI headgroups relative to their bilayer T_m and π_e values.

The monolayer phase behavior results under our conditions for a 50:50 molar mixture of DPPC/DPPC (data not shown) at 25 °C are similar to those reported previously¹² exhibiting a well-defined first-order LE/LC phase transition in the isotherm with surface phase

separation observed by FM at π_t (data not shown). Figure 6 depicts the π – A isotherms and fluorescent micrographs (Figure 7) at 25 °C of DPPC/DPPI at the three molar ratios of 50:50, 70:30, and 90:10. Inspection of these isotherms reveals a very significant effect of DPPI on DPPC at molar ratios of DPPC/DPPI of 50:50 and 70:30, both showing no apparent first-order LE/LC phase transition (Figure 6) on the isotherm nor any stable domain formation (Figure 7a,b) and each with a higher monolayer A_c than that of DPPC. In contrast, the DPPC/DPPI 90:10 monolayer isotherm (Figure 6) is quite similar to that of DPPC alone at 25 °C (Figure 3) exhibiting a well-defined first-order LE/LC phase transition with stable characteristic domain formation of the equilibrium transition (Figure 7c) but with a slight shift of the isotherm to the right (more expanded) to that of the DPPC π – A curve. The Soy PI/DPPC 50:50 isotherm (data not shown) is similar to and further supports the trends seen in the DPPC/DPPI 50:50 isotherm showing no apparent first-order LE/LC phase

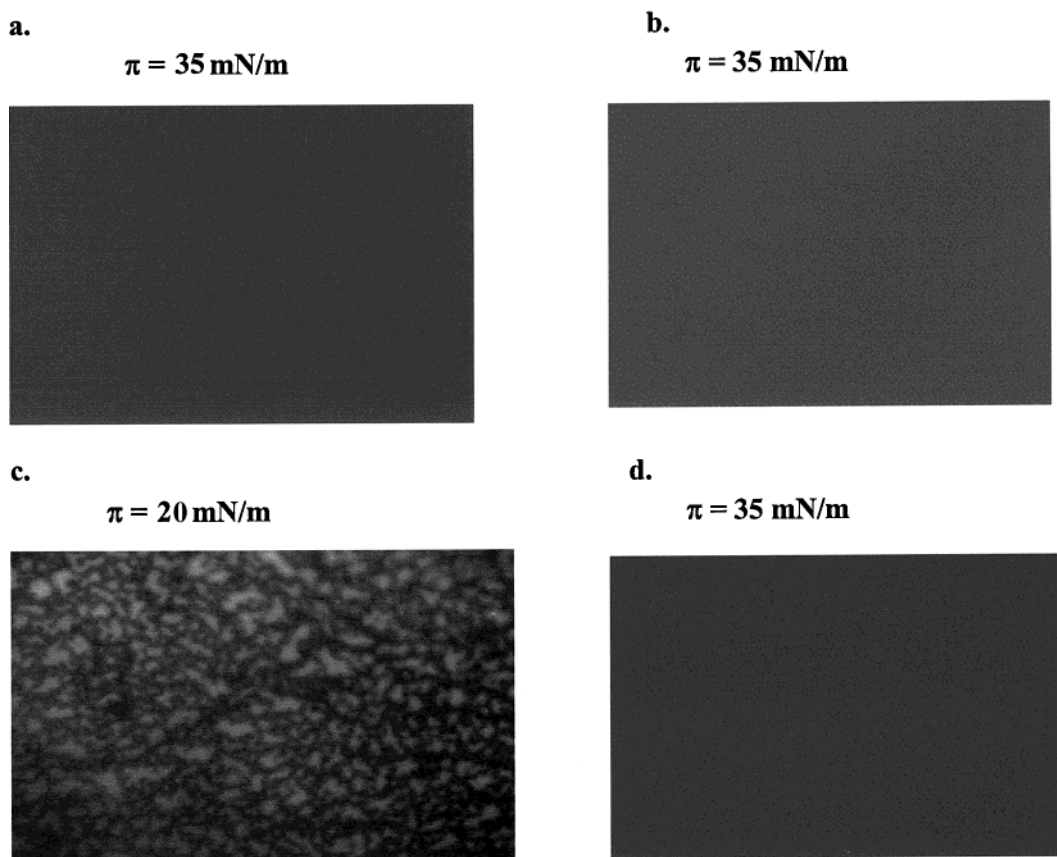


Figure 7. Phase behavior using fluorescence microscopy of various DPPC/PI monolayer binary mixtures at the air–water interface spread on pH 7.40 Tris buffer with 150 mM NaCl at 25 °C: (a) DPPC/DPPI 50:50 at 35 mN/m; (b) DPPC/DPPI 70:30 at 35 mN/m; (c) DPPC/DPPI 90:10 at 20 mN/m; (d) DPPC/Soy PI 50:50 at 35 mN/m.

transition nor any stable domain formation (Figure 7d). Another important observation in these monolayer studies is that all mixtures containing PI, at all temperatures under equilibrium conditions, collapse at $\pi_c \sim 45$ mN/m, which is the monolayer collapse surface pressure value observed for single components above T_m .

To further and more quantitatively describe the effects of adding DPPI to DPPC in binary mixtures, relative to DPPG/DPPC mixtures, one can carry out the following analysis. For monolayer binary mixtures exhibiting ideal mixing or complete immiscibility, the additivity rule relates the dependence of the average area per molecule, A_{av} , which is the ideal area per molecule, A_{ideal} , to monolayer composition as^{17–19}

$$A_{ideal} = A_{av} = N_1 A_1 + N_2 A_2 \quad (1)$$

where A_1 and A_2 are the areas per molecule of each monolayer component at constant surface pressure and N_1 and N_2 are their respective mole fractions.

Nonideal miscible monolayers represent interactions between the components leading to deviations from the additivity rule through the parameter A^E , the excess area. A^E is defined as

$$A^E = A_{12} - (N_1 A_1 + N_2 A_2) \quad (2)$$

where A_{12} is the actual area per molecule of the mixed monolayer at a given surface pressure. When $A^E = 0$, ideal

mixing or complete immiscibility occurs, as can be seen with molecules of very similar molecular shape and volume. When $A^E > 0$, positive deviations from the ideal area per molecule occur due to net repulsive interactions between the individual monolayer components, whereas $A^E < 0$ indicates negative deviations resulting from net attractive interactions between the two monolayer components.

Depicted in Figure 8 are the monolayer miscibility analyses for DPPC/DPPG and DPPC/DPPI binary mixtures at 25 °C. DPPC/DPPG shows no deviations from ideality in good agreement with previous reports,¹² which most likely may indicate ideal mixing, as is seen in the bilayer state. In contrast, DPPC/DPPI exhibits significant positive deviations from ideality, indicating a miscible monolayer with net repulsive interactions predominating between the individual DPPC and DPPI molecules. Such significant monolayer nonidealities in the mixing of DPPC and DPPI are further confirmed by similar results with DPPC/Soy PI mixtures, unlike the binary mixture of bovine liver PI/DSPC monolayers which clearly exhibits almost complete demixing for up to 20 mol % PI.³ Thus, one can conclude that whereas DPPC/DPPG mixtures tend to mix ideally, DPPC/DPPI mixtures exhibit very strong tendencies toward phase separation but remain miscible under equilibrium time-independent conditions up to collapse, at detectable levels, using fluorescence microscopy.

Discussion

Comparison of DPPG and DPPI. These studies using anionic DPPG and anionic DPPI having the same acyl chains but differing in headgroup have revealed the rather significant effects that such a substitution can have on

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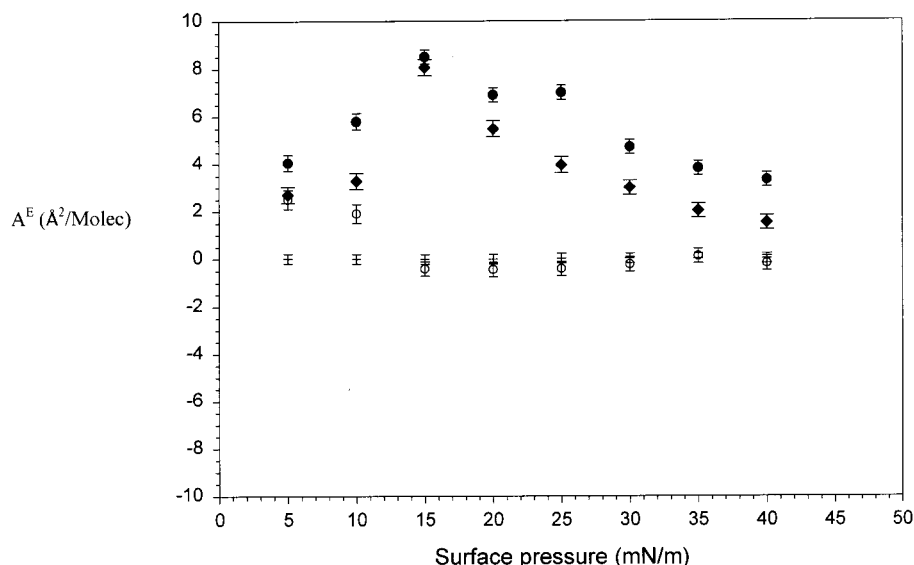


Figure 8. Two-dimensional mixing analysis of DPPC/DPPG 50:50 (+), DPPC/DPPI 50:50 (●), DPPC/DPPI 70:30 (◆), and DPPC/DPPI 90:10 (○) monolayers at the air–water interface at 25 °C spread on pH 7.40 Tris buffer with 150 mM NaCl.

some important bilayer and monolayer properties. The critical observation, in this regard, is how the bilayer gel to liquid crystalline main phase transition temperature, T_m , undergoes a shift from 40.3 °C for DPPG to −24.9 °C for DPPI. Such a marked reduction in T_m , ordinarily brought about by multiple substitutions of unsaturation on acyl chains, must reflect a very strong effect of PI on the packing of molecules in the bilayer, so as to reduce the usual strong acyl chain interactions that produce high values of T_m for dipalmitoyl acyl chains. Not surprisingly, the value of T_m for the bilayer, which is also the critical temperature for a LE/LC surface phase transition of a phospholipid monolayer to occur, T_c , in turn reflects properties that control such important events as (1) spontaneous spreading to a form a monolayer from the bilayer phase and (2) mixing tendencies with zwitterionic DPPC, within the bilayer and monolayer states, as described in this study.

Before attempting to analyze what causes DPPI to have such effects, it might be useful to briefly explore DPPG and, in particular, its relationship to DPPC. For example, anionic DPPI has a very low T_m relative to zwitterionic DPPC, but DPPG, also anionic, has a T_m that is essentially the same as that for DPPC. Consequently, mixtures of DPPC and DPPG tend to mix uniformly with close to ideal behavior, despite the net negative charge of DPPG. From the literature, it appears that the PG polar headgroup, in its anionic state, has a similar effective size to that of the PC polar headgroup, due to its negative charge and favorable formation of the hydration layers around the glycerol hydroxyls and PO^- groups through hydrogen bonding with water,²⁰ increasing the effective size of PG to a nearly identical value of PC in bilayers to about 40 Å² at temperatures below T_m ,^{21–23} whereas at temperatures above T_m , the reported headgroup size is in the range of 53–65 Å².^{23–27} The value of A_c on a time-independent π – A

isotherm reflects contributions from physical headgroup size, from hydration tendencies, and from headgroup charge. That is to say that A_c obtained under our conditions reflects the effective polar headgroup size of a phospholipid. These reported literature bilayer values are in excellent agreement with the A_c values presented in this paper above and below T_c . This increase in the effective size of PG due to hydration also may effectively shield the negative charge from adjacent DPPG molecules, providing tight enough packing to promote the condensed behavior that is characteristic of the dipalmitoyl acyl chains, as seen in the bilayer and monolayer states. Using a thermodynamic model based on the Bragg–William approximation to evaluate the excess free energy of mixing, the nonideality parameter of mixing, ρ_0 , was found to be similar for PC and PG headgroups in hydrated mixed bilayers meaning that the relative contributions of the headgroup interaction to the overall intermolecular interaction are similar for PC and PG²⁸ in the bilayer state. Moreover, the ideal mixing tendency of PC and PG in the monolayer state and the condensed nature of DPPC/DPPG monolayers have been attributed to the possibility of the formation of hydrogen bonds between the phosphate on PC and the hydroxyl groups on PG molecules.²⁹

In contrast to PG, the PI headgroup on DPPI exhibits a remarkably low T_m and highly expanded monolayer, which would seem to support a state of the headgroup through size (physical and effective), charge, and hydration properties that tends to minimize molecular packing in the bilayer and monolayer states and, hence, favor a greater degree of bilayer spreading under similar conditions relative to T_m . This is certainly consistent with the significantly greater tendency to spontaneously spread at lower temperatures, that is, 25 and 37 °C.

It has been shown in this study that at temperatures above T_m , a phospholipid monolayer most likely exists in the liquid expanded phase, where the phospholipid acyl chains are highly mobile and compressible and the acyl

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chain ends lift off the surface and interact through van der Waals interactions, but where disorder and mobility are present, up to collapse at 45 mN/m. In this case, since π_e and π_c are equal at 45 mN/m, under time-independent conditions, the collapsed bilayer must be in its thermodynamically stable liquid crystalline phase. Furthermore, this conclusion is supported, as discussed above, from the A_c values from these monolayer studies at $T > T_c$, which agree very well with values of PC and PG headgroup areas in liquid crystalline bilayers reported in the literature. Conversely, when a liquid crystalline bilayer spreads, the monolayer formed at equilibrium is in the liquid expanded phase. This equilibrium phenomenon can be attributed to similar properties shared by the monolayer liquid expanded phase and bilayer liquid crystalline phase, namely, the similar degrees of molecular disorder and molecular mobility, which allow them to coexist, at minimum surface free energy.

At temperatures below T_m , DPPC and DPPG molecules in a monolayer maintain themselves in a highly over-compressed monolayer state with collapse to a bulk bilayer phase with $\pi_c \sim 45$ mN/m most likely different than the gel phase for which $\pi_e \sim 0$ mN/m (gaseous monolayer). Since this collapsed bilayer is fluid (as revealed by the high $\pi_c \sim 45$ mN/m), it possesses liquid-crystalline-like properties, but the operating temperature is below T_m ; thus, this bilayer is a metastable liquid crystalline bilayer that exhibits kinetic stability (time-independent) but thermodynamic instability. Furthermore, as discussed above, A_c values from these monolayer studies at $T < T_c$ agree very well with values of PC and PG headgroup areas in gel bilayers reported in the literature. This is to say that this collapsed metastable liquid crystalline bilayer needs a very long amount of time, relative to experimental time scales, to nucleate and form its thermodynamically stable gel phase with a surface pressure of ~ 0 mN/m (gaseous monolayer) at temperatures below T_m . Moreover, a DPPC or DPPG gel bilayer (and their gel mixtures) spreads negligibly forming a gaseous phase monolayer, in which minimal intermolecular interactions among acyl chains and headgroups are present and molecular mobility is high, resulting in a surface pressure near 0 mN/m. This phenomenon is a classic example of the significant thermodynamic effect of entropy alone on driving a very highly ordered structure with a low degree of molecular mobility (bilayer gel phase) to coexist at equilibrium with a very highly disordered structure with a high degree of molecular mobility (monolayer gaseous phase).

What is very interesting about DPPI in the context of its mixing with DPPC is that it exhibits very significant positive deviations from ideality in miscible mixed monolayers, while showing phase separation with partial miscibility in bilayers. Clearly, therefore, some property of the PI polar headgroup, other than just net anionic charge, has a very distinct effect on the structure of DPPI at the interface, since DPPG is also negatively charged. With regard to bilayer spreading behavior from mixtures of DPPC with DPPG or DPPI, one can also see an interesting difference. Being ideally mixed both in the bilayer and monolayer states, with very close and high T_m values, no enhancement of spreading from DPPC/DPPG bilayers at all compositions is observed at 25 and 37 °C. On the other hand, being phase separated into a PC-rich and a PI-rich phase in the bilayer state yet miscible in the monolayer state, bilayer spreading from the various mixtures of DPPC/DPPI spontaneously occurs at all compositions, at all temperatures. One would conclude that it is the PI-rich phase that undergoes spontaneous bilayer spreading, since it remains in the liquid crystalline

phase at all temperatures. The fact that π_e remains constant over all compositions of PC/PI despite bilayer phase separation and monolayer miscibility up to the collapse pressure is an interesting question that can be analyzed using the two-dimensional phase rule. The surface phase rule, modified for application to insoluble monolayers in equilibrium with their three-dimensional bulk phase at π_c , is³⁰

$$F = C - P^B - P^S + 1 \quad (3)$$

where, at constant external pressure and temperature, F is the number of degrees of freedom, C is the number of chemical components, P^B is the number of bulk phases, including water, and P^S is the number of surface phases, not including water. In the case of bilayer spreading, where the components are phase separated in the bilayer state and miscible in the monolayer state (opposite phase behavior), $F = 3 - 3 - 1 + 1 = 0$. Thus, indeed, at the equilibrium point for bilayer-to-monolayer spreading, there are no degrees of freedom, and π_e is fixed at 45 mN/m, independently of bilayer composition. This immiscible bilayer, under the right conditions, spontaneously spreads to a miscible monolayer, if at least one of the bulk lipid phases in the bilayer has a T_m below the operating temperature.

Analysis of the mixing behavior in the monolayer state below collapse of DPPC with DPPI and Soy PI agrees with the results presented as a function of monolayer composition. For the DPPC/DPPI 50:50 and 70:30 miscible mixtures and the DPPC/Soy PI 50:50 miscible mixture below the monolayer collapse pressure, $F = 3 - 1 - 1 + 1 = 2$, meaning that surface pressure varies with area per molecule and phospholipid composition, as is indeed observed on the surface phase diagrams. For DPPC/DPPI 90:10, analyzing the phase transition region and beyond where two distinct surface phases exist (PI-rich LE phase and PC-rich LC phase) yields $F = 3 - 1 - 2 + 1 = 1$, indicating that only one variable, surface pressure or A_m , needs to be defined in order to know the other variable, in good agreement with its surface phase diagram.

Finally, one asks why DPPI should be so different from DPPG in producing a variety of bilayer and monolayer effects. On the basis of previous studies, it appears that the anionic PI polar headgroup has an effectively greater tendency to imbibe and bind water (which does not freeze)³¹ due to its great tendency to undergo exceptionally strong hydrogen bonding with water^{31–33} via the six hydroxyl groups on the six-carbon member ring, the interfacial carbonyl groups, and the phosphate group.^{31,32} Furthermore, it has been shown by others that PI bilayers seem to retain more water than PC bilayers which gives rise to greater interbilayer distances in PI bilayers of 54 Å at 16 atm pressure, compared to less than 10 Å in DPPC,³⁴ which may reflect the repulsive hydration force between the bilayers.^{35–37} This also, most likely, results in greater

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intermolecular distance between adjacent PI groups and, hence, more disorder and headgroup mobility. This is likely so especially since the bulky PI headgroup is intrinsically larger³⁸ (due to the presence of the six-carbon member ring and six oxygen atoms from the hydroxyls) than the PG (and PC) headgroups. Furthermore, the significant degree of conformational flexibility of the PI headgroup to adjust to environmental changes which may provide PI with an exceptional ability to adjust its physical properties for biological function, as suggested by others,³² is another unique property that may contribute to the observed bilayer and monolayer properties of PI. It has also been shown that the inositol moiety can modulate its availability at a bilayer's surface, and this is suggested to be based on a balance between steric factors, hydration forces, and intra- and intermolecular interactions.³² Specifically, the inositol ring extends away from the bilayer surface with the C2–C3 and C5–C6 bonds orienting perpendicular to the surface allowing for maximum hydration by hydrogen bonding of the six hydroxyl groups on the inositol moiety with water.³⁹

Conclusions

It was shown for the first time that the PI headgroup in DPPI, rather than the dipalmitoyl chains (as in DPPC

and DPPG), is the dominant factor in determining its bilayer and monolayer properties. Despite the charge difference, similar polar headgroup properties exist for zwitterionic PC and anionic PG, which give rise to nearly identical monolayer and bilayer phase behavior and spreading properties. In contrast, distinct differences between PG and PI headgroup properties are reflected in their physical size, state of hydration, and possible conformation, despite identical net negative charge and identical acyl chains. These polar headgroup properties of PG and PI result in very different monolayer and bilayer phase behavior and spreading tendencies, under equilibrium thermodynamic conditions, that might have significant implications for their respective roles in the normal lung surfactant system and in certain pulmonary diseases.

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