Packing Characteristics of Two-Component Bilayers Composed of Ester- and Ether-Linked Phospholipids

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ABSTRACT The miscibility properties of ether- and ester-linked phospholipids in two-component, fully hydrated bilayers have been studied by differential scanning calorimetry (DSC) and Raman spectroscopy. Mixtures of 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphocholine (DHPC) with 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) with 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) have been investigated. The phase diagram for the DPPC/DHPE mixtures indicates that these two phospholipids are miscible in all proportions in the nonrippled bilayer gel phase. In contrast, the DHPC/DPPE mixtures display two regions of gel phase immiscibility between 10 and 30 mol% DPPE. Raman spectroscopic measurements of DHPC/DPPE mixtures in the C-H stretching mode region suggest that this immiscibility arises from the formation of DHPC-rich interdigitated gel phase domains with strong lateral chain packing interactions at temperatures below 27°C. However, in the absence of interdigitation, our findings, and those of others, lead to the conclusion that the miscibility properties of mixtures of ether- and ester-linked phospholipids are determined by the nature of the phospholipid headgroups and are independent of the character of the hydrocarbon chain linkages. Thus it seems unlikely that the ether linkage has any significant effect on the miscibility properties of phospholipids in biological membranes.

INTRODUCTION

Biological membranes contain a rich diversity of phospholipid species of varying chemical composition (Ansell et al., 1973). This diversity is assumed to be related to membrane function through the differing abilities of the various phospholipids to form compositional domains, promote nonbilayer phase formation, and interact with membrane proteins. Although most membrane phospholipids contain ester-linked acyl chains, phospholipids with ether-linked alkyl moieties are important components of bacterial membranes (Snyder, 1985), the plasma membranes of cardiac muscle (Diagne et al., 1984), cells of the immune system (Müller et al., 1982), and some tumor cells (Record et al., 1984). Consequently, ascertaining the effect of the chain linkage type on the properties of membrane phospholipids is critical to an understanding of the role of ether phospholipids in membrane biochemistry. The purpose of this study was to investigate the mixing properties of ether-linked phospholipids with their ester-linked analogs in an effort to discern the individual contributions of the hydrocarbon chain linkage type and the phospholipid headgroup type to the miscibility properties of these two phospholipid classes. This has been accomplished by studying the properties of two-component, fully hydrated multilamellar bilayers composed of mixtures of 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphocholine (DHPC) with 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) with1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE). This study extends previous investigations of the mixing properties of ester-linked and ether-linked phospholipids (Kim et al., 1987; Laggner et al., 1987; Lohner et al., 1987; Devlin and Levin, 1989; Hing et al., 1991; Hing and Shipley, 1995).

These two-component bilayer assemblies were studied using differential scanning calorimetry (DSC) and Raman spectroscopy. Thermograms obtained by DSC were analyzed to determine the phase diagrams for the two phospholipid mixtures. Raman spectroscopy was used to identify the hydrocarbon chain packing characteristics of the two bilayer assemblies. The results of our findings, combined with those of others, lead to the conclusion that the miscibility properties of mixtures of ether- and ester-linked phospholipids are determined by the nature of the phospholipid headgroups and are independent of the character of the hydrocarbon chain linkages, except when DHPC is a component. DHPC-containing bilayer mixtures display significant gel phase immiscibility. Raman spectroscopic measurements of DHPC/DPPE mixtures suggest that this immiscibility arises from the formation of DHPC-rich interdigitated gel phase domains with strong lateral chain packing interactions.

MATERIALS AND METHODS

Sample preparation

DHPC, DPPC, and DPPE were obtained from Sigma Chemical Company (St. Louis, MO). DHPE was obtained from Fluka Chemical Corporation

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(Ronkonkoma, NY). Individual stock solutions of the phospholipids were dissolved in chloroform/methanol (1:2) and mixed volumetrically to give the desired combinations. The solvent was removed under a gentle stream of nitrogen while the sample was heated at 60°C to prevent selective precipitation of the phosphatidylethanolamine (PE) component. The samples were then placed in a vacuum desiccator overnight to ensure complete removal of the solvent. Samples were hydrated in 50 mM KCl and cycled several times between room temperature and 82°C. For calorimetry measurements, the desired mixture (10 mg total phospholipid) was pipetted into a 1-ml stainless steel calorimetry vial that was then tightly sealed with Vitron gaskets. After the DSC runs, aliquots of each mixture were transferred to 1-mm glass capillary tubes that were spun for 10 min in a microcapillary centrifuge, flame-sealed, and used for Raman measurements. Samples for DSC and Raman measurements were held at room temperature and used within 1 day of preparation. Immediately before analysis, all samples were heated well above their transition temperatures to avoid formation of the crystalline lamellar phase characteristic of PE bilayers.

Differential scanning calorimetry

DSC runs were performed on a Hart Scientific Model 7707 series differential scanning microcalorimeter (Provo, UT). Scans were performed in the ascending and descending temperature directions at a scan rate of 20°C/h. After baseline subtraction and correction for the thermal instrument response, the calorimetric data were analyzed to yield phospholipid excess heat content (normalized to sample mass) as a function of temperature and phase transition enthalpy changes (ΔH), using software supplied by Hart Scientific. The calorimetric data were imported into Grams/386 (Galactic Industries Corporation, Salem, NH) for baseline and offset correction and for plotting. For a given phospholipid phase transition, the transition onset temperature was taken to be the temperature at which the curve first inclines from the baseline, and the transition completion temperature was taken to be that temperature at which the curve returns to the baseline. The phase transition temperature was taken to be the point of the maximum excess heat content. Corrected onset and completion temperatures were calculated by the method of Mabrey and Sturtevant (1976). Phase diagrams (Mason, 1988) were constructed using Matlab for Windows V4.2C.1 (The Math Works Inc., Natick, MA).

Raman spectroscopy

Raman spectra were recorded on a Spex Ramalog 9I dispersive spectrometer (Spex Industries, Edison, NJ) equipped with holographic gratings. An Innova model 300 argon laser (Coherent Laser Products, Palo Alto, CA) provided 200–250 mW of 514.5-nm laser excitation at the sample. The frequencies, calibrated with atomic argon lines, are reported to ± 2 cm^{-1}. Typically, scans were obtained at an instrument resolution of 3 cm^{-1} and a scan rate of 0.5 cm^{-1}/s for the hydrocarbon stretching region (2800–3100 cm^{-1}). Temperature control, to within ± 1°C, was achieved by placing the sample capillaries in a thermally regulated cell assembly. Spectra were imported into Grams/386 for baseline and offset corrections and for the determination of Raman spectral indices. Raman spectral indices for the phospholipid mixtures were measured at equivalent reduced temperatures in the bilayer gel phase. The reduced temperature is defined as the measurement temperature relative to the onset temperature of the main phase transition of the phospholipid mixture.

RESULTS

DHPC/DPPE bilayers

DSC

Heating thermograms of DHPC/DPPE bilayers ranging in DPPE content from 0 to 100 mol% are shown in Fig. 1.

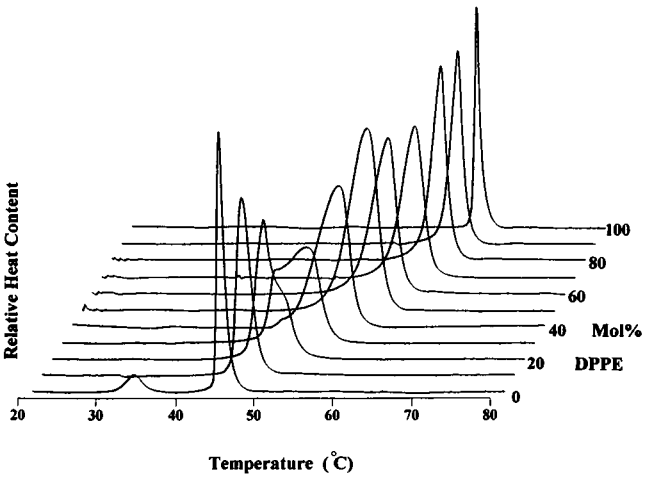

FIGURE 1 Heating thermograms of DHPC/DPPE bilayers ranging in DPPE content from 0 to 100 mol%. The individual thermograms were normalized for phospholipid concentration, baseline corrected, and offset before plotting.

Cooling thermograms (not shown) revealed that the phase transitions were reversible. The thermograms for samples containing 10–40 mol% DPPE demonstrated a complex behavior with a sharp endothermic feature centered at 44.5°C. This feature is indicative of a line of three-phase coexistence in the phase diagram at this temperature. From 50 to 100 mol% DPPE, the thermograms consisted of single peaks that progressively narrowed with increasing DPPE content. Fig. 2 shows the effect of DPPE on the pretransition of DHPC/DPPE bilayers containing 0–8 mol% DPPE. Within this concentration range, the pretransition temperature (T_p) decreased from 32.5 to 27°C, whereas the value of ΔH decreased from 0.5 to 0.08 kcal/mol. No pretransition was observed for DPPE concentrations above 8 mol%. Corrected onset and completion temperatures determined from the thermograms were used to construct the phase

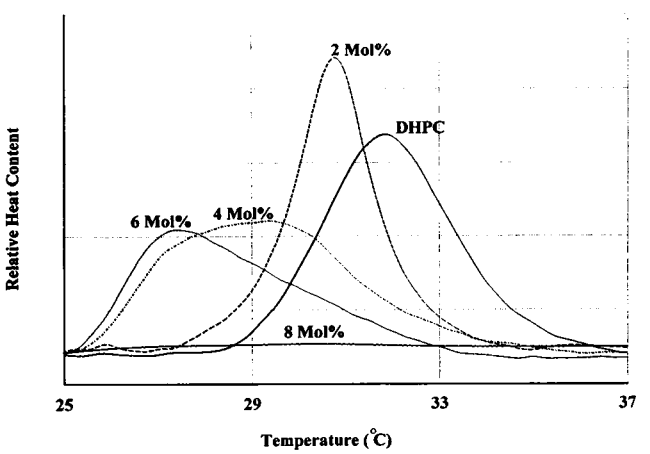

FIGURE 2 Heating thermograms of the pretransition of DHPC/DPPE mixtures containing 0, 2, 4, 6, and 8 mol% DPPE. The individual thermograms were normalized for phospholipid concentration and offset before plotting.

diagram shown in Fig. 3. The phase diagram exhibits a region of isothermal melting at 44.5°C along the solidus line between 10 and 30 mol% DPPE. This is suggestive of phospholipid lateral phase separation (Hing and Shipley, 1995). Below 10 mol% and above 30 mol% DPPE, the solidus line exhibits no region of isothermal melting, denoting gel phase miscibility of DHPC and DPPE in these two regions. The fluidus line varies in a nearly ideal manner, with DPPE concentration indicating complete miscibility of the two phospholipids in the liquid-crystalline phase. This finding is in agreement with other studies indicating miscibility of phosphatidylcholine (PC) and PE in the liquid-crystalline bilayer phase (Silvius, 1986; Kim et al., 1987; Hing et al., 1995). The value of ΔH for the main phase transition was 7.5–8.0 kcal/mol for DPPE contents below 10 mol% or above 50 mol% DPPE. The value of ΔH increased to about 9 kcal/mol for DPPE contents between 10 and 40 mol% DPPE. These elevated values of ΔH correlate with the region of gel phase immiscibility evident in the phase diagram.

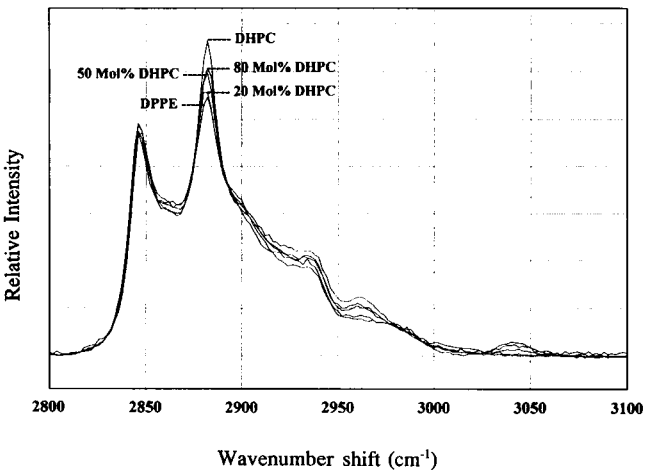

FIGURE 4 The C-H stretching mode region (2800–3100 cm^{-1}) for DHPC and DPPE bilayers and DHPC/DPPE mixtures containing 20, 50, and 80 mol% DHPC. The spectra were recorded at a reduced temperature of −20°C in the gel phase. The individual spectra were normalized for phospholipid concentration, baseline corrected, and offset before plotting.

Raman spectroscopy

The Raman C-H stretching mode region for several of the DHPC/DPPE mixtures recorded at a reduced temperature of −20°C in the gel phase is shown in Fig. 4. The spectra were normalized for concentration differences. The features at ~2850 cm^{-1} and ~2880 cm^{-1} arise from the methylene symmetrical and asymmetrical C-H stretching modes, respectively, of the hydrocarbon chains (Spiker and Levin, 1975; Levin, 1984). A relative peak-height intensity ratio, I_{2850}/I_{2880}, can be derived from these spectra, which serves as an index of the strength of the lateral interchain packing interactions in the bilayer (Lewis et al., 1986). A decrease in this empirical peak-height intensity ratio reflects stronger lateral chain-chain interactions arising from a decrease in the spacing between lipid chains. This spectral index, determined at two different reduced temperatures in the gel phase, is plotted as a function of DPPE concentration in Fig. 5.

The dashed line (with *crosses*) in Fig. 5 shows the concentration dependence of the spectral index measured at a reduced temperature of −20°C in the gel phase. The spectral index of 0.69 is characteristic of the highly ordered, fully interdigitated gel phase (L_{β}^{i}) of DHPC (O'Leary and Levin, 1984). From 2 to 10 mol% DPPE, the spectral index is constant at 0.72, a value indicative of a highly ordered gel phase. Above 34 mol% DPPE, the spectral index increases gradually from 0.79 to 0.81 with increasing DPPE concentration. These values are typical of the L_{β} gel phase of

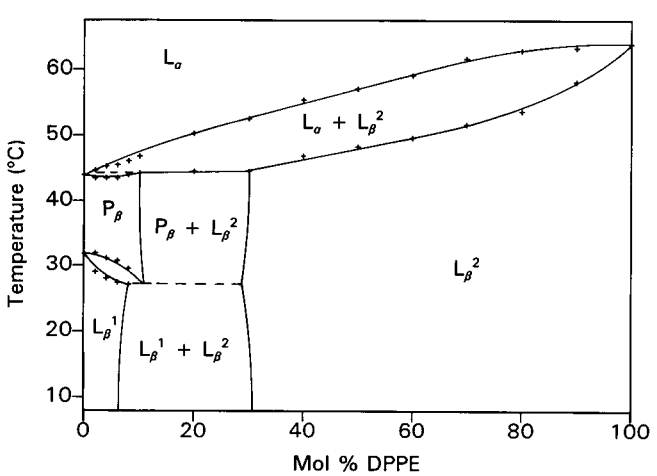

FIGURE 3 Phase diagram for the DHPC/DPPE bilayer mixtures determined from the thermograms of Fig. 1.

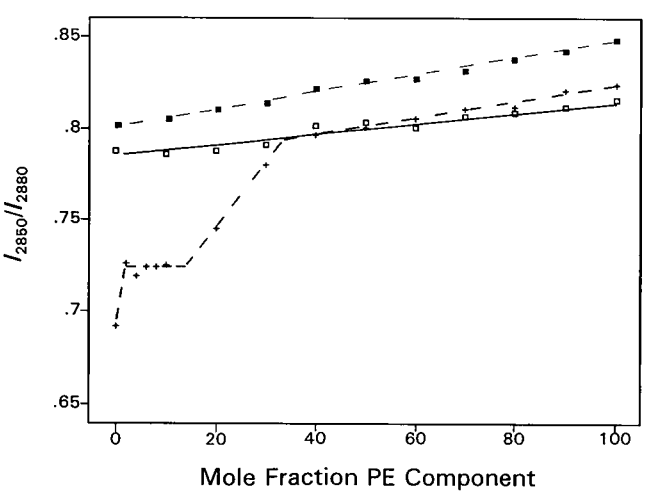

FIGURE 5 Plot of the Raman spectral index (I_{2850}/I_{2880}) versus the mole fraction of PE component (DPPE or DHPE) for DHPC/DPPE mixtures at reduced temperatures of −20°C (+) and −8°C (■) and DPPC/DHPE mixtures at a reduced temperature of −20°C (□). Lines were drawn to indicate the trends in the data. The standard deviation of the index values was ±0.02.

saturated phospholipids (Levin, 1984). From 10 to 34 mol% DPPE the spectral index increases from 0.72 to 0.79, whereas the phase diagram shows this to be a region of isothermal melting. Together these data are indicative of a shift in abundance between two distinct hydrocarbon packing arrangements as the DPPE content is increased within this region.

The dashed line (with *solid squares*) in Fig. 5 shows the concentration dependence of the spectral index measured at a reduced temperature of −8°C in the gel phase. The spectral index was found to vary nearly linearly with the molar proportions of the two phospholipid components, ranging from 0.80 for DHPC to 0.84 for DPPE. Although the above results were obtained using racemic DHPC, identical results were obtained when L-DHPC was used (not shown).

DPPC/DHPE bilayers

DSC

Heating thermograms of DPPC/DHPE bilayers ranging in DHPE content from 0 to 100 mol% DHPE are shown in Fig. 6. Cooling thermograms (not shown) revealed that the phase transitions were reversible. As above, the thermograms containing 10–40 mol% DHPE demonstrated a complex shape with a sharp endothermic feature at 43.4°C, indicative of a line of three-phase coexistence in the phase diagram at this temperature. From 50 to 100 mol% DHPE the thermograms consisted of single peaks that progressively narrowed with increasing DHPE content. The ΔH values for the main phase transition varied linearly with the molar proportions of the two phospholipid components. The pretransition of the DPPC/DHPE bilayers was found to increase in temperature and breadth with increasing DHPE content and could not be resolved from the main phase transition above about 15 mol% DHPE. Corrected onset and completion temperatures determined from the thermograms were used to construct the phase diagram shown in Fig. 7. This phase diagram indicates that the two phospholipid components are miscible in all proportions in the L_β gel phase region, but that the DHPE-rich L_β gel phase is immiscible with the DPPC-rich P_β gel phase. The intersection of the boundaries of the P_β one-phase region with the three-phase line was determined, using the procedure of Silvius (1986), to be about 22 mol% DHPE. The two phospholipid components are miscible in all proportions in the liquid-crystalline bilayer phase.

Raman spectroscopy

The solid line (with *open squares*) in Fig. 5 shows the concentration dependence of the spectral index for the DPPC/DHPE bilayers measured at a reduced temperature of −20°C in the gel phase. The spectral index was found to vary linearly with the molar proportions of the two phospholipid components, ranging from 0.79 for DPPC to 0.81 for DHPE.

DISCUSSION

The phase diagram determined here for the DPPC/DHPE two-component bilayers is almost identical to the phase diagram determined for DPPC/DPPE mixtures by Silvius (1986). The increase in the pretransition temperature of the DPPC/DHPE bilayers with increasing DHPE content indicates that DHPE prefers the L_β gel phase to the P_β gel phase, as does DPPE. This miscibility property is also responsible for the limited region of gel phase immiscibility in the phase diagram, where the DHPE-rich L_β gel phase is

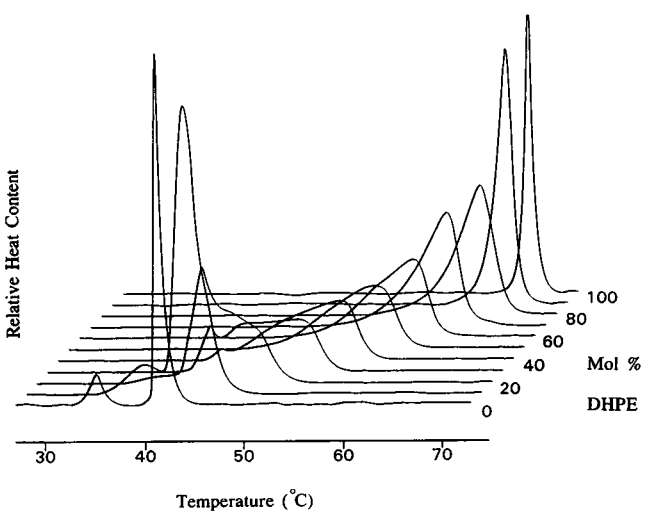

FIGURE 6 Heating thermograms of DPPC/DHPE bilayers ranging in DHPE content from 0 to 100 mol%. Other conditions were as described in Fig. 1.

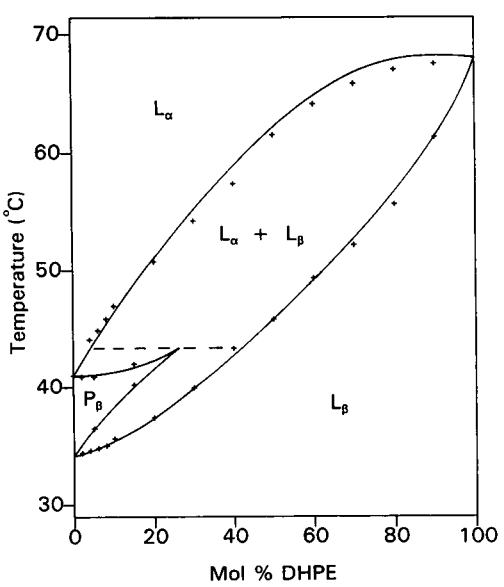

FIGURE 7 Phase diagram for the DPPC/DHPE bilayer mixtures determined from the thermograms of Fig. 6.

laterally separated from the DPPC-rich P_β gel phase. Thus the line of three-phase coexistence at 43.4°C reflects the coexistence of the L_β, P_β, and L_α phases along this line in the phase diagram. This two-phase region exists over a slightly wider compositional range in DPPC/DHPE mixtures than in the DPPC/DPPE bilayers (Silvius, 1986), indicating a lower limit of solubility for DHPE in the DPPC-rich P_β phase relative to DPPE. Outside of the two-phase region, DPPC and DHPE are completely miscible in a single L_β gel phase.

The miscibility properties of the DHPC/DPPE mixtures are different from those of the DPPC/DHPE bilayers, despite the structural similarities of the phospholipids. DHPC/DPPE mixtures display two regions of gel phase immiscibility between about 10 and 30 mol% DPPE.

The following behavior is observed below the line of three-phase coexistence at 27°C in the phase diagram. Between 2 and 10 mol% DPPE, one gel phase (L_β^1) exists exclusively, whereas between 30 and 100 mol% DPPE and a second gel phase (L_β^2) exists exclusively. Between 10 and 30 mol% DPPE, these two immiscible gel phases coexist in proportions dictated by the lever rule. This proportionality is the origin of the steep slope in the values of the Raman spectral index within this compositional range as the DHPC/DPPE mixtures change from a phase (L_β^1) with strong lateral chain packing to a phase (L_β^2) with substantially weaker lateral chain packing interactions. The line of three-phase coexistence at 27°C thus reflects the coexistence of the L_β^1, L_β^2, and P_β phases along this line in the phase diagram.

The L_β^2 phase is assigned as being a DPPE-rich noninterdigitated gel phase analogous to the L_β phase of the DPPC/DHPE mixtures. The values of the Raman spectral index for DHPC/DPPE mixtures from 34 to 100 mol% DPPE are consistent with a noninterdigitated gel phase (Levin, 1984) and are almost identical to those measured for the DPPC/DHPE bilayers over the same compositional range. The characteristics of the L_β^1 phase are suggested to be more complex. The Raman spectral index of 0.69 at 0% DPPE is characteristic of the fully interdigitated gel phase (L_β^i) of DHPC. Upon the addition of 2 mol% DPPE, the Raman spectral index jumps to 0.72 and remains at this value through 10 mol% DPPE. This spectral index indicates that the L_β^1 phase is one with strong lateral chain packing order, also consistent with an interdigitated packing arrangement (O'Leary and Levin, 1984; Batenjany et al., 1994). Based upon these observations, it is proposed that the L_β^1 gel phase of the DHPC/DPPE mixtures is analogous to the phase formed by DHPC at hydration levels between 35% and 40% (Laggner et al., 1987) or in the presence of 0.1–5 mol% cholesterol (Laggner et al., 1991). That is, the L_β^1 phase is suggested to be a single structurally coherent gel phase that contains both fully interdigitated and noninterdigitated domains organized in a nonrandom fashion and linked together by ribbons of boundary phospholipid. The fact that the $L_\beta^1 \leftrightarrow P_\beta$ phase transition occurs at progressively lower temperatures over the range of 2–10 mol% DPPE indicates that the L_β^1 gel phase also behaves as a single thermodynamic phase. A structural model for such a bilayer organization has been proposed by Laggner (Laggner et al., 1987). The aforementioned pretransition behavior also indicates that DPPE prefers the P_β (ripple) gel phase to the L_β^1 phase. This was somewhat unexpected, as DHPE was shown to prefer the L_β^i phase to the P_β phase in DHPC/DHPE mixtures (Hing and Shipley, 1995). Thus the fully interdigitated chain packing appears to be more compatible with an ether than with an ester linkage.

A second region of gel phase immiscibility exists between the two lines of three-phase coexistence at 27°C and 44.5°C in the phase diagram. This two-phase region consists of a DHPC-rich P_β gel phase and the DPPE-rich L_β^2 gel phase. Thus the line of three-phase coexistence at 44.5°C reflects the coexistence of the P_β, L_β^2, and L_α phases along this line in the phase diagram. The Raman spectral indices measured at the reduced temperature of −8°C are all above 0.8, indicating that an interdigitated gel phase does not exist in this region of the phase diagram. The failure of the Raman spectral index to map out the phase boundaries of the $P_\beta + L_\beta^2$ two-phase region is due to the low resolution of the Raman measurements and the insensitivity of the Raman C-H stretching mode region to the packing differences in these two gel phases (Levin, 1984). The phase diagram indicates the existence of a eutectic point at 27.3°C and 10.8 mol% DPPE. A sharp isothermal melting component, characteristic of a eutectic point, is not evident in the DSC data because of the breadth of the pretransition. However, the pretransition is observed to narrow slightly before its disappearance at 8 mol% DPPE, which is consistent with the presence of a eutectic point.

With the current study all possible two-component mixtures of DPPC, DHPC, DPPE, and DHPE have been investigated (Kim et al., 1987; Laggner et al., 1987; Lohner et al., 1987; Devlin and Levin, 1989; Hing et al., 1991; Hing and Shipley, 1995). The observation that can be drawn from these studies is that the miscibility properties of the phospholipid mixtures are determined by the nature of the phospholipid headgroups and are independent of the character of the hydrocarbon chain linkages, except when DHPC is a component. Two-component bilayers containing DHPC all demonstrate significant gel phase immiscibility. The presence of an ether linkage allows DHPC to form a fully interdigitated gel phase (L_β^i), whereas the ester-linked analog DPPC forms the normal noninterdigitated gel phase (L_β). The conclusion of the current study is that the ability of DHPC to form interdigitated domains is the source of the aforementioned immiscibility behavior. As is evident in Fig. 5, much stronger lateral chain packing interactions exist in the L_β^1 phase than in the noninterdigitated L_β phase. This translates into a stronger overall van der Waals cohesive energy for the L_β^1 gel phase. Subsequently, there will be a range of concentrations in DHPC-containing two-component bilayers where the van der Waals cohesive energy gained by forming a laterally separated DHPC-rich interdigitated gel phase will be sufficient to minimize the free energy of the mixture. This will occur when the gain in

cohesive energy is sufficient to overcome the entropy of demixing and any loss of interaction energy in the L_β gel phase that results from demixing.

In conclusion, the substitution of an ether for an ester linkage in phospholipids appears to have little effect on the miscibility properties of the phospholipids in normal non-interdigitated bilayers. Accordingly, it seems unlikely that the ether linkage has any significant effect on the miscibility properties of phospholipids in biological membranes.

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