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The interdigitated gel phase in mixtures of cationic and zwitterionic phospholipids

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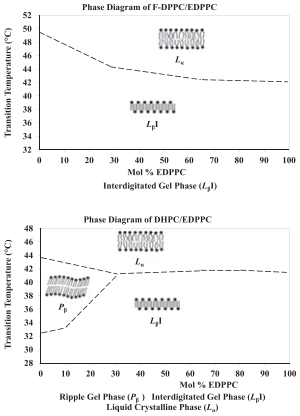
HIGHLIGHTS

- EDPPC, DHPC, and F-DPPC spontaneously form the interdigitated gel phase.
- Mixtures of F-DPPC/EDPPC are interdigitated below the main transition.
- EDPPC stabilizes the interdigitated gel phase of DHPC.
- Lower miscibility when interdigitated and non-interdigitated gel phases are present.
- Cationic and zwitterionic lipid mixtures can be compatible with interdigitation.

GRAPHICAL ABSTRACT



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ABSTRACT

To examine the phase behavior of mixtures of zwitterionic and cationic lipids we used three derivatives of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). All three lipids are uniquely capable of spontaneously forming the interdigitated gel phase (L_βI) under typical hydration conditions. The P-O-ethyl derivative, 1,2-dipalmitoyl-*sn*-glycero-3-ethylphosphocholine (EDPPC), was chosen as the cationic lipid. For the zwitterionic lipids, we use the ether-linked 1,2-di-O-hexadecyl-*sn*-glycero-3-phosphocholine, (DHPC) and the fluorine substituted 1-palmitoyl-2-(16-fluoropalmitoyl)-*sn*-glycero-3-phosphocholine (F-DPPC). Differential scanning calorimetry (DSC) and fluorescence spectroscopy were used to analyze the lipid mixtures. The F-DPPC/EDPPC mixtures are interdigitated at all lipid ratios below the main transition temperature (T_m). In addition, EDPPC stabilizes the interdigitated gel phase of DHPC until the ripple gel phase ($P_{\beta'}$) is eliminated and only the L_βI to liquid crystalline phase (L_α) main transition remains. These results demonstrate that mixtures of cationic and zwitterionic lipids can be compatible with the interdigitated phase.

Abbreviations: DSC, differential scanning calorimetry; DHPC, 1,2-di-O-hexadecyl-*sn*-glycero-3-phosphocholine; DHPE, 1,2-di-O-hexadecyl-*sn*-glycero-3-phosphoethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; EDPPC, 1,2-dipalmitoyl-*sn*-glycero-3-ethylphosphocholine; Ethyl-PC, ethylphosphocholine; F-DPPC, 1-palmitoyl-2-(16-fluoropalmitoyl)-*sn*-glycero-3-phosphocholine; L_α, liquid crystalline phase; L_βI, interdigitated gel phase; $P_{\beta'}$, ripple gel phase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; T_m, main transition temperature; T_p, pretransition temperature.

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1. Introduction

In the interdigitated gel phase (L_{β}I), the lipid hydrocarbon chains interpenetrate to maximize van der Waals interactions and to reduce head group crowding [1,2]. However, there is also increased unfavorable exposure of the hydrocarbon chains to the surrounding aqueous solution [1–3]. The interdigitated phase has unique properties that are useful for biophysical applications. For instance, the interdigitation-fusion procedure uses ethanol [4,5] or pressure [6] to create high volume vesicles or multi-compartment vesosomes [7]. When interdigitated and non-interdigitated domains coexist the instability and uneven packing at the interface can affect permeability, membrane shape, and fusogenicity [8–10]. Therefore, it is important to test the properties of lipid mixtures, especially those capable of interdigitation.

The naturally occurring lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) does not spontaneously interdigitate, but can be induced to interdigitate by the addition of chemical inducers such as alcohols or by the application of hydrostatic pressure [1,2,11,12].

The ether-linked analogue of DPPC, 1,2-di-O-hexadecyl-*sn*-glycero-3-phosphocholine, (DHPC) and the terminally fluorine substituted 1-palmitoyl-2-(16-fluoropalmitoyl)-*sn*-glycero-3-phosphocholine (F-DPPC) are unique among zwitterionic lipids as they do not require chemical inducers or pressure to interdigitate. DHPC spontaneously interdigitates but the ester-linked DPPC does not because DHPC has relatively higher head group repulsion [13,14], can pack more tightly [15–18], and has weaker attractive interactions between lipids [19,20]. DHPC exists in the L_{β}I phase below the pretransition temperature (T_p) at ~32 °C [19,21,22]. Above the pretransition, DHPC forms the non-interdigitated rippled gel ($P_{\beta'}$) phase. At the main transition (T_m) at ~43 °C, DHPC enters the non-interdigitated L_{α} phase. F-DPPC remains fully interdigitated below the main transition and has no pretransition. The primary reason F-DPPC is fully interdigitated below the main transition (~49 °C) is because the highly polar C-F bond at the end of the *sn*-2 acyl chain is less hydrophobic than DPPC [23,24]. This stabilizes interdigitation since the terminal ends of the acyl chains are exposed within the L_{β}I phase.

The cationic lipid ethyl-phosphocholine (ethyl-PC) derived from DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-ethylphosphocholine (EDPPC), shares the ability to spontaneously interdigitate [25,26]. EDPPC interdigitates because of charge repulsion and the high steric bulk of the head group [3,25,26]. Additionally, the ethyl group may reduce the unfavorable contact of hydrophobic acyl chains with the aqueous phase due to a change in the polarity of the lipid molecule [3,26]. The addition of the ethyl group to the phosphate oxygen may also reduce the attractive force of intermolecular hydrogen bonding [27,28]. Compared to the equivalent PC lipids, ethyl-PCs have greater gel phase stability, higher main transition (T_m) temperatures, and greater main transition enthalpy [3]. EDPPC forms a tightly packed L_{β}I phase below its main transition to the liquid crystalline phase (L_{α}) phase at ~42 °C [3,25].

In this study, we examine the miscibility and phase behavior of the cationic EDPPC with two zwitterionic lipids: F-DPPC and DHPC. Cationic lipids have attracted a great amount of interest for their potential as DNA transfecting lipoplexes [10,29,30], transfection reagents for RNA [31,32], co-adjuvants for vaccines [33,34] and as facilitators for drug delivery [35]. Ethyl-PC lipids are of particular interest due to their relatively low toxicity, which is likely a result of their similarity to cellular lipids and because they can be metabolized [28,36]. Intriguingly, DNA can enter into a superlattice between sheets of ethyl-PC lipid without disrupting the interdigitated phase [37,38]. The transfection efficiency of cationic lipoplexes is strongly dependent on the constituent lipids and the resulting phase behavior [10,29,30]. Therefore, it is important to understand the properties of lipid mixtures containing cationic lipids.

2. Materials and methods

All samples were hydrated using purified and double-deionized water from a Milli-Q filtration system. The lipids 1,2-di-O-hexadecyl-*sn*-glycero-3-phosphocholine, (DHPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), the fluorinated 1-palmitoyl-2-(16-fluoropalmitoyl)-*sn*-glycero-3-phosphocholine (F-DPPC), and the cationic 1,2-dipalmitoyl-*sn*-glycero-3-ethylphosphocholine (EDPPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), was obtained from Molecular Probes (Eugene, OR). Methanol (≥99.9%) was purchased from Sigma–Aldrich (St. Louis, MO). All purchased chemicals were used without further purification.

The DSC and fluorescence samples were prepared from stock solutions of lipid dissolved in methanol. All of the samples were evaporated to dryness followed by high vacuum for at least 10 h. Each sample was then hydrated, incubated at 60 °C, and vortexed periodically for 1 h. Afterwards, all samples were incubated at 25 °C for ~ 24 h after the initial preparation at 60 °C.

The calorimetry samples contained 2 mg lipid in 100 μl of water. The thermograms were obtained on a Calorimetry Sciences Corporation multi-cell DSC-HT Model 4100 DSC. Heating and cooling scans were recorded from 25 °C to 60 °C at a rate of 10 °C/h. The standard deviation of the phase transitions was ±0.1 °C.

The fluorescence samples had a lipid concentration of 1 μmol/ml with a DPH to lipid ratio of 1:500. To prevent oxygen quenching, the solutions were bubbled with nitrogen for 15 min before use. The fluorescence experiments were conducted using an ISS K2 Multi-Frequency Cross-Correlation Phase and Modulation Fluorometer with a Xenon Arc Lamp operating at 15 A. Excitation was carried out at 360 nm and the emission was measured at 430 nm. For the intensity experiments, a Hoya U-360 bandpass excitation filter and a >400 nm cutoff emission filter were used to minimize light scattering unrelated to fluorescence emissions. The filters were removed when measuring fluorescence polarization. A programmable circulating water bath maintained a scan rate of 10 °C/h from 25 °C to 60 °C.

3. Results

3.1. Differential scanning calorimetry

The DSC thermograms of the heating and cooling transitions of F-DPPC/EDPPC are shown Fig. 1A. The main transition temperature (T_m) of EDPPC is 42 °C and the T_m of F-DPPC is 49 °C. Within the temperature range studied no other thermal transitions are observed. Both pure EDPPC and F-DPPC have a large main transition hysteresis of around 4 °C between heating and cooling. This large hysteresis is preserved at all lipid ratios of the F-DPPC/EDPPC mixtures. The T_m decreases steadily up to about 50% EDPPC and approaches the T_m of pure EDPPC (Fig. 2A). Below 50% EDPPC, the transition peaks of the mixtures are broader and some thermograms have shoulder peaks. Above 50% EDPPC, there is little change in the T_m and there is only one sharp heating and cooling peak.

The thermograms of the DHPC/EDPPC system are shown in Fig. 1B. The main transition of DHPC occurs at 43 °C. Unlike the main transition of F-DPPC and EDPPC, the main transition of DHPC is highly reversible with a hysteresis of only ~0.5 °C. Up to 50% EDPPC, shoulder peaks are sometimes present in the heating or cooling thermograms. Above 50% EDPPC there is only a single sharp peak and there is an increase in the main transition hysteresis. The T_m decreases slightly in the mixtures, indicating that the gel phase is destabilized slightly relative to the pure lipids. DHPC also has a low enthalpy pretransition (T_p) that occurs at 32 °C. Fig. 3 shows the DSC thermograms of DHPC/EDPPC up to 30% EDPPC. The pretransition temperature increases with greater fractions of EDPPC (Figs. 2B and 3). Above 30 % EDPPC, the low enthalpy

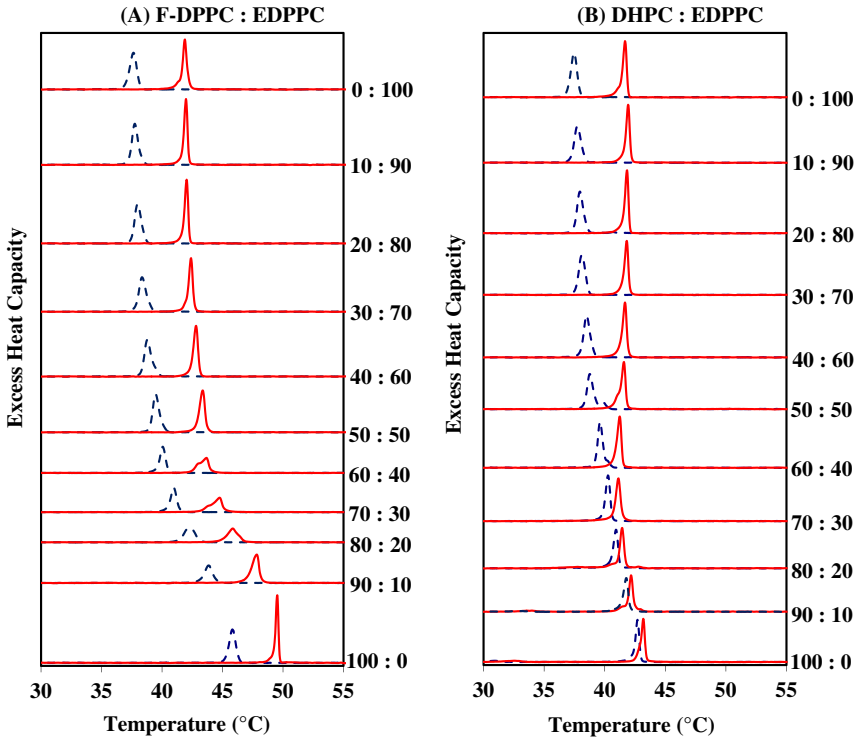

Fig. 1. Representative DSC data of (A) F-DPPC/EDPPC and (B) DHPC/EDPPC. Solid red lines show heating scans and dashed blue lines indicate cooling scans. The cooling scans have been inverted and the baselines of the thermograms are offset vertically.

pretransition merges with the main transition and is no longer detectable as a separate transition.

3.2. Fluorescence intensity

There is a substantial increase in fluorescence intensity at the main transition of F-DPPC (Fig. 4A). Similar to F-DPPC, the only phase transition present for EDPPC is the main transition, which also corresponds to an increase in DPH fluorescence intensity. In all the F-DPPC/EDPPC mixtures this single main transition change is preserved, although some of the transitions are broader. The T_m steadily decreases as the fraction of EDPPC increases.

The fluorescence intensity also increases substantially at the pretransition of DHPC at 32 °C (Fig. 4B). However, unlike F-DPPC or EDPPC, there is no increase in intensity above the T_m of DHPC. At 30% EDPPC in DHPC/EDPPC, there is a broad region where the fluorescence intensity is increasing and the pretransition is no longer clearly detectable. Above 30% EDPPC, there is only one transition and it is characterized by an increase in fluorescence intensity.

3.3. Fluorescence polarization

For each of the pure lipids (F-DPPC, DHPC, and EDPPC) the fluorescence polarization is high in the gel phase and decreases substantially above the main transition temperature (Fig. 5). The same is true of the F-DPPC/EDPPC (Fig. 5A) and DHPC/EDPPC (Fig. 5B) mixtures. The decrease in polarization corresponds to the equivalent DSC main transition. The pretransition of pure DHPC can be observed as a slight increase in polarization. However, the pretransition is obscured in the DHPC/EDPPC mixtures.

4. Discussion

Although the three DPPC derivatives studied here are capable of spontaneous interdigitation, this is no guarantee that the resulting mixtures will also be interdigitated. When cationic and zwitterionic lipids are combined there is often a balance between electrostatic interactions and steric interactions [25]. Coulombic repulsion results in the separation of like charges whereas steric interactions encourage the clustering of similar molecules [25].

In previous studies, EDPPC has been reported to be sensitive to sample preparation. High-temperature agitation leads to the formation of vesicles whereas prolonged low-temperature incubation results in a kinetically slow conversion into planar sheets [37]. Morphology can be significant because highly curved small vesicles are less likely to interdigitate due to steric interactions [39,40]. However, we find that the relatively short incubation (24 h at 25 °C) used in our procedure is sufficient to allow the interdigitated phase to form.

Our DSC results show that the main transition hysteresis is high at all lipid ratios of F-DPPC/EDPPC (Fig. 1A). Such a large thermal hysteresis is a strong indicator of the interdigitated gel phase [22,24,41]. The only phase transition present in the DSC thermograms of F-DPPC/EDPPC therefore corresponds to the L_{β}I to L_{α} main transition. Although the introduction of EDPPC destabilizes the gel phase relative to the liquid crystalline phase, the DSC results support that the membrane remains interdigitated below the T_m.

The L_{β}I phase is also favored in the DHPC/EDPPC system. Previous studies have identified the low enthalpy DHPC as a transition from the L_{β}I phase to the non-interdigitated $P_{\beta'}$ phase [21,22]. When EDPPC is introduced, the L_{β}I phase is stabilized and the T_p increases until it merges with the main transition (Figs. 2B and 3). Above ~30% EDPPC the main transition hysteresis of DHPC/EDPPC increases substantially (Fig. 1B). This supports that the main transition proceeds directly from the L_{β}I phase to the L_{α} phase with greater amounts of EDPPC.

The pattern of DPH fluorescence intensity in our results is consistent with the presence of the interdigitated phase (Fig. 4A-B) [22]. DPH becomes more exposed to the highly polar aqueous layer in the L_{β}I phase. The intensity is lower in the L_{β}I phase versus non-interdigitated phases since the fluorescence intensity of DPH is dependent on the polarity of its environment [22,42–45].

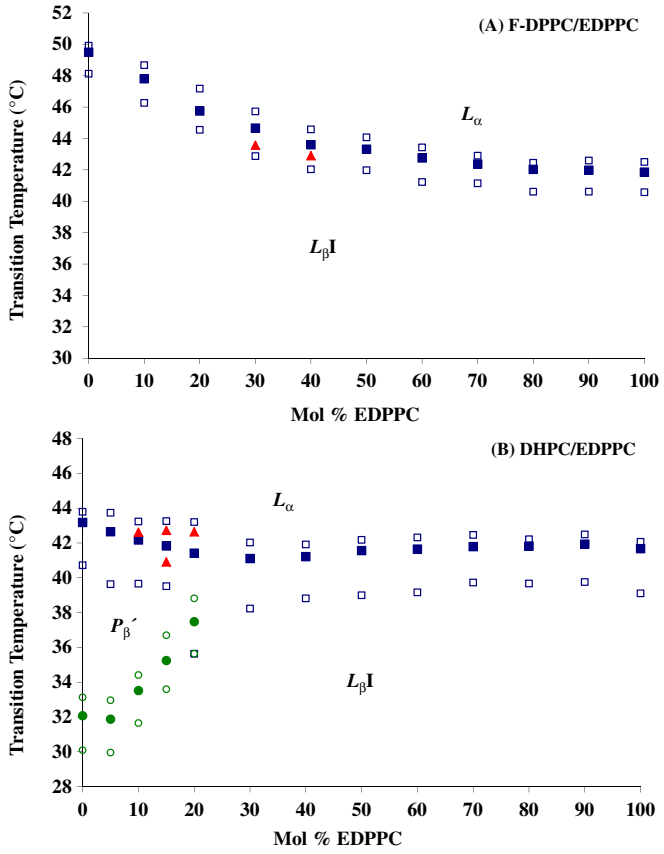

Fig. 2. Transition temperatures of (A) F-DPPC/EDPPC and (B) DHPC/EDPPC derived from DSC data. Blue squares (■) show the main transition peak and smaller unfilled blue squares (□) indicate the onset and completion of the transition. Red triangles (▲) indicate shoulder peaks. Green circles (●) show the pretransition peak and smaller unfilled green circles (○) indicate the onset and completion of the transition.

The DPH intensity of F-DPPC/EDPPC increases in all the mixtures at the main transition. This provides strong evidence that the main transition is from the L_βI phase to the non-interdigitated L_α phase (Fig. 4A).

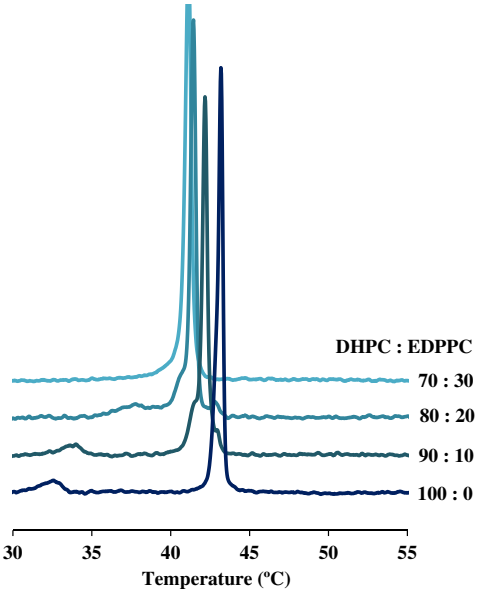

Fig. 3. Selected DSC thermograms of DHPC/EDPPC. Only heating scans are shown.

The fact that there are no other changes in the fluorescence intensity supports that the L_βI phase is the only gel phase present.

The phase diagram of DHPC/EDPPC is more complex than F-DPPC/EDPPC due to the presence of the non-interdigitated P_β' phase. The L_βI to P_β' pretransition of DHPC is characterized by a substantial increase in fluorescence intensity (Fig. 4B). The decrease in intensity at the P_β' to L_α main transition of DHPC reflects that the transition is between two non-interdigitated phases. Above 30% EDPPC in DHPC/EDPPC, the non-interdigitated P_β' phase is eliminated and only a single transition remains. This is similar to F-DPPC/EDPPC, where the L_βI phase transitions directly into the L_α phase and there is an increase in fluorescence intensity.

The fluorescence polarization of DPH is not sensitive to interdigitation as the interdigitated gel phases of F-DPPC, DHPC, and EDPPC have similar polarization values compared to the non-interdigitated gel phase of DPPC [22]. However, fluorescence polarization is highly dependent on the increased fluidity in the L_α phase. Our polarization data corroborate the phase transition temperatures detected by DSC and fluorescence intensity (Fig. 5A–B).

It is instructive to compare our results to previously studied systems involving interdigitation. When only one lipid favors interdigitation, the resulting phase diagrams are often complicated due to the great disparity between molecule packing in interdigitated versus non-interdigitated gel phases. Sometimes, this conflict can result in the coexistence of interdigitated and non-interdigitated domains. This occurs in the DPPC/F-DPPC system, where there is coexistence between interdigitated and non-interdigitated gel phases for a large portion of the phase diagram [24,46]. Similarly, in the DHPC/DPPE [47] and DHPC/DHPE [48] systems there is also immiscibility due to the separation of DHPC-rich interdigitated gel phase domains. Unlike DHPC, the ether lipid DHPE does not interdigitate due to intermolecular hydrogen bonding and reduced headgroup repulsion of the phosphatidylethanolamine (PE) head group [20,48]. In other mixtures, such as DPPC/DHPC [49–51] and DPPC/EDPPC [25], there is little or no phase coexistence except perhaps at the phase transition regions.

The F-DPPC/EDPPC system is unique among lipid mixtures because the L_βI phase transitions directly into the L_α phase at all lipid ratios. A similar phenomenon can be induced by the addition of alcohols [2,11,12,21]. Above the threshold concentration for interdigitation, the main transition is characterized by high thermal hysteresis and there is no pretransition.

The closest analogue of the DHPC/EDPPC system is DHPC/F-DPPC. In both phase diagrams the non-interdigitated P_β' phase of DHPC is replaced with the L_βI phase. The main transition hysteresis of DHPC/F-DPPC is highest where only the L_βI phase remains [22]. The stabilization of the L_βI phase is analogous to the addition of alcohols [21] or the application of hydrostatic pressure to DHPC [52].

The distinguishing feature of F-DPPC/EDPPC and DHPC/EDPPC is that both of the constituent lipids are able to spontaneously interdigitate. Only the L_βI phase is found below the T_m of F-DPPC and EDPPC. Therefore, it is reasonable that miscibility is high in F-DPPC/EDPPC since there is no inherent conflict between interdigitated and non-interdigitate gel phases. In DHPC/EDPPC, the non-interdigitated P_β' phase is present when the percentage of EDPPC is below ~30%. Consequently, the miscibility is lower when the P_β' phase remains in the phase diagram. DHPC and EDPPC are most miscible at higher concentrations of EDPPC, suggesting that miscibility is highest once the interdigitated phase predominates.

5. Conclusions

In the F-DPPC/EDPPC and DHPC/EDPPC systems, we find that zwitterionic and cationic lipids can coexist within the interdigitated phase. By entering the interdigitated gel phase the charge density is reduced and the headgroup crowding is lessened. It is likely that the charge interaction between cationic and zwitterionic lipids is different within the interdigitated phase since there is a significantly larger spacing

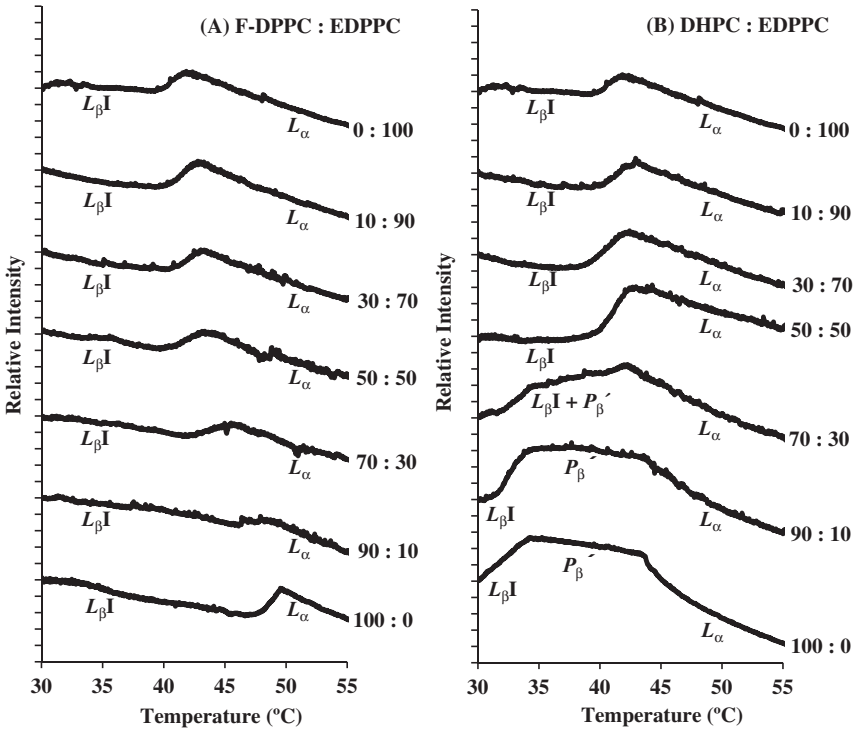

Fig. 4. Relative change in fluorescence intensity of the DPH probe (vs. 30 °C) in (A) F-DPPC/EDPPC and (B) DHPC/EDPPC. Only heating scans are shown. For clarity, the baselines are offset vertically. Each tick mark indicates a 10% change in the fluorescence intensity.

between headgroups. If zwitterionic lipids are inserted between interdigitated cationic lipids the membrane can maximize the distance between cationic charges.

We can conclude that a major factor in gel phase mixing is the drastic difference in molecule packing between the interdigitated and non-interdigitated gel phases.

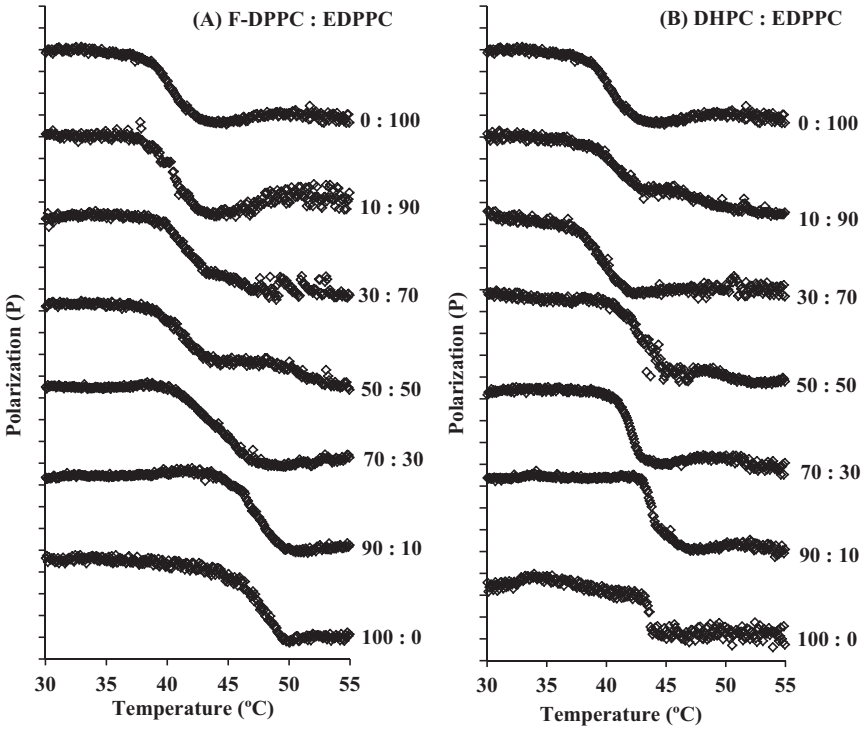

Fig. 5. Fluorescence polarization of the DPH probe in (A) F-DPPC/EDPPC and (B) DHPC/EDPPC. Only heating scans are shown. For clarity, the baselines are offset vertically. Each tick mark indicates a 0.1 change in the fluorescence polarization value.

The mutual proclivity for interdigitation results in improved miscibility. Conversely, there is greater gel phase immiscibility when there is competition between interdigitated and non-interdigitated phases.

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