Characterization of Complexes Formed in Fully Hydrated Dispersions of Dipalmitoyl Derivatives of Phosphatidylcholine and Diacylglycerol

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ABSTRACT The phase diagram of fully hydrated binary mixtures of dipalmitoylphosphatidylcholine (DPPC) with 1,2-dipalmitoylglycerol (DPG) published recently by López-García et al. identifies regions where stoichiometric complexes of 1:1 and 1:2 DPPC:DPG, respectively, are formed. In this study, the structural parameters of the 1:1 complex in the presence of pure DPPC was characterized by synchrotron low angle and static x-ray diffraction methods. Structural changes upon transitions through phase boundaries were correlated with enthalpy changes observed by differential scanning calorimetry in mixtures of DPPC with 5, 7.5, 10, and 20 mol% DPG dispersed in excess water. Phase separation of a complex in gel phase could be detected by calorimetry in the mixture containing 5 mol% DPG but was not detectable by synchrotron low angle x-ray diffraction. Static x-ray measurements show evidence of phase separation, particularly in the reflections indexing chain packing. In the mixture containing 7.5 mol% DPG, two distinct lamellar repeat spacings could be seen in the temperature range from 25 to 34°C. The lamellar spacing of about 6.6 nm was assigned to pure gel phase DPPC because the change in the spacing corresponds with thermal transition of the pure phospholipid, and a longer repeat spacing of about 7.2 nm was assigned to domains of the 1:1 complex of DPPC-DPG. In the temperature range from 34 to 42°C, i.e., in the region of coexistence of the ripple phase of DPPC and the gel phase of the complex, a single, rather broad lamellar reflection appears because of superposition of two reflections of DPPC and the complex; the lamellar spacing of DPPC in the ripple phase is similar to that of the gel phase of complex. In the coexistence region of the liquid-crystalline phase of DPPC and the gel phase of complex (~42–48°C), the lamellar reflections of the both phases are present. The fluidus boundary lies between the coexistence region and the fluid region. In the fluid region (~48–55°C), the gel state of complex persists up to the fluidus boundary, whereupon the liquid-crystalline state of complex replaces the gel state of the complex. This indicates that the complex is also immiscible with DPPC even above the fluidus boundary at least in the temperature range close to the phase boundary. For mixtures comprising 10 and 20 mol% DPG in DPPC, complex formation is clearly detectable in both the gel region and the coexistence region by x-ray diffraction. Synchrotron x-ray measurements indicate phase separation between pure DPPC and liquid-crystalline complex just above the fluidus boundary. Static, wide angle x-ray measurements also suggest phase separations of the 1:1 complex not only from the gel phase but also the liquid-crystalline phase of pure DPPC. Two distinct diffraction peaks were detected for the mixture of DPPC with 5, 10, and 20 mol% DPG. One is due to the chain spacing of the complex, and the other is due to that of the pure DPPC. In the coexistence region of the liquid-crystalline phase of DPPC and the gel phase of complex, two kinds of diffraction peaks of the hydrocarbon chain of the gel phase complex and the broad scattering profile for the chain melting of DPPC were observed in the wide angle region. Electron density reconstructed from the lamellar reflections indicates that the thicknesses of both the bilayer and the water layer of the gel phase complex are greater than those of the respective thicknesses of gel phase DPPC.

INTRODUCTION

An important mechanism of signal transduction in biological membranes is the production of hydrolytic products of phospholipase action, including diacylglycerols generated by phospholipase C-type enzymes acting on membrane phospholipid substrates. The action of these enzymes is known to be augmented by numerous hormones, neurotransmitters, and growth factors, which results in a transient accumulation of diacylglycerol in the membrane (Kikkawa and Nishizuka, 1986; Berridge, 1987). The products of enzymatic action are believed to play an important role in signal transduction mechanisms (Dennis et al., 1991).

An examination of the molecular species of 1,2-diacylglycerol products suggests that phosphatidylcholine is the major substrate for phospholipase C action over relatively long time scales (Pelech and Vance, 1989), whereas phosphatidylinositol 4,5-bisphosphate is the major substrate yielding diacylglycerol in an acute response (Nishizuka, 1984, 1992). Several consequences have been described when diacylglycerols are present in relatively small amounts in phospholipid bilayers and membranes. These include activation of protein kinase C (Bell, 1986), potentiation of the action of various phospholipases on the their respective phospholipid substrates (Dawson et al., 1983, 1984), and destabilization of phospholipid bilayer membranes so as to facilitate fusion (Siegel et al., 1989).

The molecular mechanisms that underlie these different processes are presently unknown, but there is general agreement that the presence of relatively small amounts of diacylglycerols can influence the phase behavior of membrane lipid bilayers. Accordingly, mixtures of diacylglycerols and phospholipids have been subject to numerous studies to define the way that diacylglycerols modify the phase behavior

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Abbreviations used: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPG, 1,2-dipalmitoylglycerol; DSC, differential scanning calorimetry.

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of membrane phospholipids and thereby to gain a clearer understanding of the mechanism of their biological functions. For example, studies of mixed monomolecular films of 1-palmitoyl-2-oleoyl-phosphatidylcholine and varying proportions of 1,2-dioleoylglycerol have shown that stable complexes of the two components are formed at a molar fraction of 0.25 diglyceride (Samby and Brockman, 1985). Further evidence of the formation of stable complexes was obtained by calorimetric studies of these mixtures in aqueous dispersions (Cunningham et al., 1989), which confirmed conclusions based on earlier thermal and fluorescent probe studies of mixtures of various diacylglycerols with phospholipids (Ortiz et al., 1988). Further evidence of phase immiscibility and separations were obtained from partial phase diagrams constructed from x-ray diffraction studies of mixtures of various diacylglycerols with phospholipids (Das and Rand, 1984, 1984). More recently, the formation of complexes between dimyristoyl derivatives (Heimburg et al., 1992) and dipalmitoyl derivatives (López-García et al., 1994) of diacylglycerol and phosphatidylcholine (PC) have been identified from phase diagrams of fully hydrated binary mixtures examined in the temperature range from 20 to 80°C. The complexes appear to consist of approximately 1 and 2 mol of DG per 1 mol of PC. Thermal evidence indicates that the 1:1 complex coexists with pure phospholipid in the gel phase in mixtures containing less than 50 mol% 1,2-dipalmitoylglycerol (DPG). The essential features of the phase diagram of the dipalmitoylphosphatidylcholine (DPPC)-DPG mixture for the DPG concentration range studied in this work are as follows. For the DPPC-DPG mixture with less than 12.5 mol% DPG, the pretransition of DPPC was observed in differential scanning calorimetry (DSC) traces. At temperatures below the main transition of DPPC, two regions can be defined. A lower temperature region where there is coexistence between the gel phase of pure DPPC and the gel phase of complex. In the higher temperature region, the ripple phase of DPPC coexists with the gel phase of complex. Furthermore, the liquid-crystalline phase of DPPC coexists with the gel phase of complex at temperatures above the main transition of pure DPPC. López-García et al. (1994) have defined the fluidus boundary where the complex undergoes a transition to the fluid phase but have found no evidence for any immiscibility in the lamellar phase in this region of the phase diagram.

Structural evidence for the coexistence of stoichiometric complexes with pure phospholipid is presently lacking, and no characterization of the compounds formed in these mixtures has yet been performed. In the present study, we have used synchrotron low angle and static x-ray diffraction to examine the region of phase coexistence of phospholipid with stoichiometric complex comprising of 1:1 DPG/DPPC in a molar fraction. The resolution of the method is sufficient to distinguish the coexistence of the gel phase of complex from the gel phase of DPPC and the gel phase of complex with the liquid-crystalline phase of DPPC. The coexistence of the liquid-crystalline phase of complex with the liquid-crystalline phase of DPPC is detectable by synchrotron, low angle x-ray diffraction for the region near the fluidus line.

MATERIALS AND METHODS

Sample preparation

DPPC was purchased from Avanti Polar Lipids (Birmingham, AL), and DPG was obtained from Sigma Chemical Co. (St Louis, MO). No chromatographic analyses were performed, but narrow (<0.2°C) main transition of the phospholipid and melting transition temperatures of the DPG indicated that the purity of the lipids was comparable with that of lipids used in previous studies and consistent with the supplier's claim of 99% authentic compound. DPPC and DPG were dissolved in chloroform and mixed in appropriate proportions to achieve the desired molar fractions. The solvent was evaporated under a stream of oxygen-free dry nitrogen and stored for 16 h in vacuum to remove remaining traces of solvent. These conditions were known to result in minimization of the weight of lipid. Multilamellar suspensions of the lipid mixtures were obtained for calorimetry by adding 14 ml of bidistilled deionized (pH 5.3, conductivity < 2 mS) per g of lipid and 1 ml per g of lipid for samples used in x-ray examination. The lipid mixtures were hydrated at 70°C for at least 1 h and dispersed by sonication in an ultrasonic bath. The lipid dispersions were stored at ~0–4°C until required for examination. The method of preparation and storage gave reproducible phase behavior when samples prepared at different times were examined by calorimetry or x-ray diffraction.

Calorimetry

DSC was performed using a DSC10 with SSC580 (SEIKO I & E, Tokyo, Japan) and with scan rates of 0.8°C/min. Lipid dispersions (15 ml) were placed in sample pans (P/N 50–023P/023C, SEIKO) and hermetically sealed. Transition enthalpies were determined by integration of the area under the transition curve, and the position of the peak maximum was used to determine the transition temperature.

X-ray diffraction

Static x-ray diffraction patterns were recorded using a rotating anode generator (Rigaku RU200, Tokyo, Japan) producing CuKα with a Ni filter (λ = 0.1542 nm). The sample was sandwiched between polyimide windows to give a path length of 1 mm and held in a water-jacketed sample holder through which water was circulated at designated temperatures using a Thermomix (Braun Biotech) circulating water bath. The sample temperature was monitored by a thermocouple placed in the sample, and the stability was within ±0.1°C. The x-ray beam was focused using a Franks optical system, and the sample-to-detector distance was 370 mm. low angle x-ray diffraction profiles were detected by a position-sensitive proportional counter (PSPC, Rigaku) with a length of 100 mm divided into 2815 channels. Counts were accumulated over ~3–10 h and were uncorrected for background scattering. X-ray data including both low angle and wide angle (S = 0.1 ~ 3 nm^{-1}) were also recorded on a two-dimensional imaging plate (Type BA-III, Fuji Photo Film Co. LTD., Japan) mounted at a distance of 187 mm from the sample. Each pattern was averaged by azimuthally integrating two-dimensional Debye-Scherrer patterns (Takahashi et al., 1991). In these measurements, the exposure times were typically ~3–16 h. The x-ray diffraction data used for the reconstruction of electron density profiles were accumulated over ~16–18 h, and the intensities of these reflections were determined by fitting the observed reflection peaks to Lorentzian line shapes accompanied with a linear function ($y = ax + b$). These data used for the above analysis were corrected by subtracting the background scattering, the scattering for the sample cell containing no lipid.

Synchrotron x-ray diffraction studies were undertaken at station 15A of the Photon Factory (Amemiya et al., 1983). Monochromatic x-rays (λ = 0.1506 nm) produced by a germanium (111) crystal with a Frankuchen cut were focused horizontally and collimated with two pairs of slits to give a beam dimension of 2.0 × 1.0 mm (width × height). The photon flux of the incident beam was monitored continuously by an ionization chamber located in front of the sample holder and was typically in the range of 2–4 × 10^8 photons/s. The photon flux was relatively invariant during individual temperature scans. The photon flux incident on the sample was reduced by an estimated 30-fold amount

by the water jacket in which the sample was immersed, thus giving a flux incident of $\sim 10^7$ photons/s on the sample.

Diffraction patterns were recorded using a one-dimensional, position-sensitive proportional counter with 512 channels (PSPC, Rigaku) linked to the station computer. The detector was positioned with the center of the beam aimed at the midpoint of the detector so that two symmetric profiles of the powder pattern on either side of the beam stop were recorded concurrently, each containing the first- and second-order Bragg reflections of the phospholipid bilayers. The active length of the PSPC was 170 mm (462 channels). The sample-to-detector distance was 1629 mm, and the maximum angle of x-ray scattering that could be detected was 3°. The channel-to-channel dimension of the PSPC was 0.368 mm, giving an angular resolution of 0.013°, equivalent to a resolution of approximately 0.07 nm for spacings in the range ~6–7 nm.

The sample was housed in a holder described in detail elsewhere (Tenchov et al., 1989). The temperature of the sample was monitored continuously by a chromel-alumel thermocouple mounted adjacent to the sample. Linear heating and cooling temperature scans of 0.8°C/min between 2 5 and 90°C were produced by electronically controlled flow of water through the sample holder. The sample stage was translated vertically after each scan so that the maximum time of exposure to the beam was 45 min. This time of exposure produced no detectable change in the diffraction pattern or thermal phase behavior judged by calorimetry. Up to 90 successive diffraction patterns were recorded, each of 30-s exposure. The patterns were subjected to a smoothing routine, and Bragg spacings were determined from the position of the diffraction maximum.

EXPERIMENTAL RESULTS

Phase separation between DPPC and a complex of DPG and DPPC is illustrated in thermal behavior of 5 mol% DPG in DPPC mixed aqueous dispersion shown in Fig. 1 *A*. The main and pretransitions were observed at 41.5 and 34.5°C (peak temperatures), respectively, for pure DPPC as shown in (*a*) of Fig. 1 *A*. The main transition is seen in both heating and cooling scans of the mixtures shown in (*b*) and (*c*) of Fig. 1 *A*, respectively, but the transitions in the latter appear to be less cooperative as judged by a slight broadening of the peaks. The peak temperatures of the main transition of the mixtures are 41.6 and 40.4°C, for heating and cooling scan, respectively. This is also true of the pretransition, which is also broader in the mixture and takes place at a slightly lower temperature (heating, 33.5°C; cooling, 30°C). The anomalies in heating and cooling scans near 48 and 47°C for heating and cooling scans, respectively, are due to an order-disorder transition of hydrocarbon chains of a stoichiometric complex of DPG and DPPC. It is of interest that this transition peak in cooling scans is sharper than that in heating scans. The enthalpy of the main transition of DPPC is the same as the enthalpy of DPPC plus DPG-DPPC recorded in the mixture (31.2 kJ/mol). No differences could be detected in lamellar repeat spacings obtained from static or synchrotron x-ray diffraction patterns recorded under identical conditions as those used to record the calorimetric behavior, with the exception of the coexistence region of the liquid-crystalline phase of DPPC and the gel phase of the complex. The plots, (*a*) and (*b*), of Fig. 1 *B* show static diffraction patterns of DPPC and a 5 mol% DPG in DPPC mixture recorded at 42.5°C, respectively. The lamellar repeat spacing is increased from 6.70 to 7.09 nm in the presence of 5 mol% DPG. In this mixture, there was no evidence of a phase separation in either the static or synchrotron x-ray diffraction patterns recorded over the temperature range 25–50°C. No evidence for a the ripple repeat spacing was observed in diffraction patterns recorded in the mixed aqueous dispersion.

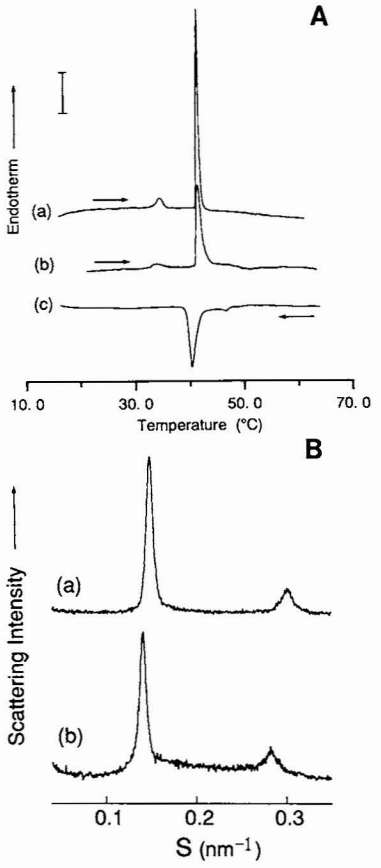

FIGURE 1 Thermal and structural parameters of aqueous dispersions of DPPC and 5 mol% DPG in DPPC. (*A*) Differential scanning calorimetric curves of (*a*) DPPC during heating at 0.8°C/min, (*b*) heating, and (*c*) cooling thermograms of a mixture of DPG and DPPC at temperature rates of 0.8°C/min. The bar indicates 10 kJ/mol deg. (*B*) Static x-ray diffraction profiles obtained from aqueous dispersions of (*a*) DPPC and (*b*) 5 mol% DPG in DPPC recorded at 42.5°C.

Clear evidence of immiscibility of a complex of DPG and DPPC was observed in mixtures containing 7.5 mol% DPG or more in DPPC. Immiscibility between the complex of DPG-DPPC in the gel phase and DPPC in the gel phase can be seen, for example, in the x-ray scattering patterns recorded at temperatures up to about 34°C in the heating scan of 7.5 mol% DPG in DPPC (Fig. 2). A first-order lamellar repeat spacing of the complex centered at 7.27 nm is seen together with the lamellar gel phase of pure DPPC, which has a repeat spacing of 6.57 nm. The lamellar repeat of the gel phase of pure phospholipid increases approximately to that of the gel phase of complex when the ripple structure (~34–42°C) is formed, and the lamellar spacing of the two phases cannot be distinguished. Heating through the main transition of the phospholipid, however, again shows the structure of the complex with an unchanged lamellar repeat spacing together with the more prominent lamellar repeat of the liquid-crystalline

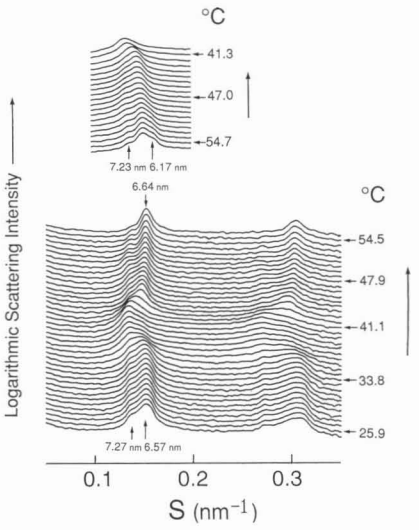

FIGURE 2 Plots of successive x-ray scattering intensity profiles versus reciprocal spacing as a function of temperature of a fully hydrated binary mixture of DPPC and 7.5 mol% DPG in DPPC heated from 25°C at a rate of 0.8°C/min. Inset shows the sequence of diffraction patterns obtained for the first-order diffraction maxima during a subsequent cooling scan at 0.8°C/min. Each diffraction pattern represents x-ray scattering accumulated during 30 s.

phase of DPPC centered at 6.64 nm. The reflections of the complex around 7.2 nm diminish above 48°C, which delineates the fluidus boundary. The scattering profiles at higher temperatures indicate a major lamellar liquid-crystalline phase with a second lamellar phase with a slightly shorter spacing that replaces the phase-separated DPG-DPPC complex in the gel phase at temperatures below the phase boundary. This transition we assign, on the basis of corresponding enthalpy changes observed in calorimetric scans (López-García et al., 1994), to a gel-liquid-crystalline transition of the DPG-DPPC complex. The transition can be seen more clearly in the subsequent cooling scan (Fig. 2). A shoulder in the wide angle side at 6.17 nm clearly replaces the lamellar reflection around 7.23 nm upon heating through the fluidus boundary. This result indicates immiscibility of the liquid-crystalline phase of complex from the liquid-crystalline phase of pure phospholipid.

Phase separations between DPPC and the complex of DPPC-DPG are more clearly evident in the mixed dispersions containing 10 mol% DPG. Dynamic x-ray diffraction data recorded during heating and cooling after heating scan of this sample showed that at the commencement of the initial heating scan (35°C) there were two lamellar repeat spacings; one structure is presumed to correspond to the complex with hydrocarbon chains in the gel configuration, and the other lamellar structure is assigned to the pure DPPC either in an expanded lamellar-gel phase or a modified ripple phase. A similar pattern was obtained from a static x-ray diffraction profile recorded from a sample under equilibrium conditions at 25°C. A transition of the latter phase occurs at about 42°C to liquid-crystalline phase with a spacing of 6.89 nm at 43°C decreasing to 6.60 nm at 52°C. This spacing is slightly longer than that obtained from dispersions of pure DPPC. The peak assigned to the complex in the gel phase remains throughout the transition region, but the intensity greatly decreases. A third lamellar component appears at temperatures just above the transition of the pure DPPC, and the lattice spacing of this component increases gradually to that identical with the complex. The spacings of the lamellar liquid-crystalline phase of pure DPPC and the gel phase of the complex coexist up to the fluidus boundary, where the first-order lamellar diffraction peak for the complex at 7.4 nm disappears. The question of immiscibility between pure DPPC and the liquid-crystalline phase of the DPPC-DPG complex is problematic. Representative diffraction patterns in the transition through the fluidus boundary are shown in Fig. 3. At temperatures above the fluidus line, the shape of the diffraction bands are asymmetric, which is particularly evident in the second-order reflection, with a shoulder on the wide angle side of the lamellar repeat spacings of the liquid-crystalline phase of the pure phospholipid. It is possible that phase separations between phospholipid and complex of DPPC-DPG persist at least in regions close to the fluidus boundary.

Changes in diffraction patterns during heating and cooling a dispersion containing 20 mol% DPG in DPPC are illustrated in Fig. 4, *A* and *B*. The heating scan shows persistence of the dominant x-ray scattering peak centered at 7.29 nm during transitions between gel and liquid-crystalline phases in the pure phospholipid, again providing strong evidence for phase separation of complex in the gel phase. Heating the mixed dispersions to temperatures above the fluidus line causes broadening of the peaks. A static diffraction pattern of the 20 mol% DPG in DPPC mixture recorded at 70°C is compared with pure DPPC in Fig. 4 *C*. A single lamellar spacing is observed in both cases. The only distinguishing feature between the two diffraction patterns is an increase in lamellar repeat spacing from 6.3 to 7.1 nm due to the presence of the DPG. Thus, there is no evidence of phase immiscibility between the complex and pure phospholipid at 70°C from the low angle static diffraction measurements.

The gel phase immiscibility between DPPC and the complex of DPPC-DPG was also observed clearly in the profiles

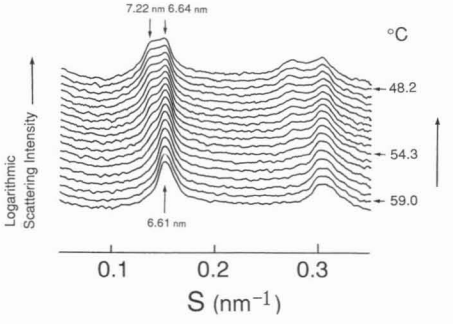

FIGURE 3 Plots of successive x-ray scattering intensity profiles versus reciprocal spacing as a function of temperature of a fully hydrated binary mixture of DPPC and 10 mol% DPG during cooling from 60 to 48°C at 0.8°C/min through the fluidus line. Each diffraction pattern represents x-ray scattering intensity accumulated during 30 s.

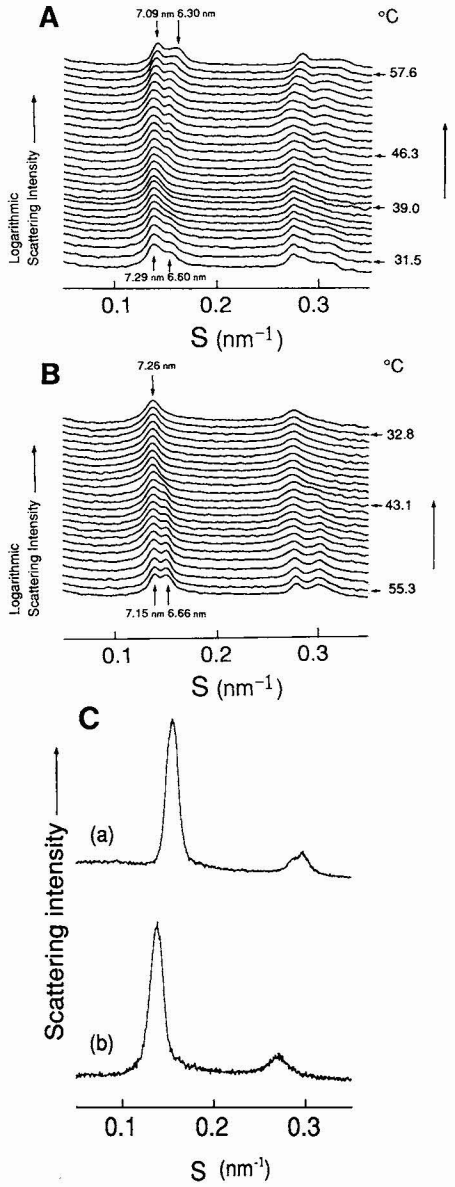

FIGURE 4 Plots of successive x-ray scattering intensity profiles versus reciprocal spacing recorded during heating (*A*) and cooling (*B*) an aqueous dispersion of DPPC containing 20 mol% DPG. Each diffraction pattern represents x-ray scattering accumulated during 30 s. (*C*) Static x-ray diffraction profiles of (*a*) pure DPPC and (*b*) 20 mol% DPG in DPPC recorded at 70°C.

of the wide angle scattering region. Wide angle diffraction provides information on the arrangement of the hydrocarbon chains of lipid. Fig. 5 shows wide angle diffraction profiles at 20°C for pure DPPC and the mixture of DPPC with 5, 10, and 20 mol% DPG and the 1:1 complex of DPPC-DPG. The profile for pure DPPC consists of a sharp peak at 0.422 nm, and a broad shoulder on the higher angle side implies that the hydrocarbon chains are packed in a quasi-hexagonal lattice and are tilted with respect to the bilayer plane (Tardieu et al., 1973). On the other hand, a sharp symmetrical peak at 0.411 nm for the 1:1 complex of DPPC-DPG indicates that the chains are packed in the hexagonal lattice and are normal to the bilayer plane. The profiles for the mixture of DPPC with

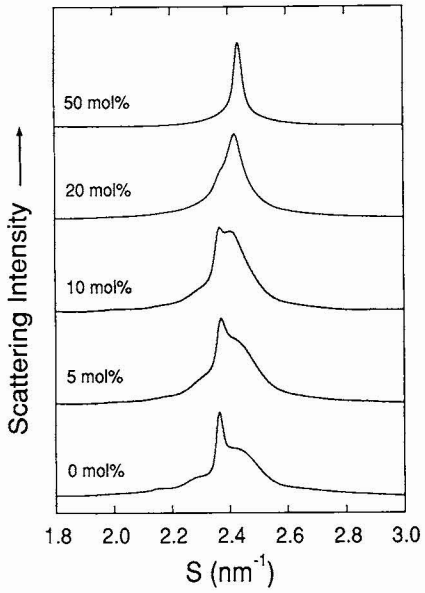

FIGURE 5 Static wide angle x-ray diffraction patterns of pure DPPC and mixtures of DPPC with DPG at proportions of 5, 10, 20, and 50 mol% recorded at 20°C.

5, 10, and 20 mol% DPG appear to be superpositions of the peaks of pure DPPC and the 1:1 complex. The increase in the intensity of a band centered at 0.416 nm with increasing proportion of DPG in the mixture is consistent with the formation of 1:1 complex that is phase separated from pure DPPC. Likewise, in the coexistence region of the liquid-crystalline phase of DPPC the presence of gel phase complex can be observed from scattering peaks in the wide angle region. Thus, in Fig. 6 it can be seen that a broad diffraction

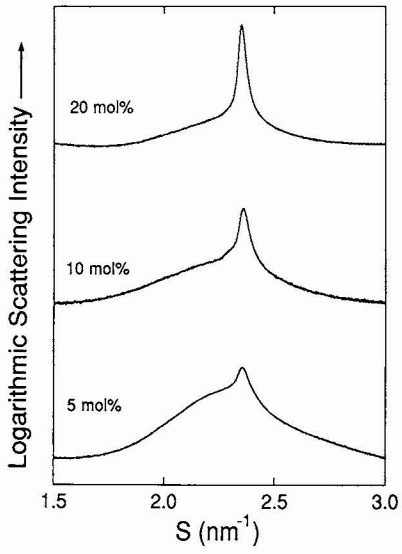

FIGURE 6 Static wide angle x-ray diffraction profiles of mixtures of DPPC with 5, 10, and 20 mol% DPG recorded at 43, 45, and 50°C, respectively. These measurement temperatures are midway between the solidus and fluidus phase boundaries in the DPPC-DPG phase diagram (López-García et al., 1994).

band from the chains of pure DPPC in the liquid-crystalline phase coexists with a sharp symmetrical peak centered at a spacing of about 0.423 nm due to the gel phase complex. At temperatures above the fluidus boundary, this sharp peak disappears and only a single diffuse wide angle scattering band is observed in all mixtures (data not shown). From the results, no conclusion can be drawn about the miscibility of DPPC and DPG from the wide angle data at temperatures above the fluidus boundary.

To estimate the thickness of the bilayer and the water layer, the electron density profile of the gel phase 1:1 complex of DPPC-DPG was calculated and was compared with the electron density profile of pure DPPC in the gel phase. The thickness of the water layer is established by the balance of attractive and repulsive forces between the neighboring surfaces of the bilayers, i.e., the thickness of the water layer is reflection of interbilayer interactions. The x-ray diffraction patterns for the 1:1 complex and pure DPPC at 20°C are shown in Fig. 7 *A*. The DPPC-DPG complex gives rise to five orders of the lamellar reflection. On the other hand, the higher order lamellar reflections up to the eighth order were observed for pure DPPC; however, the intensities of seventh- and eighth-order reflections were very weak, and the sixth reflection was not detected. Fig. 7 *B* shows the one-dimensional electron density profiles for pure DPPC and the 1:1 complex of DPPC-DPG in the gel phase. To obtain comparable resolution for the two samples, the five peak intensities were used to calculate the Fourier reconstruction of the electron density profiles. These profiles were calculated using the phase combination −,−,+,−,−. This phase combination has been reported consistently for the DPPC in the gel phase using the water swelling method (Torbet and Wilkins, 1976; Franks and Lieb, 1979; McIntosh and Simon, 1986). The same phase combination has been applied in the Fourier reconstruction of the 1:1 complex. The calculated electron density profile is consistent with that of a hydrated lipid bilayer (see Appendix). The electron density profiles show that the thickness of the bilayer and the water layer of the 1:1 complex are greater than those obtained for pure DPPC. From the electron density profiles, we estimate the thickness of the bilayer (i.e., the distance of between the headgroups) of pure DPPC and the 1:1 complex are 4.11 and 4.54 nm, respectively. The difference of the bilayer thickness is 0.43 nm. This value may be interpreted by considering the tilt angle of hydrocarbon chain. The length of hydrocarbon chain of DPPC in the gel phase was estimated by Nagle and Wiener (1988) to be 2.03 nm. If the chains of the 1:1 complex are not tilted, the tilt angle of the chains of DPPC is calculated to be about 27° ($\cos^{-1}((2 \times 2.03 - 0.43)/2 \times 2.03) = 26.6°$). This value is in agreement with reported values of chain tilt (Tardieu et al., 1973; Tristram-Nagle et al., 1993). The difference of the thickness of the water layer would indicate the difference of the interbilayer interaction between pure lipid and the complex. It may not be unexpected that inserting DPG between molecules of DPPC alters the balance of forces between adjacent polar headgroups in the surface layer thereby altering the interbilayer interacting forces.

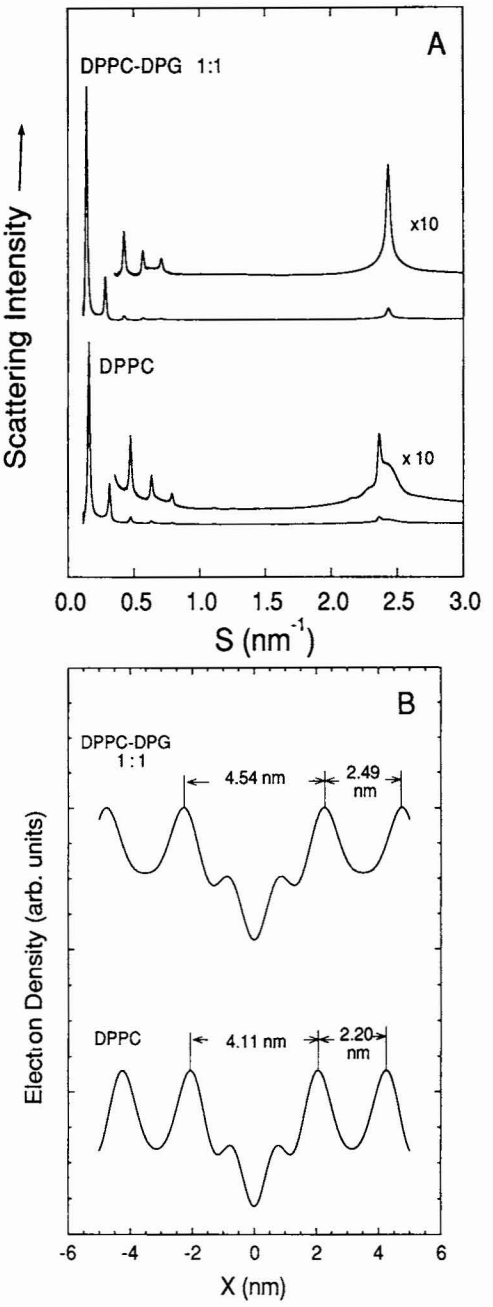

FIGURE 7 (*A*) Static x-ray diffraction profiles obtained from aqueous dispersions of DPPC and the 1:1 complex of DPPC-DPG recorded at 20°C. For $S > 0.3$ nm^{-1}, the plots with a 10-fold expanded scale are also shown. (*B*) One-dimensional electron density profiles constructed from the x-ray diffraction patterns for the gel phases of pure DPPC and the 1:1 complex of DPPC-DPG.

DISCUSSION

The phase diagram for the fully hydrated DPPC-DPG mixtures has already reported by López-García et al. (1994). The formation of complexes has been suggested from the phase diagram. In mixtures containing less than 50 mol% DPG, the complex composition is 1:1 (mol:mol) for DPPC:DPG. In temperature regions below the main transition of DPPC, immiscibility between pure DPPC and stoichiometric

complex of DPPC-DPG has been observed using the freeze-fracture electron microscopy (López-García et al., 1994). Cunningham et al. (1989) and Ortiz et al. (1988) have also pointed out the immiscibility of complex and PC for diacylglycerol-PC mixtures from the results of DSC.

In the temperature region below the main transition of pure DPPC, two distinct sets of lamellar reflections are present in the low angle scattering region for the mixtures. The coexistence of two diffraction peaks was also observed in the wide angle region for the mixtures. These results confirm that DPPC in either the gel or ripple phase is immiscible with the gel phase of DPPC-DPG complex. Furthermore, two different sets of lamellar spacings arising from pure DPPC and DPPC-DPG complex were also observed in the temperature region above the main transition of pure DPPC. The diffraction peak in the wide angle region corresponding to the packing of the hydrocarbon chains of the complex appears together with the board-scattering profile, indicating the chain-melting of pure DPPC. Therefore, the DPPC-DPG complex is immiscible with the DPPC bilayer in both the gel and the liquid-crystalline phases. The most likely explanation of why the phase separation could not be detected for the mixture with 5 mol% DPG in DPPC is that the amount of the complex present is not sufficient to produce a detectable diffraction peak.

In the previous work of López-García et al. (1994), the pretransition of DPPC was detected clearly in thermograms of mixtures containing up to 12.5 mol% DPG. In the temperature region between the pretransition and the main transition, we observed a large lamellar spacing that is characteristic of the ripple phase (see, for example, the diffraction patterns from a mixture containing 7.5 mol% DPG (Fig. 2)). However, we could not detect the reflection corresponding to the ripple repeat periodicity. This might be because the lateral domain size of DPPC in the mixtures is not large enough to give detectable scattering intensity and also that the ripple structure is not well ordered. The pure DPPC domain might also include small amounts of DPG that perturb the formation of a coherent ripple structure. There is evidence, however, that the characteristically large lamellar repeat of the ripple phase is stabilized by DPG. This is seen in x-ray diffraction patterns as a difference in heating and cooling scans for the mixture with 20 mol% DPG. The lamellar reflection has a shoulder on the wide angle side of the diffraction peak around 30°C in heating scan. By contrast, there is only a single symmetrical peak corresponding to the lamellar repeat spacing in the cooling scan (cf. Fig. 4, *A* and *B*). As seen in the thermal data (Fig. 1 *A*), the pretransition has a hysteresis in the cooling scan. The pretransition in cooling mode occurs at a lower temperature than heating scan; therefore, the DPPC domain in the mixture of 20 mol% DPG still remains in the ripple phase even at 30°C. Accordingly, only a single peak could be observed in the cooling scan at low temperature.

The lamellar repeat spacing of the complex is longer than that of pure DPPC in the gel phase. A similar finding has already been reported for phospholipid-diacylglycerol mixtures (Das and Rand, 1986; López-García et al., 1994). The lamellar repeat spacing is the sum of the thickness of the bilayer and the water layer. The Fourier reconstructions of the electron density profiles show that both of the bilayer thickness and thickness of water layer increase. This result implies that DPG molecules affect the packing and the tilt of hydrocarbon chains of DPPC in the complex. In this case, the presence of gel phase DPG alters the tilt angle of the chains of DPPC with respect to the bilayer normal (Tardieu et al., 1973; Tristram-Nagle et al., 1993). Such an effect has also been observed in binary mixtures of DPPC and palmitic acid (Koynova et al., 1988).

One of the most significant results is that from the x-ray diffraction patterns of samples containing of 7.5 mol% or more of DPG where there is direct evidence of immiscibility between pure phospholipid in the liquid-crystalline phase and the stoichiometric complex. Immiscibility above the fluidus boundary remains unresolved in mixtures containing DPG, but immiscibility has been reported for binary mixtures of dielaidoylphosphatidylcholine and dipalmitoylphosphatidylethanolamine above the fluidus boundary using spin-label electron spin resonance spectroscopy (Wu and McConnell, 1975). The spin-label method, however, is sensitive to the presence of relatively small lateral domains within the bilayer. low angle x-ray diffraction, however, demands correlation between domains in successive bilayers. Judging from two different lamellar spacings, the domains of the DPPC-rich region and the DPPC-DPG complex must separate three-dimensionally as a consequence of lateral two-dimensional phase separation brought about by intermolecular interactions between DPPC and DPG. This suggests that the free energy associated with the interaction between bilayers in the case of the surface of the complex juxtaposed with a surface of DPPC bilayer is high compared with that existing between the same neighboring surfaces. In fact, the electron density profiles (Fig. 7 *B*) show that the water layer, which reflects the interaction between bilayers, is different between pure DPPC and the complex.

The formation of complexes and domain structures of DPPC and DPG, which are detectable even at low molar ratios of DPG, may underlie the biological function of diacylglycerol in cell membranes. Thus, the creation of domains rich in diacylglycerol in the fluid bilayer matrix could give rise to structural features responsible for activation of various enzymes related by signal transduction mechanisms in biomembranes (Dennis et al., 1991; Dawson et al., 1984).

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APPENDIX

The phase determination required to calculate the electron density profile of the DPPC-DPG complex (1:1) at 20°C was done in two ways. The bilayer arrangement is considered to be a centrosymmetric structure. In this case, the values of the structural factors are real numbers, i.e., the phase angles are limited to 0 or π. For the DPPC-DPG complex, five lamellar reflections are observed; therefore, the variety of the phase combinations is expected to be 32. All possibilities of the phase combination were examined. Only one set, (−, −, +, −, −, i.e., π, π, 0, π, π), gave an electron density profile that was not inconsistent with a hydrated lipid bilayer. All other phase sets gave anomalous results, such as large positive electron density peaks in the center of the bilayer.

In a second approach, we interpreted the intensities of lamellar reflection using a strip model. Strip models have been used to analyze the structure of lipid bilayers (Worthington, 1969; Furuya et al., 1976; Wiener et al., 1989). The strip model represents the electron density of bilayer structures as a sequence of constant electron density. We considered a four-strip model for the electron density of the unit cell of multilamellar bilayer. The bilayer is divided into by four parts with constant electron density, namely, terminal methyl, methylene, headgroup, and interlamellar water (Fig. 8 *A*). In the strip model, the structure factor can be calculated analytically (Worthington, 1969). A four-strip model has seven parameters, i.e., the value of electron density of each of four parts and three values of boundary position of each strip. The electron density of the interlamellar water is assigned a value of 335 e/nm^3. The electron density of the methylene part was estimated by same method used by Wiener et al. (1989). The projected separation of methylenes along an all-*trans* chain is 0.127 nm. The cross sectional area of the chains can be calculated from the Bragg spacing of the wide angle diffraction maxima. Furthermore, judging from the shape of the wide angle diffraction, the hydrocarbon chains of the 1:1 complex of DPPC-DPG are oriented normal to the bilayer plane. From these parameters, the volume of each methylene residue of the 1:1 complex is estimated to be 2.48×10^{-2} nm^3. Because methylene has eight electrons, the electron density of methylene is estimated to be 323 e/nm^3. It is assumed that the position of the boundary between terminal methyl and methylene and the electron density of terminal methyl part are the same for DPPC and the DPPC-DPG complex. These values were estimated to be 0.47 nm and 210 e/nm^3, respectively, for DPPC at 20°C from the analysis of the four-strip model using our low angle intensity data. The origin is the center of the bilayer. Similar values have been estimated for parameters (see Fig. 6 in Wiener et al., 1989). The optimal values of three remaining parameters (the value of electron density of headgroup, and the positions of the methylene-headgroup boundary and the headgroup-water boundary) were determined by searching the minimum of the residue R defined by

$$R = \sum \{|F_c(h)| - K|F_m(h)|\}^2, \quad (1)$$

where, $F_c(h)$ is the structure factor calculated from the four-strip model and $|F_m(h)|$ is the absolute value of the measured normalized structure factor. This value is the square root of the integrated intensity and is normalized as $|F_m(1)| = 1.00$. h is the diffraction order. Because a one-dimensional system is considered in this study, the unit of the structure factor is e/nm^2. Σ is sum of h. K is a scale factor. The scale factor K is also one of the parameters. Nonlinear least-squares fitting was performed to establish the minimum of the residue R. The optimal value obtained for the electron density of the headgroup was 445 e/nm^3. The positions of the methylene-headgroup boundary and the headgroup-water boundary were 1.72 and 2.96 nm, respectively. Fig. 8 *B* shows a comparison of measured and calculated structure factors. The agreement of both factors indicates that the four-strip model is a reasonable model of the 1:1 complex of DPPC-DPG. The resulting phase set derived from this analysis of the complex (−, −, +, −, −) is the same as that obtained from examination of all possible phase sets.

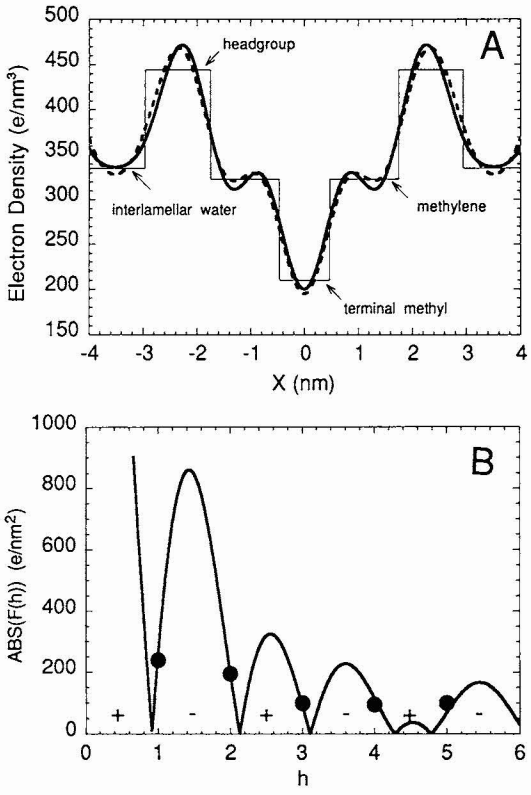

FIGURE 8 (*A*) Electron density profiles and the four-strip model of the 1:1 complex of DPPC-DPG at 20°C. The thin solid line shows the best fit of the four-strip model of the 1:1 complex. The thick solid line indicates the electron density profile constructed from the measured structure factors multiplied by the scale factor K at best fit. The phase set (−, −, +, −, −) and value of $F(0)$ were derived from the analysis of the four-strip model for the 1:1 complex of DPPC-DPG at 20°C. The dashed line shows the model electron density profile with same resolution, calculated from the four-strip model. (*B*) Comparison of the calculated structure factor $F_c(h)$ from the four-strip model (——) and the measured structure factor $F_m(h)$ (●). For both factors, the absolute values are shown. The phases assigned to the different regions are indicated by + and − on the figure.

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