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Miscibility of Phosphatidylcholine Binary Mixtures in Unilamellar Vesicles: Phase Equilibria

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Summary. Miscibility among phospholipids with different lipid chain-lengths or with different head groups has attracted a number of research efforts because of its significance in biological membrane structure and function. The general consensus about the miscibility of phosphatidylcholines with varying lipid chain-lengths appears to be that binary mixtures of phospholipids with a difference of two carbon atoms in the lipid chain mix well at the main phase transition. Miscibility between phosphatidylcholines with differences of four carbon atoms appears to be inconclusive. Previous reports on the phase transition of binary phospholipid mixtures are concerned mainly with multilamellar vesicles and are mostly limited to the main transition. In the present study, unilamellar vesicles were used and miscibility in binary systems between dimyristoyl-, dipalmitoyl- and distearoyl-phosphatidylcholines at pretransition as well as main transition temperatures was evaluated by constructing phase diagrams. Two methods were used to monitor the phase transitions: differential scanning microcalorimetry and optical absorbance methods. The optical method has the advantage that unilamellar vesicles of dilute phospholipid concentrations can be used. The liquidus and solidus phase boundaries were determined by the onset temperature of heating and cooling scans, respectively, because the completion temperature of a phase transition has no meaning in binary solutions. Dimyristoyl- and distearoyl-phosphatidylcholines, where the difference in the lipid chain-length is four carbon atoms, mixed well even at pretransition temperature.

Key Words phospholipid bilayer · phase transition · phase diagram · phospholipid binary mixture · pretransition

Introduction

Phase equilibria of phospholipid binary mixtures in bilayer systems have significance for the functions of biological membranes. Structural changes caused by the transition between gel and liquid-crystalline phases are implicated as the basis of biological activities, such as ion transport and membrane fusion. Membrane structures are a function of interactions between component lipids, and hence are characterized by phase diagrams.

A number of phase diagrams has been presented for binary mixtures of phospholipids, consisting of different acyl chains or of different polar head groups (Shimshick & McConnell, 1973; Blume & Ackermann, 1974; Chapman et al., 1974; Wu & McConnell, 1975; Lentz et al., 1976; Mabrey & Sturtevant, 1976; Jacobs et al., 1977; Kremer et al., 1977; Lee, 1978; Lentz & Litman, 1978; Marcelja & Wolfe, 1979; Scott & Cheng, 1979; Freire & Snyder, 1980; Chen & Sturtevant, 1981). Phospholipids with a difference in acyl-chain length of two carbon atoms, such as dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC), appear to mix well, although not ideally, at the main phase transition. Pretransition phase diagrams have not been established. Reports on the phase diagram of the mixture between DMPC and DSPC, however, have been inconclusive. Shimshick and McConnell (1973) concluded that the lipids are immiscible in a gel phase. Lee (1978) also analyzed the phase diagrams of binary mixtures of DMPC and DSPC under the immiscible condition. On the other hand, Mabrey and Sturtevant (1976) reported that DMPC and DSPC are miscible in both gel and liquid-crystalline phases. These two conflicting conclusions were reached in the presence of similar contours of the solidus curves, although their precise locations were not identical.

These studies are performed mainly with multilamellar vesicles for the main transition. Two problems remain to be clarified for the analysis of miscibility of phospholipids. One is the phase behavior of unilamellar vesicle membranes; the other is the pretransition phase diagram. It is probably justified to assume that unilamellar vesicles with large diameter would represent biological membranes better than multilamellar vesicles. Despite many reports about

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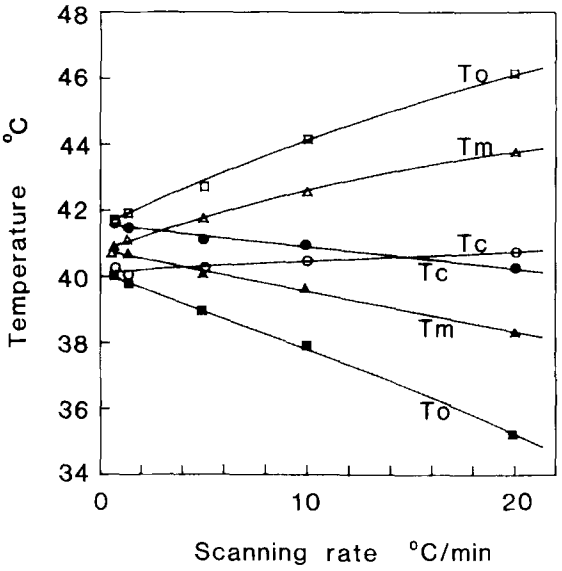

Fig. 1. Effect of temperature scanning rate on the phase transition temperature. Heating: filled signs. Cooling: open signs. Onset temperature: □; midpoint temperature: △; completion temperature: ○

the main phase transition of mixtures in a lipid bilayer, little is known about the pretransition. The present study reports the phase behavior of binary mixtures of DMPC-DPPC, DPPC-DSPC, and DMPC-DSPC systems in large unilamellar vesicles with relatively uniform vesicle size distribution. The effect of temperature scanning velocity in estimating the temperature of the thermotropic phase transition is also presented.

Materials and Methods

Synthetic dimyristoylphosphatidylcholine (1,2-ditetradecanoyl-*sn*-glycero-3-phosphorylcholine, DMPC), dipalmitoylphosphatidylcholine (1,2-dihexadecanoyl-*sn*-glycero-3-phosphorylcholine, DPPC), and distearoylphosphatidylcholine (1,2-dioctadecanoyl-*sn*-glycero-3-phosphorylcholine, DSPC) were obtained from Sigma. The purity was checked by thin-layer chromatography, and single spot has been confirmed. Water was purified by distillation followed by passage through ion-exchanger and activated charcoal columns in a Millipore water purifying system (Bedford, Mass.). Specific resistance of the obtained water was maintained at better than 16 MΩ cm. The absence of surface-active impurities was verified with a dynamic surface tension balance by confirming that a 10 : 1 compression of the water surface area produced less than 0.1 dyne cm^{-1} change after standing for 10 min, as previously reported (Shibata et al., 1981).

Phospholipid binary mixtures were prepared by premixing the phospholipids in methanol, followed by evaporation of the solvent in a 10-ml flask of a Microrotary Evaporator (Cole-Parmer, Chicago, Ill.) under nitrogen gas. After adding an appropriate amount of water to make the phospholipid concentration about 1 mM for a dilute solution, phospholipids were suspended by a Vortex Mixer (Fisher, Pittsburgh, Pa.). Unilamellar vesicles were prepared from the vortex suspension by ultrasonic irradiation in a cup-horn of a Branson Sonifier W185 (Danbury, Conn.) at above the phase-transition temperature as previously reported (Kamaya et al., 1984). The preparation was aged at 4°C for one week to obtain relatively homogeneous size distribution by fusing vesicles, as described by Wong et al. (1982). When observed by electron microscopy after negative staining by 0.5% ammonium molybdate, the aged preparation showed an average diameter of 0.7 μm.

Two methods were used to monitor the phase transition: differential scanning microcalorimetry and optical absorbance. For calorimetry, a Perkin-Elmer Differential Scanning Microcalorimeter DSC-2 with auto zero (Norwalk, Conn.) was used with samples sealed in an aluminum pan. For optical absorbance, a Perkin-Elmer 554 spectrophotometer, equipped with a digitally controlled programmable electronic heat exchanger and a cuvette microstirrer, was used at a 440 nm wavelength. The suspension temperature was monitored by a calibrated thermistor thermometer (United Systems, Dayton, Ohio) which has a 0.01°C resolution and 0.5 sec response time. The thermistor tip was inserted into the cuvette just above the light path. The temperature was scanned at a rate of 0.1°C · min^{-1}. The DSC-2 system requires a concentrated phospholipid suspension, typically above 100 mM. Therefore, the phospholipid mixture, after evaporation of the solvent, was suspended in water at 100 mM by vortex mixing for differential scanning microcalorimetry. The advantage of the optical method is that unilamellar vesicle suspensions of dilute phospholipid concentrations, in the range of 1 mM, can be used. The scanning speed does not affect the sensitivity of the optical system in detecting phase transitions; hence a slow scan can be used, whereas microcalorimetry sensitivity is strongly dependent upon the scanning speed and decreases with a decrease in the scanning speed.

The dilute phospholipids suspension in the form of unilamellar vesicles with relatively homogeneous vesicle-size distribution is probably superior to the concentrated multilamellar preparation in modeling biological membranes. For this reason, the optical method was mainly used to analyze the phase behavior. The agreement between differential scanning microcalorimetry and the optical method was excellent as previously reported (Kamaya et al., 1984), when compared at 100 mM phospholipid suspensions.

Results and Discussion

When expressing the transition temperature, the onset and midpoint (which approximates the peak in differential scanning microcalorimetry) of the phase transition have been arbitrarily used. To examine the adequacy of the choice of these temperatures in expressing the phase transition, the effect of scanning velocity on the onset (T_o), midpoint (T_m) and completion (T_c) temperatures in heating and cooling scans was checked with DPPC single-component vesicles by differential scanning microcalorimetry.

Figure 1 shows the plots of T_o, T_m and T_c temperatures of phospholipid vesicles against the speed of the temperature scan in the heating and cooling

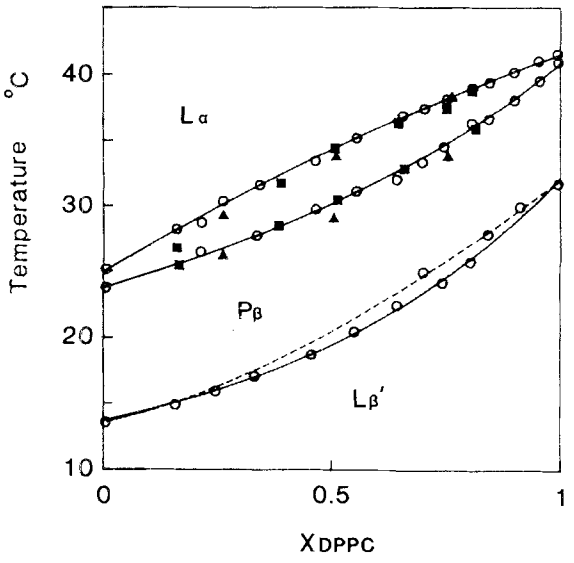

Fig. 2. Phase diagram of DMPC-DPPC mixture. Present data: ○. Data by Shimshick and McConnell (1973) ▲ and Mabrey and Sturtevant (1976) ■ are also shown. The dotted line in the pretransition is estimated from Eq. (1) in the text and the low-temperature boundary of the pretransition

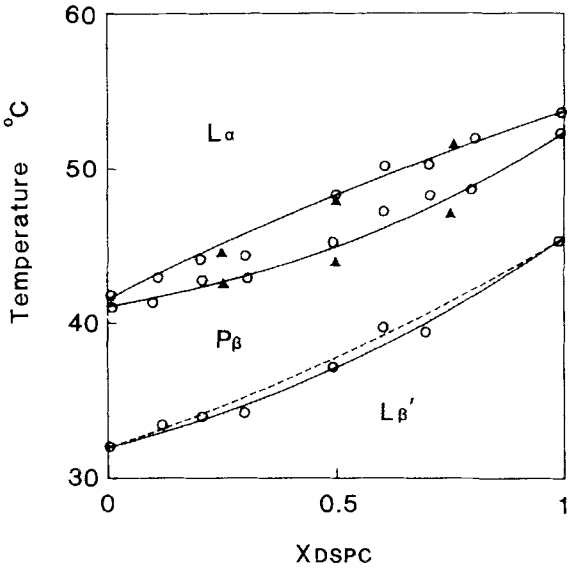

Fig. 3. Phase diagram of DPPC-DSPC mixture. Signs are the same as in Fig. 2

runs. The scanning rate showed large effects on the transition temperature. Among the three, T_o was less influenced by the scanning rate when compared with T_m and T_c. As seen in Fig. 1, the changes in the transition temperature were a linear function of the scanning rate at the limit of the present experimental condition. When the T_c of the cooling scan was extrapolated to the zero scanning rate, the value was identical to the extrapolation of T_o of the heating scan. Similarly, extrapolations of T_c of the heating scan and T_o of the cooling scan intersected the zero scanning rate axis at the same point. T_m values of heating and cooling scans extrapolated to the same point at the zero scanning axis.

From these results, it may be reasonable to express the phase-transition temperature by the extrapolated value of T_m to the zero scanning rate as the average of the vesicles, which have a notable distribution in size inhomogeneity in the single component vesicles.

With binary mixtures, however, T_m values may fall into an arbitrary point between solidus and liquidus curves when two lipids are miscible in a bilayer. This is because the melting process from gel to liquid-crystalline states would expel a component that has a higher melting temperature from the liquid-crystalline domain of the vesicle membranes. These conditions make it difficult to obtain precise values for T_m and T_c, because rearrangement of lipids in the membrane is a slow process. For these reasons, we chose T_o as the thermotropic phase-transition temperature for binary mixtures.

Experimentally determined phase diagrams for the mixtures of DMPC-DPPC, DPPC-DSPC, and DMPC-DSPC are shown in Figs. 2–4. According to the nomenclature of Tardieu et al. (1973), L_α is the liquid-crystalline phase, $L_{\beta'}$ is the gel phase with tilted acyl chains, and P_β is the gel phase with rippled structure. The results of Shimshick and McConnell (1973) and Mabrey and Sturtevant (1976) are also included in these figures. The present phase diagrams of DMPC-DPPC and DPPC-DSPC systems agree well with the two previous reports, demonstrating complete miscibility of the component lipids with almost ideal mixing.

With the DMPC-DSPC system, however, the present result was in conflict with those reported by Shimshick and McConnell (1973) and Mabrey and Sturtevant (1976). Their solidus curves showed an almost constant transition temperature from 0 to 50 mol% of DSPC. The discrepancy may be caused by the method of sample preparation. We observed that with insufficient ultrasonic irradiation, the preparation often showed a melting temperature similar to that of DMPC, which has a lower melting point.

If we assume that the water can be ignored and that the system behaves as a binary mixture, consisting of two lipids where they form mixed crystalline phases at temperatures above and below the phase transition, the liquidus and solidus curves are described by (Kirkwood & Oppenheim, 1961):

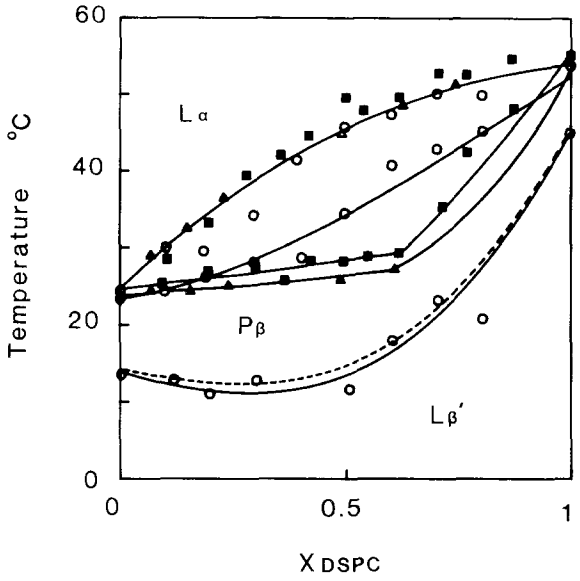

Fig. 4. Phase diagram of DMPC-DSPC mixture. Signs are the same as in Fig. 2

$$x_2^l = \frac{\exp(\lambda_1)\gamma_1^l\gamma_2^s - \gamma_1^s\gamma_2^s}{\exp(\lambda_1)\gamma_1^l\gamma_2^s - \exp(-\lambda_2)\gamma_1^s\gamma_2^l} \quad (1)$$

and

$$x_2^s = \frac{\exp(\lambda_1)\gamma_1^l\gamma_2^l - \gamma_1^s\gamma_2^l}{\exp(\lambda_1 + \lambda_2)\gamma_1^l\gamma_2^s - \gamma_1^s\gamma_2^l} \quad (2)$$

where

$$\lambda_1 = \frac{\Delta H_1}{R}\left(\frac{1}{T} - \frac{1}{T_1^0}\right) \quad (3)$$

$$\lambda_2 = \frac{\Delta H_2}{R}\left(\frac{1}{T_2^0} - \frac{1}{T}\right). \quad (4)$$

Superscripts l and s denote liquidus and solidus phase, respectively, and subscripts 1 and 2 denote the two mixing lipids, respectively. R is the gas constant, T is the absolute temperature, T^0 is the melting temperature of the lipids, ΔH is the enthalpy of fusion of pure lipids, and γ is the activity coefficients. Assuming that both mixed crystalline phases are represented by the regular solution, where

$$\gamma_1^l = \exp\{\alpha(x_2^l)^2/RT\},\ \gamma_2^l = \exp\{\alpha(x_1^l)^2/RT\},$$
$$\gamma_1^s = \exp\{\beta(x_2^s)^2/RT\} \text{ and } \gamma_2^s = \exp\{\beta(x_1^s)^2/RT\}. \quad (5)$$

The regular solution theory was applied to phase diagrams of lipid membranes by Mabrey and Sturtevant (1976), Kremer et al. (1977) and Lee (1978).

The two constants, α and β, relating to the interaction between two mixing molecules, are estimated by a curve-fitting procedure of experimentally obtained solidus and liquidus curves, where the onset temperatures of both heating and cooling scans are corrected for the contribution from the width of the transition temperature of the single component. The estimated values of α were 0.25, 1.7 and 0.25 kJ · mol^{-1} and those for β were 1.1, 2.2 and 1.7 kJ · mol^{-1} for DMPC-DPPC, DPPC-DSPC and DMPC-DSPC systems, respectively. The calculated curves are shown in Figs. 2–4 as solid lines.

In the present phase diagrams, we constructed liquidus and solidus phase boundaries by the onset points of the heating and cooling scans, respectively. These points almost coincide with the onset and completion temperatures of a single scan in single component vesicles. The width of the transition temperature of the main transition (or the steepness of the phase transition) has attracted considerable attention, and often is interpreted as a cooperativity of the transition. This concept defines a cooperative unit as a cluster of phospholipid molecules changing their state synchronously at the phase transition. The number of phospholipid molecules in a cluster is estimated by taking the ratio between the enthalpy change at the phase transition, derived from the van't Hoff equation, and the experimentally obtained excess enthalpy.

The effect of acyl chain-length (Shimshick & McConnell, 1973; Chapman et al., 1974; Lentz et al., 1976; Mabrey & Sturtevant, 1976; Jacobs et al., 1977; Kremer et al., 1977; Lee, 1978; Marcelja & Wolf, 1979; Scott & Cheng, 1979; Freire & Snyder, 1980; Chen & Sturtevant, 1981), polar head group (Blume & Ackerman, 1974; Chapman et al., 1974; Wu & McConnell, 1975; Lentz & Litman, 1978), or perturber molecules such as anesthetics (Jain & Wu, 1977; Mountcastle et al., 1978; Heyn et al., 1981) upon the width of the phase transition, have been reported. The theory on the cooperativity of lipid phase transition in bilayer systems is inconsistent with the idea that the thermotropic phase transition of lipid bilayers follows thermodynamically first-order kinetics. The first-order phase transition is supported by the experimental facts that the latent heat of the transition is clearly observable, and the Clapeyron-Clausius relation holds for the main transition (Nagle & Wilkinson, 1978).

A phase transition always has a cooperative aspect even in two-dimensional monolayers. The negative interaction energy between nearest neighbor molecules in the same state should favor a large number of molecules to be in the same state. The cooperativity cannot be evaluated with the van't

Hoff equation by arbitrarily assuming a two-state model. The first-order transition suggests that the change should occur abruptly in an all-or-none fashion, if the scanning is performed slowly enough in a quasistatic fashion. This idea of first-order phase transition is also supported by the experimental studies on the effect of the curvature of vesicles on main transition temperatures, reported by Lichtemberg et al. (1981); the width in the transition temperature between the onset and completion may be caused by the inhomogeneity of the vesicle size.

In Figs. 2–4, we have plotted only onset points of the heating scans for the pretransition. The change in absorbance of the lipid dispersion at pretransition in the heating scan was larger than that of the main transition in the present preparation. This is probably caused by the aggregation of phospholipid vesicles at the pretransition temperature. By studies of the optical properties of the aqueous dispersion of phosphatidylcholines, Yi and McDonald (1973) suggested that the pretransition change is associated with aggregation-disaggregation of vesicles. Peterson and Chan (1978) also reached the same conclusion, that the aggregation-disaggregation of the vesicles is associated with the pretransition, by optical measurements of an added salt effect on the transition temperature. In a cooling scan, the absorbance change occurs below the onset temperature of the heating scan. The structural change at the pretransition exhibits pronounced hysteresis, similar to that of subtransition (Nagle & Wilkinson, 1982). Apparently, the rearrangement of the phospholipid molecules at the pretransition in a cooling scan is a slow process. Because of the hysteresis, it was difficult to obtain an equilibrium onset temperature of pretransition in the cooling scan, which corresponds with the onset temperature in the heating scan.

Nevertheless, the plotted transition temperature curves for the heating scan show that these lipid mixtures form mixed crystalline phases at temperatures below the solidus curves of the main transition. The differential scanning microcalorimetry measurements also showed latent heat at the pretransition temperature. Thus, the phase boundary can be estimated by the above equations used for the calculation of the main transition. In this case, experimental data for the high-temperature boundary are lacking, but one can use the numerical value of the constant α, obtained with the main transition for estimation of the activity coefficients γ_1^l and γ_2^l at the pretransition temperature. Then, x_2^l and β are estimated by computing the best-fit values to the experimentally obtained pretransition data for the low-temperature boundaries. In Figs. 2–4, the pretransition high-temperature boundaries calculated by this method are shown. The estimated numerical values were 1.5, 2.3 and 2.8 kJ · mol^{-1} for DMPC-DPPC, DPPC-DSPC and DMPC-DSPC systems, respectively.

The present experimental data on binary mixtures with different acyl chain-length and an identical head group show the pretransition low-temperature boundaries with a contour similar to that of the solidus curves of the main transition. Long-chain fatty acids with only one carbon chain-length difference show nonideal miscibility in a three-dimensional bulk phase. For example, the equilibrium between solid and liquid solutions for tetradecanoic acid and pentadecanoic acid is reportedly similar to a positive azeotrope, corresponding to nonideal behavior (Motomura et al., 1977). On the other hand, the two-dimensional phase diagram for these two fatty acids at an air/water interface, where expanded and condensed monolayers are in equilibrium, shows typical ideal behavior (Motomura et al., 1977). Monolayers of phosphatidylcholines, spread on a water surface, would be expected to show similar ideal behavior on the π-A isotherms because the large phosphatidylcholine head group may interfere with lateral cohesion between acyl chains; then the discrepancy caused by acyl chain-length would diminish.

The present results suggest that the change at the pretransition involves interaction between hydrocarbon chains of the component lipids. Because the present study was limited to the determination of the transition temperature, detailed structural changes associated with the transition cannot be assessed. Nevertheless, for binary mixtures of phosphatidylcholines, it is probably justified to conclude that the pretransition is an equilibrium point where two phospholipid forms coexist and these lipids are freely miscible even at temperatures as low as the pretransition. These two forms may be the tilted carbon chains and rippled forms, analogous to the case of the pure DPPC-excess-water system (Tardieu et al., 1973; Guldbrand et al., 1982). Apparently, the structural change in the lipid conformation triggers the aggregation-disaggregation process in unilamellar vesicles, and displays the abrupt change in the absorbance at the pretransition.

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