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Lipid arrangement in fluid model membranes: analysis by cross-linking of phosphatidylethanolamines

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Binary mixtures of fluid phase phosphatidylethanolamines at pH 10 were treated with the bifunctional cross-linking reagent dimethylsuberimidate. Analysis of the dimeric species formed demonstrated that the phospholipid species in dimyristoylphosphatidylethanolamine / dielaidoylphosphatidylethanolamine mixtures at 52°C and dielaidoylphosphatidylethanolamine / dilauroylphosphatidylethanolamine mixtures at 41°C were randomly arranged. Analysis of the dimeric species formed in dipalmitoylphosphatidylethanolamine / dioleoylphosphatidylethanolamine mixtures at 68°C showed that this mixture was very close to being randomly arranged, with just a slight propensity of like phospholipid species to cluster.

Introduction

The existence of non-random lateral distributions (domains) of lipids in fluid-phase bilayers is a subject of some controversy. Perhaps because it is technically difficult to detect multiple domains in fluid-phase lipid mixtures, there have been relatively few convincing demonstrations of the existence of fluid phase immiscibility. The existence of domains of lipid in fluid-phase model systems was suggested by Wu and McConnell [1] who interpreted discontinuties in an electron paramagnetic resonance spectral parameter above a horizontal fluidus line in their phase diagram of DEPC/DPPE as evidence for the existence of two mutually immiscible fluids with different compositions. However, a careful DSC study of this system by Silvius demonstrated that the fluidus line was not truly horizontal and that these lipids mixed ideally in the fluid phase [2]. Lentz et al. [3] also interpreted the presence of a horizontal fluidus line in their phase diagram of dipentadecanoylphosphatidylglycerol/DMPC, obtained using DSC and fluorescence polarization measurements on diphenyl-1,3,5-hexatriene, as evidence of compositional inhomogeneities in the fluid phase. Melchior [4] investigated fluid DSPC/DMPC mixtures by 'quick-freezing' samples which had been equilibrated at 85°C and using DSC to examine the samples as they were heated. Melchior found that the quickly frozen samples exhibited two distinct endotherms, while conventionally cooled samples exhibited only a single endotherm at an intermediate temperature. He interpreted these results to suggest that, in the fluid phase, DSPC and DMPC are clustered into separate domains; the lipids are presumably crystallized so rapidly with the quick-freeze method that the lateral mobility of the lipids is too slow to allow co-crystallization.

From previous studies such as the ones described, it is unclear what lipid characteristic is associated with lateral separation in the fluid phase. The lipids used by Lentz et al. [3] differed in chain length by one carbon, differed in head group, and differed in gel-to-fluid transition temperature by about 10°C. The lipids employed by Melchior to demonstrate fluid-phase immiscibility have the same head group, but differ in chain length by four carbons and in gel-to-fluid phase transition temperature by about 35°C [5].

Abbreviations: DSC, differential scanning calorimetry; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DEPC, dielaidoyl PC (di-9,10-*trans*-octadecenoyl PC); DEPE, dielaidoyl PE (di-9,10-*trans*-octadecenoyl PE); DMPC, dimyristoyl PC (ditetradecanoyl PC); DMPE, dimyristoyl PE (ditetradecanoyl PE); DSPC, distearoyl PC (dioctadecanoyl PC); DLPE, dilauroyl PE (didodecanoyl PE); DOPE, dioleoyl PE (di-9,10-*cis*-octadecenoyl PE); DPPE, dipalmitoyl PE (dihexadecanoyl PE).

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In this report, we examine the arrangement of PE species in fluid lipid bilayers. In order to do this, we conducted our experiments at pH 10, since at this pH, phosphatidylethanolamines tend to adopt a bilayer, rather than hexagonal, conformation in the fluid phase, except, perhaps, at very high temperatures [6,7]. We examined mixtures of DPPE/DOPE, DMPE/DEPE, and DEPE/DLPE. DPPE, with 16-carbon, saturated fatty acyl groups, is similar in chain length to DOPE, with its *cis*-monounsaturated 18-carbon fatty acyl groups, while the gel-to-fluid transition temperatures of these lipids differ by at least 68°C. DMPE differs from DEPE, with its *trans*-monounsaturated 18-carbon chains, by four carbons in chain length and by 14°C in transition temperature, while DEPE and DLPE differ by 6 carbons in chain length, but by only 3°C in transition temperature.

The method used in this work to determine the arrangement of PE species in the fluid phase involved cross-linking of the binary mixtures of PEs in vortexed vesicles by the bifunctional reagent, dimethylsuberimidate, using conditions under which about 10% of the PE molecules are cross-linked. The three dimeric species formed were then resolved by high-performance liquid chromatography [8]. The fraction of each dimeric species present was compared with the fraction expected based on random mixing of the two PE species.

Materials and Methods

Materials. PEs were obtained from Avanti, Alabaster, AL. Dimethylsuberimidate-2 HCl was obtained from Pierce, Rockford, IL.

Cross-linking reaction. Solutions of the PEs in chloroform/methanol (2 : 1, v/v) were mixed to give the indicated ratios of the PE species. Each sample contained a total of 4 μmol PE. The solvent was removed from the sample by evaporation under a nitrogen stream, followed by 30 min under vacuum. 2 ml of 100 mM sodium chloride, 50 mM sodium bicarbonate (pH 10) was added to each sample, the sample was equilibrated to the reaction temperature and then vortexed. After allowing further equilibration of the sample at the reaction temperature, 40 μl of freshly-made 10 mM dimethylsuberimidate-2 HCl (0.4 μmol) with 20 mM NaOH in the same buffer was added, the sample was vortexed, and the reaction was carried out for 30 min. The reaction was terminated by the addition of 24 μl glacial acetic acid, which lowered the pH to approximately 4. The termination of the reaction under these conditions could be demonstrated by cross-linking a control sample in which the PE species in the binary mixture were present in separate vesicles which had been mixed after formation. In this case, no heterodimer formation was observed, indicating that no reaction occurred during the extraction procedure.

Extraction and separation of the dimeric species. 4 ml methanol and 2 ml chloroform were added to each sample, and the single-phase sample was mixed. Then 2 ml chloroform and 2 ml 0.15 M sodium chloride were added, followed by vigorous mixing. The phases were separated by a brief centrifugation. The organic (lower) phase was removed. 2 ml chloroform was added again, the sample was mixed and centrifuged, and the organic phase was removed. This process was repeated one more time. The combined organic phases were dried under a stream of nitrogen. Dimeric species from DMPE/DEPE and DEPE/DLPE mixtures were isolated from other reaction products by two-dimensional TLC. The dried organic phases from these mixtures were redissolved in 100 μl chloroform/methanol (2 : 1, v/v) and applied to a 20 × 20 cm silica gel G plate with a 250 μm layer. The plate was developed first in chloroform/methanol/acetic acid (65 : 25 : 8, v/v/v), dried thoroughly, and then developed in the second dimension in chloroform/methanol/water (65 : 25 : 4, v/v/v). The spot corresponding to the PE dimers [8] was scraped, and the silica gel was extracted three times with chloroform/methanol/water (5 : 5 : 1, v/v/v). This material was dried under a stream of nitrogen. This material or the dried organic phase after the extraction of the reacted DPPE/DOPE mixtures was dissolved in 4 ml chloroform and filtered through a 0.45 μm filter. The solvent was again removed by evaporation under a nitrogen stream, the entire sample was dissolved in 50 μl chloroform/methanol (2 : 1, v/v), and the sample was injected onto a reversed phase (Altex-ODS, 4.6 mm × 25 cm) HPLC column(s). Dimeric mixtures derived from DPPE/DOPE mixtures were resolved into their three component species by isocratic chromatography in 20 mM choline chloride in ethanol/water/hexane (77 : 13 : 10, v/v/v) on two identical columns in series with a flow rate of 0.5 ml/min. Dimeric species derived from DMPE/DEPE mixtures were resolved by chromatography in the same solvent on a single column with a flow rate of 1 ml/min. Dimeric species derived from DEPE/DLPE mixtures were resolved by gradient chromatography on a single column at a flow rate of 0.8 ml/min. Elution was begun isocratically with 20 mM choline chloride in ethanol/water/hexane (78 : 16 : 6, v/v/v) with 60 μl chloroform/l for 15 min. Over the next 60 min, the solvent was changed from the initial solvent to 100% 20 mM choline chloride in ethanol/water/hexane (79 : 11 : 10, v/v/v) in a linear gradient. Elution was continued for 135 additional min with the second solvent.

Quantitation of the dimers and statistical analysis. Detection of the dimers was by absorbance at 205 nm, monitored continuously, and recorded on a chart. Dimers of various PE molecular species have similar extinction coefficients at this wavelength, since most of the absorbance is due to incorporation of the dimethylsuberimidate [8]. Peaks corresponding to the dimers were reproduced photostatically, cut, and weighed. The percentage of each dimer in each sample was calculated. The mean of these percentages for triplicate samples ± the standard deviation is reported. Experimental data were fit with a second order regression curve.

Calculation of the theoretical curves for randomly-mixed PEs. The fraction of each homodimer expected for random mixing is simply the square of the fraction of the corresponding PE in the mixture. For $y =$ the homodimer of the higher melting lipid (mol fraction),

$$y = x^2 \tag{1}$$

where x is the mol fraction of higher melting lipid in the mixture being investigated. For $y =$ the homodimer of the lower melting lipid (mol fraction),

$$y = (1-x)^2 = x^2 - 2x + 1 \tag{2}$$

where $1 - x$ is the mol fraction of the lower melting lipid in the mixture under investigation. The fraction of heterodimer expected for random cross-linking is two times the product of the mol fraction of each PE in the mixture. For $y =$ the heterodimer (mol fraction),

$$y = 2(x)(1-x) = -2x^2 + 2x \tag{3}$$

where x is the mol fraction of higher melting lipid in the mixture being investigated and $1 - x$ is the mol fraction of the lower melting lipid.

Results

The gel-to-fluid transition temperatures of the PEs at pH 10, as determined by steady-state fluorescence polarization of *cis*-parinaric acid, are DPPE, 52°C; DMPE, 37°C; DEPE, 23°C; and DLPE, 20°C [9]. These transition temperatures are about 10 C° lower than the corresponding transition temperatures in water. While the transition temperature of DOPE was not determined at pH 10, it has been reported to be −16°C in water [10,11].

Analysis of the dimers formed from the cross-linking of DMPE and DEPE mixtures of various compositions at 52°C are shown in Fig. 1. The experimental data are very similar to the theoretical curve, based on random mixing of the lipids. Similarly, analysis of the dimers formed from cross-linking of DEPE and DLPE mixtures at 41°C is shown in Fig. 2. Again, the experimental data are similar to the data which would be expected for random mixing of the PE species.

Fig. 3 shows the mole fractions of each dimer obtained from the dimethylsuberimidate cross-linking of DPPE/DOPE mixtures at 68°C. While the experimental data are similar to the theoretical data for random cross-linking, the slightly lower then expected values for

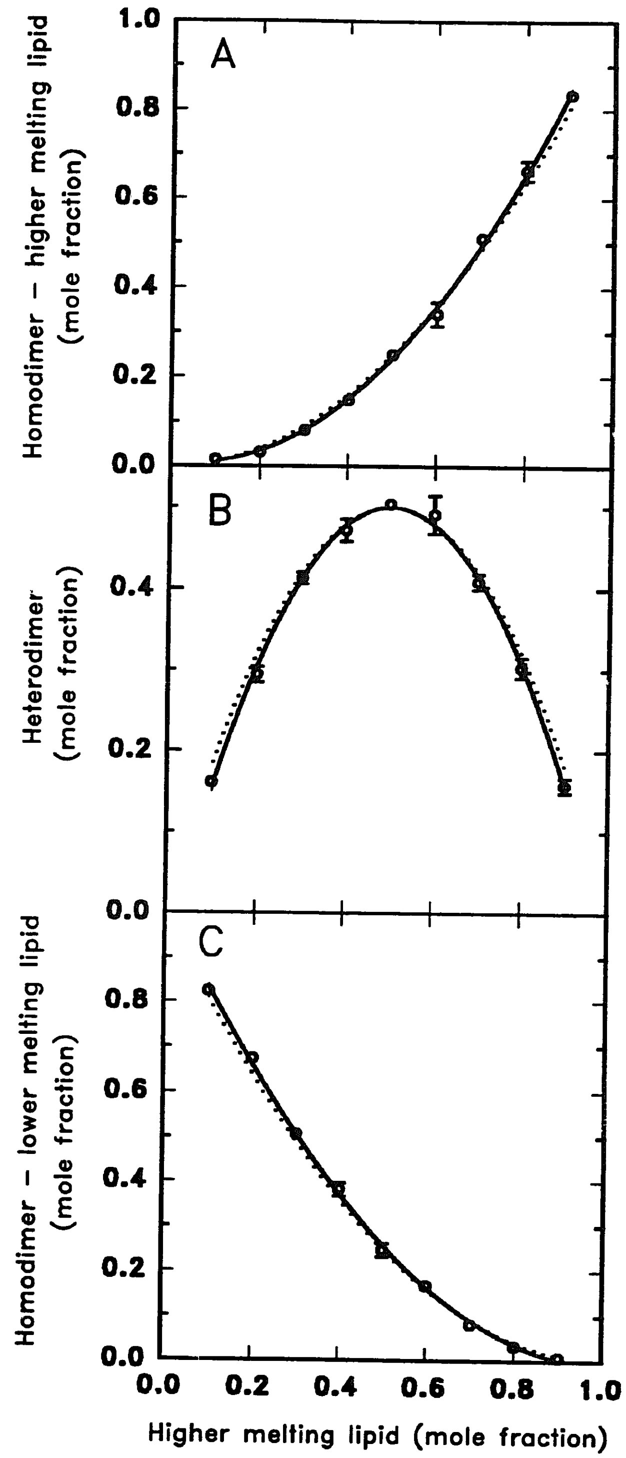

Fig. 1. Analysis of the fraction of each dimeric species formed from dimethylsuberimidate cross-linking of DMPE and DEPE mixtures at 52°C. Panel A represents the mole fraction of DMPE-DMPE dimers of the total dimers, panel B represents the mole fraction of DMPE-DEPE, and panel C represents the mole fraction of DEPE-DEPE. In each case the points represent the mean of triplicate determinations and the error bars represent standard deviations. If error bars are not seen, it is because they are smaller than the size of the points. The solid line is a calculated second-order regression curve through the experimental data. The dotted line represents the theoretical yield of each dimer if the PE species were randomly mixed.

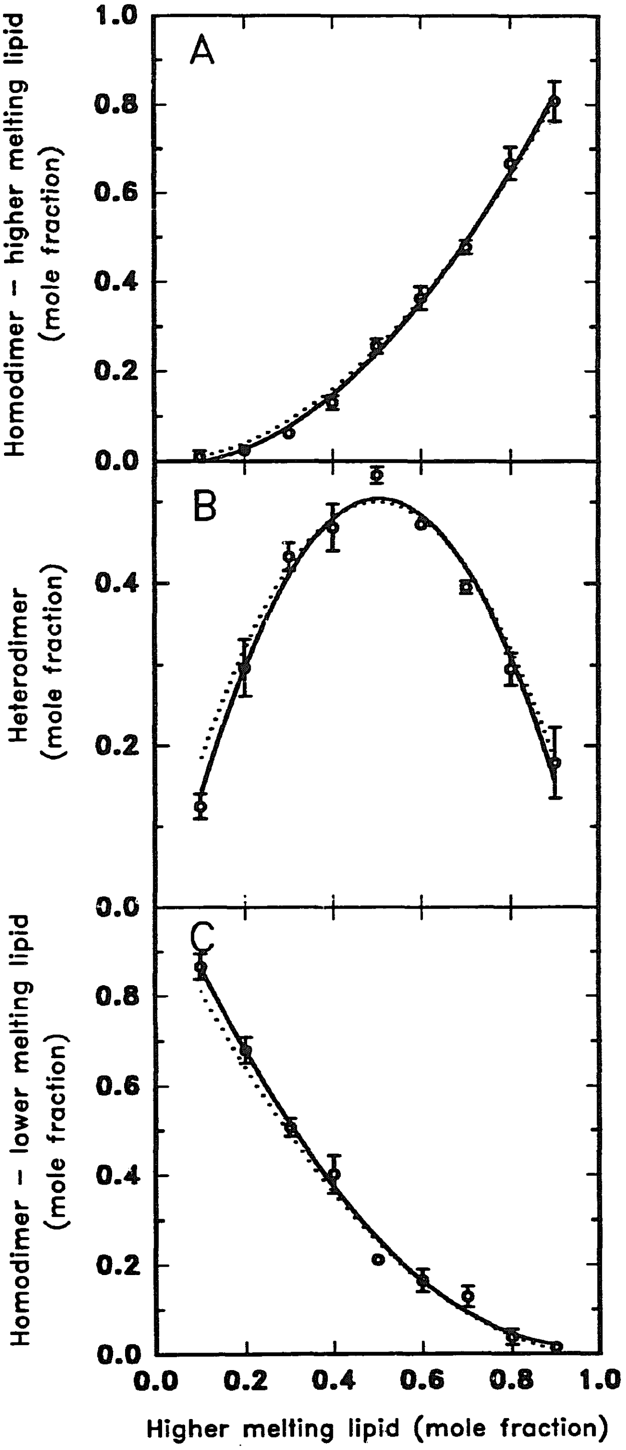

Fig. 2. Analysis of the fraction of each dimeric species formed from dimethylsuberimidate cross-linking of DEPE and DLPE mixtures at 41° C. Panel A represents the mole fraction of DEPE-DEPE dimers of the total dimers, panel B represents the mole fraction of DEPE-DLPE, and panel C represents the mole fraction of DLPE-DLPE. In each case the points represent the mean of triplicate determinations and the error bars represent standard deviations. If error bars are not seen, it is because they are smaller than the size of the points. The solid line is a calculated second order regression curve through the experimental data. The dotted line represents the theoretical yield of each dimer if the PE species were randomly mixed.

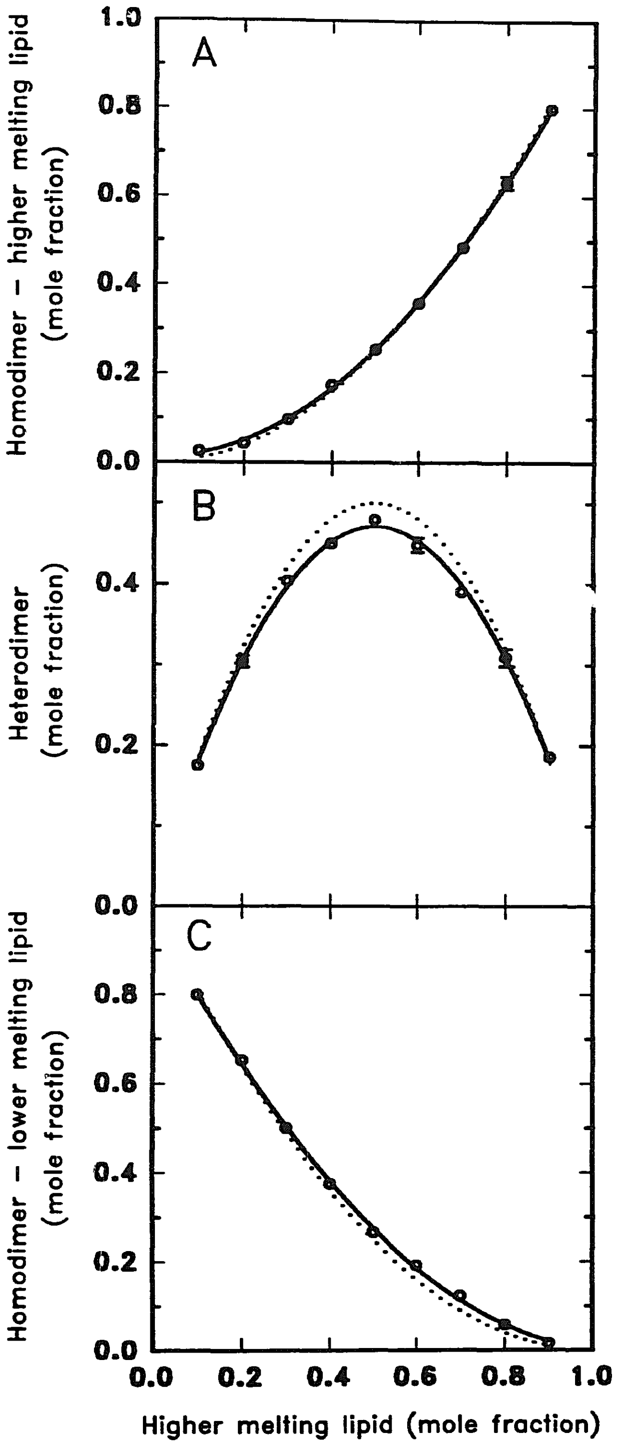

Fig. 3. Analysis of the fraction of each dimeric species formed from dimethylsuberimidate cross-linking of DPPE and DOPE mixtures at 68° C. Panel A represents the mole fraction of DPPE-DPPE dimers of the total dimers, panel B represents the mole fraction of DPPE-DOPE, and panel C represents the mole fraction of DOPE-DOPE. In each case the points represent the mean of triplicate determinations and the error bars represent standard deviations. If error bars are not seen, it is because they are smaller than the size of the points. The solid line is a calculated second-order regression curve through the experimental data. The dotted line represents the theoretical yield of each dimer if the PE species were randomly mixed.

heterodimer formation suggest a slight inhomogeneity in the fluid phase of this mixture. For example, in the equimolar mixture of DPPE and DOPE, the mol fraction of DPPE-DPPE cross-links is about 0.26; DPPE-DOPE cross-links, about 0.48; and DOPE-DOPE cross-links, about 0.26. (The theoretical values for random cross-linking would be mol fractions of 0.25, 0.50, 0.25, respectively.) One illustration of the degree of immiscibility which would produce the experimental data which we have obtained would be a equimolar mixture of Fluid A with a composition of 60 mol% DPPE, 40 mol% DOPE, randomly mixed, and Fluid B with a composition of 40 mol% DPPE, 60 mol% DOPE, randomly mixed. A second example of a mixture which would produce the experimental data which we have obtained upon cross-linking would be a mixture of 2 mol% Fluid A composed of pure DPPE, 2 mol% Fluid B composed of pure DOPE, and 96 mol% Fluid C composed of an equimolar mixture of DPPE and DOPE, randomly mixed.

Discussion

We have used a chemical method to examine the arrangement of lipid species in fluid, binary PE mixtures without altering the temperatures of the mixtures. Our results suggest little, if any, fluid-fluid immiscibility under our conditions. Each of the mixtures was examined 15 to 18 C° above the gel-to-fluid transition temperature of the higher melting lipid.

Our method is based on the ability of dimethylsuberimidate to cross-link neighboring PE molecules. Obviously, cross-linking is a two-step reaction, with the formation of a dimethylsuberimidate-PE compound, followed by reaction with a second PE molecule. Thus the validity of our results depends on the similarity of the interaction of a mono-reacted PE and its neighbors with the interaction of an unreacted PE and its neighbors. It seems it would require several coincidences for a difference in these interactions to lead to random cross-linking in a system with a nonrandom arrangement. For example, both monoreacted species would have to partition equally between the domains, regardless of their acyl chains. In addition, their rate of dimer formation would have to be very slow compared to their lateral mobility, so that their initial location would not be a factor in determining the cross-linked products. That neither monoreacted species would have a preference for a domain in a system where domain formation would have been dictated by acyl chains seems highly unlikely to us.

While we used different lipids, our results stand in sharp contrast to the interpretation put forth by Melchior [4] for his results using DSPC/DMPC mixtures. Melchior quickly froze DSPC/DMPC mixtures from 85°C, or about 27°C above the gel-to-liquid transition temperature of the higher-melting lipid (DSPC), and then subjected the mixtures to DSC. Melchior found that the quickly frozen samples exhibited two distinct endotherms, while conventionally cooled samples exhibited only a single endotherm at an intermediate temperature. He interpreted these results to suggest that, in the fluid phase, DSPC and DMPC are clustered into separate domains; the lipids are presumably crystallized so rapidly with the quick-freeze method that the lateral mobility of the lipids is too slow to allow co-crystallization. Perhaps the different lipids used in our study account for the different results obtained as compared to Melchior [4]. Because PCs are not amenable to dimethylsuberimidate cross-linking as they lack a free amino group, it would be interesting to investigate the behavior of the PEs which we have studied with the 'quick-freeze'/DSC method.

Does fluid-fluid immiscibility of lipids exist? Our data indicate that such inhomogeneity is not a general phenomenon. Perhaps, however, immisicibility in the fluid phase exists in systems like the one we studied at temperatures closer to the gel-to-fluid transition temperature. Fluid-fluid lipid inhomogeneities may also be more common in mixed head group lipid mixtures and, certainly, in mixtures of lipids and proteins.

Acknowledgments

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