Insight into the Fluid-Phase Miscibility of Ester and Ether Phospholipids through Analysis of Nearest-Neighbor Recognition

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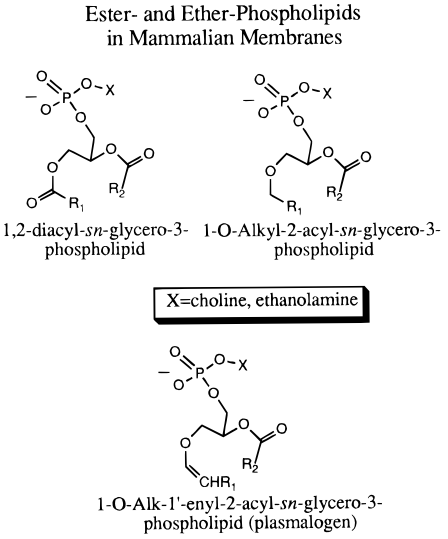
Abstract: The mixing behavior of three saturated ester phospholipids with one ether analog has been examined in the bilayer state by use of nearest-neighbor recognition methods [Vigmond, S. J.; Dewa, T.; Regen, S. L. *J. Am. Chem. Soc*. **1995**, *117*, 7838]. Specifically, the miscibility of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) with 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) has been investigated by analyzing mixtures of corresponding dithidipropionyl-based dimers that have been chemically equilibrated via thiolate−disulfide interchange reactions. In the fluid phase, those membranes derived from DMPE plus DHPE were found to exhibit lateral heterogeneity. In contrast, fluid membranes that were derived from DPPE plus DHPE, and also DSPE plus DHPE, were randomly arranged. Inclusion of cholesterol did not significantly alter the lateral distribution of any of these phospholipid mixtures in the fluid phase. Comparison with results obtained from previous nearest-neighbor recognition studies has revealed a synergistic effect between an ester/ether and chain length mismatch, which yields a nonrandom state. Lateral heterogeneity within bilayers derived from DMPE plus DHPE was also evident in the gel−fluid coexistence region from NNR and from high-sensitivity differential scanning calorimetry (*hs*-DSC) analyses; such heterogeneity, however, was eliminated by the presence of 40 mol % cholesterol and was significantly reduced in the presence of 50 mol % DPPC. Analogous gel−fluid membranes derived from DPPE plus DHPE, and DSPE plus DHPE, were found to favor a random arrangement in the gel−fluid phase. The biological significance of these findings are briefly discussed.

Introduction

Phospholipids and cholesterol represent the two major lipid components of mammalian membranes.[1] While most phospholipids are of the diacyl type (i.e., having two fatty acids covalently linked to the glycerol backbone), significant quantities of ether analogs are also present, i.e., 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phospholipids and 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phospholipids (plasmalogens) (Chart 1).[2] What role these ether lipids play with respect to membrane function, and how they are distributed, laterally, throughout the bilayer are two fundamental and important issues that remain to be clarified.[2] The fact that relatively high levels of ether lipids have been found in a variety of human brain tumors suggests that high local concentrations may be present; it also suggests that the clustering of such lipids may contribute to the malignant state.[3−6]

Most studies that have provided insight into the supramolecular properties of ether lipids have been based on simple model systems. In previous work, for example, it has been shown that dialkylphospholipids behave quite differently than diacylphospholipids in the gel phase. Specifically, whereas ester lipids such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) form the common bilayer structure, ether analogs such as 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC) form

Chart 1



chain-interdigitated "monolayers".[7−9] Thus, the replacement of two ester carbonyl groups by two methylene units can have a profound effect on the organizational properties of a phospholipid. How such structural differences affect the membrane properties of phopholipids in the *physiologically-relevant fluid phase*, however, remains to be established. In principle, the ester carbonyl could play an important role in controlling how ester lipids mix with ether lipids. It is also possible that the

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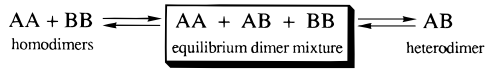
presence of cholesterol, which is known to have a strong condensing effect on the fluid phase, may significantly affect the mixing of ether and ester lipids.[2,10,11] Unfortunately, such issues have been extremely difficult to resolve even in the simplest of model systesm, due to the absence of experimental methods that can provide unambiguous structural information in the fluid phase.

Nearest-Neighbor Recognition Method. We have recently introduced a chemical approach for investigating phospholipid mixing.[12−14] We have termed this technique nearest-neighbor recognition (NNR) analysis since it measures the thermodynamic preference for one phospholipid molecule to become a covalently attached nearest-neighbor of an identical phospholipid in a mixed bilayer. One unique feature of this method is that *it can lead to definitive conclusions regarding two-dimensional structure in the fluid phase*. Moreover, the fact that it can detect lateral heterogeneity that results from free energy differences between random and nonrandom states as small as ca. 100 cal/mol makes it one of the most sensitive techniques that is presently available for investigating lateral organization. An additional virtue of the NNR method is that it can be applied to the study of complex, multicomponent systems. In principle, it should even be applicable to the study of reconstituted biological membranes.

In a typical NNR experiment, two phospholipid molecules of interest (A and B) are first converted into exchangeable homodimers and heterodimers (AA, BB, and AB). Subsequent formation of vesicular membranes, using an equimolar mixture of homodimers (and also pure heterodimer), followed by monomer interchange affords a chemically equilibrated product mixture (Scheme 1). When such mixtures are found to be statistical (i.e., when the molar ratio of AA/AB/BB is 1/2/1), this finding establishes that the phospholipid dimers as well as the monomer units are randomly distributed throughout the bilayer.[12] When there is a thermodynamic preference for homodimer formation, and when such "recognition" can be reduced or eliminated through the addition of a nonexchangeable phospholipid due to improved mixing, the existence of lateral heterogeneity is indicated.[14]

Scheme 1



NNR Applied to the Question of Ester/Ether Miscibility. In this paper, we report the results of a study that provide insight into the mixing behavior of ester- and ether-based phospholipids in the *fluid* phase. In essence, this work was aimed at addressing three specific questions: (I) Can the replacement of both ester carbonyl groups of a phospholipid with two methylene units provide a driving force for lateral heterogeneity? (II) Can an ester/ether mismatch combine, synergistically, with a chain-length mismatch to produce lateral heterogeneity? (III) Can the presence of cholesterol significantly affect the mixing behavior of ester- and ether-based phospholipids? Although these questions have less biological relevance in the gel−fluid coexistence region, the fact that direct comparisons can be made with calorimetry measurements (high-sensitivity differential scanning calorimetry, *hs*-DSC) makes such comparisons meaningful in terms of confirming that the NNR method is capable of detecting lateral heterogeneity in such systems.[15,16] For this reason, NNR analysis in the gel−fluid phase was also deemed worthy of investigation.

Experimental Section

General Methods. All of the general methods that have been used in this study were similar to those previously described.[12] Each of the ester phospholipid homodimers (**14-14**, **16-16**, and **18-18**) was prepared using published procedures;[12] the analogous ether homodimer (**DH-DH**) and corresponding heterodimers (**14-DH**, **16-DH**, and **18-DH**) were prepared by similar methods. In all cases, the thiol monomers that were used as synthetic intermediates were purified by preparative TLC prior to use. The ester-based phosphoethanolamines (Avanti Polar Lipids, Birmingham, AL), 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine, DHPE (Fluka, Ronkonkoma, NY), and *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP, Pierce, Rockford, IL) were used as obtained.

1,2-Dimyristoyl-1′,2′-bis(hexadecyl)-*N*,*N*′-(dithiodipropionyl)bis-[*sn*-glycero-3-phosphoethanolamine] (14-DH). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.86 (t, 12H), 1.10−1.40 (br m, 92 H), 1.45−1.60 (br m, 8H), 2.28 (virtual q, 4H), 2.61 (br t, 4 H), 2.99 (br t, 4H), 3.30−3.60 (br m, 11H), 3.90 (br m, 8H), 4.12 (d of d, 1H), 4.40 (d, 1H), 5.20 (m, 1H), 7.81 (br s, 2H). HRMS for $(C_{76}H_{148}O_{16}N_2P_2S_2Na_3)^+$: calcd, 1539.9439; found, 1539.9360.

1,2-Dipalmitoyl-1′,2′-(hexadecyl)-*N*,*N*′-(dithiodipropionyl)bis[*sn*-glycero-3-phosphoethanolamine] (16-DH). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.86 (t, 12H), 1.05−1.40 (br m, 100 H), 1.46−1.61 (br m, 8H), 2.29 (virtual q, 4H), 2.62 (br t, 4 H), 2.99 (br t, 4H), 3.30−3.60 (br m, 11H), 3.89 (br m, 8H), 4.12 (d of d, 1H), 4.39 (d, 1H), 5.20 (m, 1H), 7.81 (br s, 2H). HRMS for $(C_{80}H_{156}O_{16}N_2P_2S_2Na_3)^+$: calcd, 1596.0065; found, 1596.0061.

1,2-Distearoyl-1′,2′-bis(hexadecyl)-*N*,*N*′-(dithiodipropionyl)bis[*sn*-glycero-3-phosphoethanolamine] (18-DH). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.86 (t, 12H), 1.05−1.40 (br m, 108 H), 1.46−1.61 (br m, 8H), 2.29 (virtual q, 4 H), 2.61 (br t, 4H), 2.98 (br t, 4H), 3.30−3.62 (br m, 11H), 3.89 (br m, 8H), 4.13 (d of d, 1H), 4.39 (d, 1H), 5.20 (m, 1H), 7.80 (br s, 2H). HRMS for $(C_{84}H_{164}O_{16}N_2P_2S_2Na_3)^+$: calcd, 1652.0691; found, 1652.0620.

***N*,*N*′-(Dithiodipropionyl)bis[1,2-bis(hexadecyl)-*sn*-glycero-3-phosphoethanolamine] (DH-DH).** ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.87 (t, 12H), 1.10−1.32 (br m, 104 H), 1.51 (br m, 8H), 2.63 (t, 4H), 2.99 (t, 4 H), 3.30−3.65 (br m, 18H), 3.90 (br m, 8H), 7.90 (br s, 2H). HRMS for $(C_{80}H_{160}O_{14}N_2P_2S_2Na_3)^+$: calcd, 1568.0479; found, 1568.0529.

Preparation of Liposomes and Initiation of Thiolate−Disulfide Interchange Reaction.[17,18] In a typical preparation, 0.45 μmol of **14-14** and 0.45 μmol of **DH-DH** in chloroform were transferred to a test tube. The chloroform was then evaporated by passing a stream of argon over the solution, thereby leaving a thin film of the lipid mixture. Diisopropyl ether (0.40 mL) and chloroform (0.15 mL) were added and the resulting solution mixed by vortexing. Subsequent addition of 50 μL of a 3.3 mM borate buffer (47 mM NaCl and 0.7 mM NaN_3, pH 7.4) resulted in an emulsion, which was subjected to mild (bath-type) sonication, followed by the removal of the organic phase under reduced pressure at 60 °C; a white gel formed in the bottom of the test tube. After the gel was collapsed by vortex mixing for 3 min, 3.0 mL of a 10 mM borate buffer (140 mM NaCl and 2 mM NaN_3, pH 7.4) was added dropwise with vortex mixing. The vesicle dispersion was then degassed with an aspirator for 20 min, and the residual traces of organic solvent were removed by dialysis under an argon atmosphere using three 200 mL portions of degassed 10 mM borate buffer (pH 7.4) over the course of 18 h.

The thiolate−disulfide interchange reaction was initiated, after the sample had thermally equilibrated at the desired reaction temperature,

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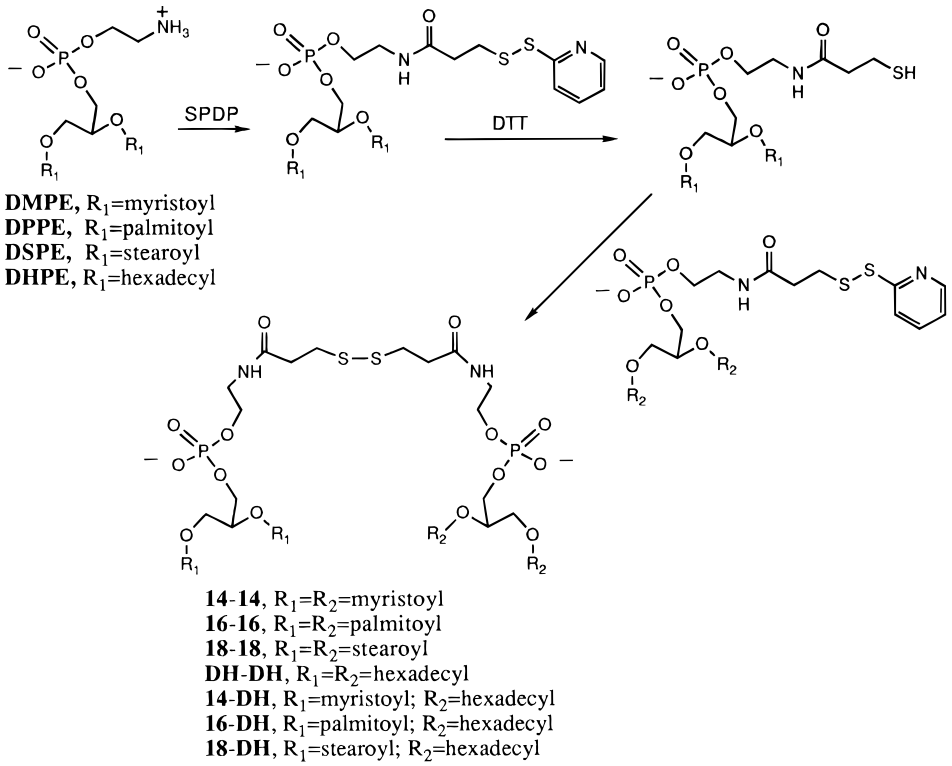
Scheme 2



by increasing the pH to 8.5 (addition of 35 μL of 0.15 M NaOH) followed by injection of 300 μL of an aqueous solution of 4.8 mM dithiothreitol (1.6 equiv relative to moles of lipid) and brief vortex mixing. All dispersions were maintained under an argon atmosphere throughout the course of the interchange reaction. Aliquots (0.3 mL) were removed at desired time intervals and quenched with 0.1 mL of 0.01 M HCl. After removal of water under reduced pressure, the resulting white salt was triturated with 2 mL of $CHCl_3$ and centrifuged, and the $CHCl_3$ was then removed under reduced pressure to yield a clear film. Samples were dissolved in 4 μL of chloroform plus 80 μL of mobile phase (HPLC) prior to injection.

Analysis of Dimer Distributions by High-Performance Liquid Chromatography. Mixtures of lipid dimers were analyzed by HPLC using a Beckman Ultrasphere C18 reverse-phase column (4.6 × 250 mm, 5 μm particle size). In general, the premixed mobile phase contained 77% 12 mM tetrabutylammonium acetate (TBA) in denatured ethanol, 13% water, and 10% hexane (v/v/v). For the analyis of **14-14**, **14-DH**, and **DH-DH** dimers, a 9 mM TBA solution was used when cholesterol was present. The flow rate was 0.9 mL/min, and the column was maintained at 31.2 °C. The UV detector was set at 205 nm. Data were collected and processed using a Maxima 820 workstation (Millipore Corp.).

Differential Scanning Calorimetry. All calorimetry measurements were performed using a Microcal MC-2 calorimeter with a DA-2 data acquisition and analysis software. Multilamellar vesicles were prepared by dispersing a thin lipid film (1 μmol) in 2.0 mL of 10 mM borate buffer (140 mM NaCl and 2 mM NaN_3, pH 7.4) and their melting behavior measured after four freeze−thaw (−196/60 °C) cycles, using the same buffer solution as a reference. Heating scans were recorded between 10 and 60 °C at a scan rate of 30 deg/h. Three DSC runs were performed for each sample; no difference was observed among the scans. A borate buffer baseline was also collected and subtracted from each thermogram.

Results

Ester and Ether Phospholipid Dimers. In this work, we have investigated the mixing behavior of three saturated ester phospholipids with one ether-based analog by use of NNR methods. Specifically, the miscibility of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) with 1,2-bis(hexadecyl)-*sn*-glycero-3-phosphoethanolamine (DHPE) has been investigated by analyzing mixtures of corresponding dithidipropionyl-based dimers, which have been chemically equilibrated via thiolate−disulfide interchange reactions.[12,19]

Synthetic procedures that were used to prepare the requisite dimers were similar to those previously described.[12] In brief, a given phosphoethanolamine (PE) was first derivatized with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Scheme 2). Subsequent deprotection with dithiothreitol (DTT) and coupling with either its precursor or a homolog afforded the desired homodimer or heterodimer, respectively. For purposes of convenience, we will use an abbreviated nomenclature throughout this paper. Thus, homodimers that are derived from DMPE, DPPE, and DSPE will be referred to as **14-14**, **16-16**, and **18-18**, respectively, where each number corresponds to the number of carbon atoms that are present in the saturated fatty acids of each lipid monomer. Since DHPE bears the same number of carbon atoms in its hydrocarbon chains as DPPE, we will distinguish its corresponding homodimer by referring to it as **DH-DH**. Similarly, the heterodimers will be designated as **14-DH**, **16-DH**, and **18-DH**.

An analysis of NNR within membranes composed of **16-16**/**16-DH**/**DH-DH** was intended to provide insight into the consequences of replacing ester carbonyl groups with methylene units (question I). Similar analysis of equilibrated bilayers composed of **14-14**/**14-DH**/**DH-DH** and **18-18**/**18-DH**/**DH-DH** was expected to address the issue of whether or not an ester/ether mismatch can combine, synergistically, with a chain length mismatch in producing lateral heterogeneity (question II). Finally, a comparison of NNR, in the absence and in the presence of cholesterol, was expected to reveal the effects of cholesterol on the miscibility of ester and ether phospholipids (question III).

Thermotropic Phase Behavior of the Lipids. The temperature at which a phospholipid bilayer is half-converted from a

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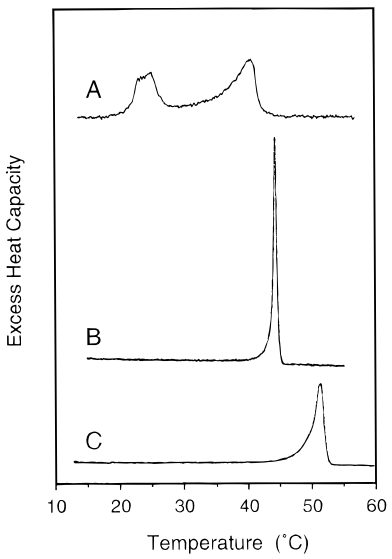

Figure 1. High-sensitivity excess heat capacity profiles of (A) **14-14**/**DH-DH**, 1/1, (B) **16-16-DH-DH**, 1/1, and (C) **18-18**/**DH-DH**, 1/1.

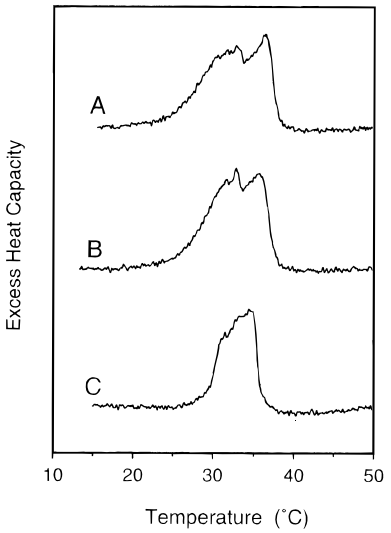

Figure 2. High-sensitivity excess heat capacity profiles of (A) **14-14**/**14-DH**/**DH-DH**, 1/1.59/1, (B) **14-14**/**14-DH**/**DH-DH**, 1/2/1, (C) DMPC/DHPC, 1/1. The *hs*-DSC thermograms that are shown were for unilamellar vesicles prepared by reverse evaporation methods; similar profiles were observed using multilamellar vesicles.

gel into a fluid phase is, by definition, its characteristic gel to liquid-crystalline phase-transition temperature (T_m). At temperatures below the onset of such melting, the hydrocarbon chains are in an ordered, all-*anti* configuration. When melting does occur gauche conformations are introduced into the hydrocarbon chains, and they become disordered and fluidlike in character.[15,16] In general, higher T_m values reflect stronger intermolecular forces between neighboring lipids. Examination of each of the dimers used in the present work by *hs*-DSC revealed sharp endotherms having widths at half-maximum specific heat capacity of $\leq$0.5 °C. The T_m values that were measured for the dimers were as follows: 22.7 °C (**14-14**), 41.9 °C (**16-16**), 46.2 °C (**DH-DH**), 55.4 °C (**18-18**), 32.3 °C (**14-DH**), 44.2 °C (**16-DH**), and 50.7 °C (**18-DH**). As noted previously, the **14-14**, **16-16**, and **18-18** dimers have melting temperatures that are very similar to those of analogous phosphocholines; i.e., 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, 24.0 °C), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, 41.5 °C), and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC, 54.3 °C).[12] The T_m value that has been measured for the ether phospholipid dimer **DH-DH** is higher than that of its ester analog, **16-16**. A similar trend was also apparent for the analogous phosphocholines; i.e., the T_m of 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC, 43.7 °C) is higher than that of DPPC.

In Figure 1 we show the *hs*-DSC thermograms that characterize the melting behavior of bilayers derived from 1/1 molar mixtures of **14-14**/**DH-DH**, **16-16**/**DH-DH**, and **18-18**/**DH-DH**. In contrast to the latter two cases, where only a single endotherm was present (i.e., at 44.5 and 51.5 °C, respectively), the melting of bilayers composed of **14-14**/**DH-DH** was characterized by two distinct endotherms, one having a T_m at 24.9 °C and the second having a T_m at 40.7 °C. When this membrane was diluted with 44 mol % of the corresponding heterodimer (**14-DH**), however, only a broadened endotherm was observed at an intermediate temperature, which was suggestive of two separate phase transitions (Figure 2A). This level of **14-DH** content corresponds to that which is produced when such bilayers are allowed to reach chemical equilibrium at 33 °C (*vide infra*). An analogous membrane that was composed of a 1/2/1 molar ratio of **14-14**/**14-DH**/**DH-DH** (i.e., dilution with 50 mol % of **14-DH**) resulted in a slightly narrower endotherm (Figure 2B). A similar broadened endotherm is also evident for a 1/1 mixture of DMPC/DHPC (Figure 2C). Thus, whereas the binary system composed of **14-14**/**DH-DH** (homodimers) tends to favor lateral-phase separation [gel-phase domains that are richer in the higher melting homodimer (**DH-DH**) and fluid-phase domains that are richer in the lower melting homodimer (**14-14**)], the inclusion of **14-DH** *counterbalances* the effects of dimerization by promoting mixing.[20] Exactly analogous results have previously been observed for bilayers composed of **14-14**/**14-16**/**16-16**.[12,21] It is also noteworthy that the high miscibility of **16-16** with **DH-DH**, and also **18-18** with **DH-DH**, is similar to the high miscibility that can be found for the corresponding phosphocholines; i.e., the peak widths at half-maximum excess specific heat capacity ($\Delta T_{1/2}$) that were observed for 1/1 mixtures of **16-16**/**DH-DH**, DPPC/DHPC, **18-18**/**DH-DH**, and DSPC/DHPC were 0.6, 0.3, 1.8, and 1.6 °C, respectively (Figures 1 and 3).

Incorporation of 40 mol % cholesterol, and also 50 mol % DPPC, resulted in enhanced mixing of **14-14** and **DH-DH** in the gel−fluid coexistence region (Figure 4); the miscibilizing effect of cholesterol, however, was found to be greater than that of DPPC. Incorporation of 40 mol % cholesterol in bilayers of **16-16**/**DH-DH** afforded a single endotherm, which was characterized by a depressed T_m (40.8 °C) and a larger $\Delta T_{1/2}$ (1.5 °C) (not shown). Similarly, introduction of 40 mol % in bilayers of **18-18**/**DH-DH** lowered the T_m of the endotherm to 48.9 °C and increased the $\Delta T_{1/2}$ to 2.9 °C.

Nearest-Neighbor Recognition in the Gel-Fluid Coexistence Region. Chemical equilibration of a gel−fluid bilayer (33 °C) of **14-14**/**14-DH**/**DH-DH** resulted in significant NNR; i.e., the hetero/homodimer ratio was equal to 1.59 $\pm$ 0.03 (Table 1). When 40 mol % cholesterol was included in the membrane, however, the dimer distribution was found to be statistical (1.98 $\pm$ 0.03). Similar incorporation of 50 mol % DPPC led to only a partial reduction in NNR; i.e, the hetero/homodimer ratio was 1.71 $\pm$ 0.05. Thus, the greater effectiveness of cholesterol in reducing NNR, relative to DPPC, can be correlated with its greater effectiveness in promoting mixing between **14-14** and

(20) The fact that the pure lipid dimers show neither a subtransition nor a pretransition, together with the similarity in the phase transition temperature of each homodimer with that of the corresponding endotherm in the **14-14**/**DH-DH** mixture (and also the similarity in enthalpy for the two endotherms of the mixture), argues strongly against the possibility that sub- or pretransitions are indicated in Figure 1A.

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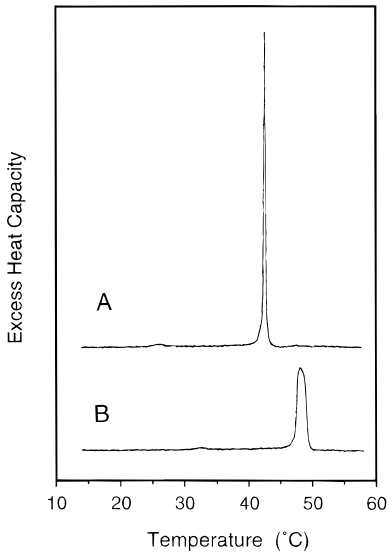

Figure 3. High-sensitivity excess heat capacity profiles of (A) DHPC/DPPC, 1/1, and (B) DHPC/DSPC, 1/1.

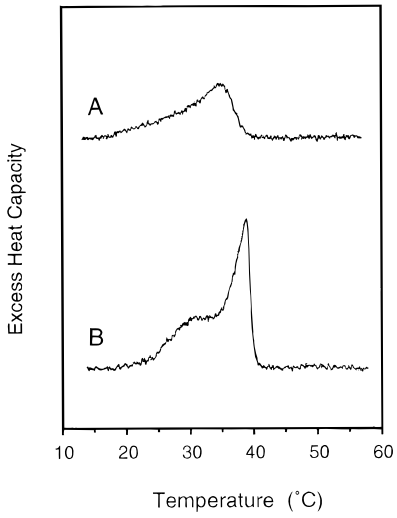

Figure 4. High-sensitivity excess heat capacity profiles of (A) **14-14/DH-DH**, 1/1, plus 40 mol % cholesterol, and (B) **14-14/DH-DH**, 1/1, plus 50 mol % DPPC.

DH-DH, as judged by *hs*-DSC. Similar gel−fluid membranes made from **16-16/16-DH/DH-DH** and **18-18/18-DH/DH-DH** were found to be statistical in dimer composition and were not affected by the presence of cholesterol.

Nearest-Neighbor Recognition in the Fluid Phase. When bilayers composed of **14-14/14-DH/DH-DH** were chemically equilibrated at a temperature that was in excess of the T_m of the highest melting dimer, **DH-DH** (i.e., a temperature at which the membrane was fully melted), NNR was evident; i.e., the observed heterodimer/homodimer ratio was 1.84 ± 0.04. In contrast to the gel−fluid phase, where cholesterol was more effective than DPPC in promoting lipid mixing, the situation was reversed in the fluid phase; i.e., inclusion of 40 mol % cholesterol had a negligible effect on NNR, but the presence of 50 mol % of DPPC completely eliminated NNR. For fluid bilayers composed of **16-16/16-DH/DH-DH**, and also **18-18/18-DH/DH-DH** (with and without cholesterol), only statistical product mixtures were observed.

Discussion

Miscibility in the Gel-Fluid Coexistence Region. The results of NNR measurements within gel−fluid bilayers of **16-16/16-DH/DH-DH** and **18-18/18-DH/DH-DH** (with and without cholesterol) are in complete agreement with those obtained by *hs*-DSC analyses; i.e., each of these lipid systems reveal a high degree of mixing. The presence of single endotherms for 1/1 mixtures of the homodimers clearly reflects a high misibility. The fact that the equilibrium dimer distributions are statistical further shows that monomeric components are randomly distributed throughout each monolayer leaflet. Thus, for each of these systems, the difference in intermolecular forces that result from a combination of chain length and ester/ether mismatch is insufficient to produce lateral heterogeneity. In the case of gel−fluid bilayers (33 °C) of **14-14/14-DH/DH-DH**, however, such a driving force exists. The observation of significant NNR, and the shape of the endotherm that characterizes a 1/1.59/1 mixture of **14-14/14-DH/DH-DH** (Figure 2A), clearly reflect a laterally heterogeneous membrane. The greater effectiveness of cholesterol in enhancing the mixing of gel−fluid bilayers of **14-14/DH-DH** (shown by *hs*-DSC) and **14-14/14-DH/DH-DH** (shown by NNR), relative to DPPC, is a likely consequence of the fluidizing effect that this sterol is known to have on the gel state *and* also its condensing effect on the fluid phase.[22]

Table 1. Nearest-Neighbor Recognition within Ester/Ether Phospholipid Membranes

equilibrating lipid dimers	cholesterol[a] (mol %)	temp (°C), phase[b]	heterodimer/homodimer[c]
14-14/14-DH/DH-DH	0	33, *G*−*F*	1.59 ± 0.03
	40	33, *G*−*F*	1.98 ± 0.03
	0	60, *F*	1.84 ± 0.04
	40	60, *F*	1.88 ± 0.02
14-14/14-DH/DH-DH/DPPC[d]	0	33, *G*−*F*	1.71 ± 0.05
	0	60, *F*	2.03 ± 0.02
1616/16-DH/DH-DH	0	44, *G*−*F*	2.00 ± 0.03
	40	39, *G*−*F*	2.00 ± 0.01
	0	60, *F*	1.98 ± 0.01
	40	60, *F*	1.98 ± 0.01
18-18/18-DH/DH-DH	0	50, *G*−*F*	2.01 ± 0.05
	40	47, *G*−*F*	2.00 ± 0.06
	0	60, *F*	2.03 ± 0.03
	40	60, *F*	1.95 ± 0.06

[a] The mol % of cholesterol is based on total lipid that is present, where each phospholipid "counts" as two lipid molecules. [b] Gel−fluid (*G*−*F*), fluid (*F*). Appropriate reaction temperatures that were needed were determined by *hs*-DSC analysis; see text. [c] Equilibrium ratio of heterodimer/homodimer ± two standard deviations from the mean. In all cases, homodimers were present in equal molar quantities at equilbrium. [d] 50 mol % DPPC.

Miscibility in the Fluid Phase. Analysis of NNR for each of the above bilayers in the fluid phase provides insight into the three questions that were posed in the Introduction. Specifically:

An ester/ether mismatch, by itself, is insufficient for creating lateral heterogeneity. Examination of the equilibrium dimer distribution for fluid membranes composed of **16-16/16-DH/DH-DH** clearly shows that the monomer units are randomly distributed throughout each monolayer leaflet. Thus, the replacement of two carbonyl moieties by two methylene units does not provide a driving force for producing lateral heterogeneity in the fluid phase.

An ester/ether mismatch can combine, synergistically, with a chain length mismatch in producing lateral heterogeneity. Results that have been obtained from NNR measurements for bilayers of **14-14/14-DH/DH-DH** (60 °C) and **18-18/18-DH/DH-DH** (60 °C) reveal an intriguing difference. Specifically, NNR is observed only in the former case. The fact that such recognition can be eliminated by the addition of DPPC (having an intermediate T_m) also provides compelling evidence for the presence of lateral heterogeneity.[14] Although the two exchang-

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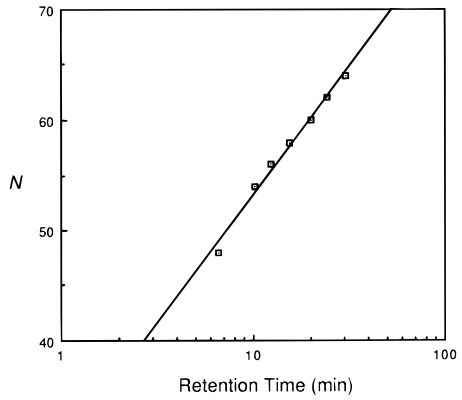

Figure 5. Plot of HPLC retention times of the phospholipids listed in Table 2 as a function of the total number of methylene groups (*N*) that are contained in each dimer.

Table 2. Retention Times of the Lipid Dimers Using Reverse Phase HPLC[a]

dimer	retention time (min)	dimer	retention time (min)
14-14	6.6	DH-DH	20.0
14-DH	10.2	18-DH	24.4
16-16	12.4	18-18	30.6
16-DH	15.4		

[a] Specific HPLC conditions are described in the Experimental Section.

ing monomer units in each of these systems differ by two carbons, the difference in the number of methylene groups is not the same; i.e., **14**, **DH**, and **18** monomers contain 12, 15, and 16 methylene groups, respectively. In essence, therefore, conversion of the ester carbonyl group into a methylene unit "moves" the hydrophobicity of the hydrocarbon chains of **DH** closer to that of the **18** monomer. Such a change in hydrophobicity is not only evident by the higher T_m that is observed for **DH-DH** compared with **16-16** but also by their relative retention times in the reverse phase chromatograms (Table 2); the more hydrophobic the dimer, the greater its retention time. In Figure 5, we show a plot of the retention times for all of the phospholipids that are listed in Table 2 as a function of the total number of methylene groups (*N*) that are present. The exponential increase in the retention time that is observed with increasing *N* is consistent with the assumption that each added methylene unit contributes equally to the ΔG of partitioning and that the retention times are directly proportional to the partitioning coefficients. Since the difference in van der Waals forces between identical pairs of monomer is a major contributor to NNR, the absence of NNR in membranes composed of **18-18**/**18-DH**/**DH-DH** can be accounted for in terms of a smaller difference in hydrophobicity between the exchanging monomers.

In order to place this NNR into perspective, it is instructive to compare it with previous results obtained with fluid bilayers of **14-14**/**14-18**/**18-18**. In the latter system, no evidence of NNR was observed; only a random mixture of dimers was found at 60 °C.[12] The fact that **14** and **DH** have a smaller difference in hydrophobicity than **14** and **18** (i.e., a three versus a four methylene unit difference, which is also reflected by a smaller difference in the retention times of the homodimers) indicates that the absence of carbonyl groups in **DH** plays an important role in inducing lateral heterogeneity in the **14-14**/**14-DH**/**DH-DH** system. Since an ester/ether mismatch, by itself, does not lead to lateral heterogeneity (*vide ante*), and since saturated diacylphospholipids that differ by four methylene groups distribute themselves randomly in the fluid phase, the ester/ether mismatch in bilayers of **14-14**/**14-DH**/**DH-DH** must be combining, synergistically, with the three-methylene unit difference to produce a heterogeneous state. Although we do not yet fully understand this synergistic effect, we suspect that hydration of the ester carbonyl groups may be an important factor. Previous monolayer studies of fluid-phase ester and ether phosphocholines have revealed differences in their packing behavior. Specifically, the area that was occupied by the ester lipids was found to be greater than that of the ether lipids.[23] This difference was attributed to hydrated water that increases the effective size of the ester carbonyl groups. On the basis of these previous findings, we hypothesize that the existence of lateral heterogeneity in bilayers composed of **14-14**/**14-DH**/**DH-DH** is the result of a combination of (i) differences in van der Waals forces between identical monomers and (ii) differences in packing efficiency between the ester and ether lipids.

The presence of cholesterol does not enhance lateral heterogeneity derived from an ester/ether mismatch. Previous studies with bilayers of **14-14**/**14-18**/**18-18** have shown that inclusion of 40% cholesterol significantly enhances NNR and lateral heterogeneity in the fluid state.[14] Here, it was presumed that cholesterol "moves" the liquid-crystalline phase closer toward a gel−fluid-like state (condensing effect) where increased compactness and stronger van der Waals forces create a driving force for NNR.[13,14] The inability of cholesterol to enhance NNR in the present systems implies that its condensing effect cannot be detected through either the ester/ether or the hydrophobic mismatches that exist. Apparently, a hydrophobic mismatch of at least four methylene unit is required in order for the condensing effect of cholesterol to result in NNR.

Biological Significance. Nearest-neighbor recognition experiments that have been carried out in the present work have shown that the *replacement of ester carbonyl groups by methylene units, in combination with a chain-length mismatch (or more precisely, hydrophobic mismatch), can lead to lateral heterogeneity in the physiologically-relevant fluid phase*. This finding, together with our recent demonstration that saturated and unsaturated phospholipids can also lead to lateral heterogeneity, suggests that other and more pronounced differences among phospholipids should contribute, significantly, to their overall two-dimensional structure.[24] In particular, it seems likely that differences in head group charge (e.g., negatively charged versus zwitterionic), the presence of hydrogen bonding elements within and beneath the head group region (e.g., phosphoethanolamines and sphingomyelins, respectively), should play an important role in defining the organizational state of a fluid membrane. It also seems likely that the presence of integral and peripheral proteins should make significant contributions to membrane suprastructure.[25−27] Efforts aimed at clarifying each of these variables through NNR analysis of simple model and reconstituted membranes are continuing in our laboratory.

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