Influence of Head Group Mismatch on the Miscibility of Phospholipids in the Physiologically-Relevant Fluid Phase: A Nearest-Neighbor Recognition Analysis

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Abstract: The mixing behavior of phosphoethanolamine (PE)-like and phosphoglycerol (PG)-like lipids have been examined in the fluid bilayer state by use of nearest-neighbor recognition methods [Vigmond, S. J.; Dewa, T.; Regen, S. L. *J. Am. Chem. Soc.* **1995**, *117*, 7838]. Disulfide-based phospholipid dimers derived from 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) have been synthesized, containing PE- and PG-like head groups. Subsequent exchange and equilibration of the monomer units, via thiolate−disulfide interchange, produced a nonstatistical distribution of dimers in the fluid bilayer phase (60 °C); i.e., a heterodimer/homodimer ratio of 1.76 ± 0.05 was formed. Analogous membranes that were rich in cholesterol (i.e., 29 mol %) produced a statistical mixture of dimers. Equilibration experiments that were carried out using PE-like and PG-like lipids derived from 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and DMPE, respectively, gave similar results, except that a greater level of nearest-neighbor recognition was evident in the absence of cholesterol. The biological implications of these findings are briefly discussed.

Introduction

Although a considerable body of information is currently available concerning the mixing properties of phospholipids in the gel−fluid coexistence region, very little is known about lipid miscibility in the *physiologically-relevant fluid phase*.[1−5] The primary difficulty has been the lack of experimental methods that are directly applicable to the fluid state.[2,3] Thus, whereas techniques such as differential scanning calorimetry, electron paramagnetic resonance spectroscopy, and freeze-fracture electron microscopy can detect "islands" of gel phase that are rich in a high melting lipid, which are surrounded by a "sea" of fluid phase that is rich in a lower melting component, these methods do not provide structural insight when the membrane has completely melted.[2,3]

We have recently introduced a chemical approach for analyzing phospholipid mixing that is based on the lipid's thermodynamic preference for forming homodimers in the bilayer state. We have termed such a preference “nearest-neighbor recognition” (NNR).[6−10] In a typical NNR experiment, two phospholipids (A and B) are converted into exchangeable, disulfide-linked homodimers AA and BB and heterodimer AB. Subsequent vesicle formation, using an equimolar mixture of the two homodimers, followed by monomer interchange, leads to an equilibrium mixture. In order to ensure that an equilibrium point has been reached, equilibration is also carried out using vesicles that have been prepared from pure heterodimer. When

$$\underset{\text{homodimers}}{AA + BB} \rightleftharpoons \boxed{\underset{\text{equilibrium dimer mixture}}{AA + AB + BB}} \rightleftharpoons \underset{\text{heterodimer}}{AB}$$

the resulting dimer composition is statistical (i.e., when the molar ratio of heterodimer to each homodimer is 2.0), and when transmembrane exchange of the phospholipids (“flip-flop”) is negligible, this finding establishes that (i) the monomeric components are randomly distributed throughout the membrane and (ii) there is no thermodynamic preference for one phospholipid to be a nearest-neighbor of another; i.e., there is complete mixing of the phospholipids. When homodimers are favored, however (i.e., when the molar ratio is less than 2.0), the dimers may be either *randomly or nonrandomly* arranged. In the former case, dimer stability is dominated by intramolecular interactions between monomer units. In the latter case, intramolecular and intermolecular interactions contribute, similarly, to dimer stability, and NNR reflects the presence of lateral heterogeneity. Experimentally, one can distinguish between these two situations by introducing a nonexchangeable diluent into the membrane, provided that it functions as a mixing agent for A and B and that it does not alter the phase properties of the bilayer. Specifically, a laterally heterogeneous state is indicated by the reduction or elimination of NNR.[8] In the limiting case, where the molar ratio is zero (absence of heterodimers), and where the introduction of a diluent promotes heterodimer formation, complete segregation of the phospholipids is indicated.

In this paper, we report the synthesis of disulfide-based phospholipid dimers that bear monomeric units, which resemble phosphoethanolamines (PEs). We also report the miscibility of such lipids with analogous molecules that mimic phosphoglycerols (PGs) by use of nearest-neighbor recognition analysis. The specific aim of this work is to clarify whether or not a mismatch of phospholipid head groups (i.e., those resembling PEs and PGs) can provide a driving force for the formation of

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lateral heterogeneity within the physiologically-relevant fluid phase, in the absence and in the presence of cholesterol.

Experimental Section

General Methods. All of the general methods that have been used in this study are similar to those previously described.[6] Unless stated otherwise, all reagents were obtained from commerical sources and used without further purification. All synthetic transformations were carried out under an argon atmosphere. Dimethyl-3,3'-dithiobis(propionimidate) dihydrochloride (DTBP; Pierce Chemical Co.) was extensively washed with dry diethyl ether and then dried (6 h, 23 °C, 0.1 mmHg) prior to use. Each of the PG-like homodimers was prepared using published procedures.[6] Thiol monomers that were used as synthetic intermediates were purified by preparative TLC prior to use. All phosphoethanolamines (Avanti Polar Lipids, Birmingham, AL) were used as obtained. All ^{1}H NMR spectra were recorded on a Bruker 360 MHz instrument; chemical shifts are reported in parts per million and were referenced to residual solvents. Experimental procedures that were used for recording surface pressure−area isotherms are similar to those previously described.[11] Monolayers were spread using $CHCl_3$ as a solvent and lipid concentrations of 1 mg/mL; $CHCl_3$, which was used as a solvent for DPPG, contained ca. 1% CH_3OH. Dynamic light scattering measurements were carried out by use of a Nicomp 270 submicrometer particle size analyzer.

***N,N′*-[Dithiobis(1-iminopropyl)]bis[1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine] ($PE^{14}PE^{14}$)**. To a solution that was composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (200 mg, 0.315 mmol), triethylamine (0.053 mL, 0.378 mmol), and $CHCl_3$ (8.0 mL) was added, dropwise, a second solution that was made from dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride (43.2 mg, 0.137 mmol), triethylamine (0.042 mL, 0.30 mmol), and $CHCl_3$ (4.0 mL). The total addition time was ca. 3 min. After the reaction mixture was stirred for 13 h at room temperature, the solvent was removed under reduced pressure. Subsequent column chromatography using $CHCl_3/CH_3OH/H_2O$ (13/6/1, v/v/v) as an eluting solvent afforded 162 mg of crude product, which was further purified by reversed phase preparative TLC (Aldrich C-18, $C_6H_{14}/CH_3CH_2OH/CH_3CO_2H/H_2O$ (50/30/1/1), and then regular phase preparative TLC (silica, $CHCl_3/CH_3OH/H_2O$, 13/6/1), to give 64 mg (32%) of $PE^{14}PE^{14}$ as a colorless solid: R_f 0.64; ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.85 (t, J = 6.6 Hz, 12 H), 1.23 (br s, 80 H), 1.56 (br s, 8 H), 2.26 (q, J = 7.1 Hz, 8 H), 2.94 (br s, 4 H), 3.12 (br s, 4 H), 3.53 (br s, 4 H), 3.8−4.2 (m, 10 H), 4.36 (d, J = 9.8 Hz, 2 H), 5.20 (br s, 2 H); HRMS for $(C_{72}H_{141}O_{16}N_4P_2S_2)^+$, calcd 1443.9259, found 1443.9220.

***N,N′*-[Dithiobis(1-iminopropyl)]bis[1,2-palmitoyl-*sn*-glycero-3-phosphoethanolamine] ($PE^{16}PE^{16}$)**. Using procedures similar to those that were used for the synthesis of $PE^{14}PE^{14}$, a 32% yield of $PE^{16}PE^{16}$ was obtained: R_f 0.62 (silica, $CHCl_3/CH_3OH/H_2O$, 13/6/1); ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.85 (t, J = 6.5 Hz, 12 H), 1.22 (br s, 96 H), 1.55 (br s, 8 H), 2.25 (q, J = 7.8 Hz, 8 H), 2.96 (br s, 4 H), 3.12 (br s, 4 H), 3.51 (br s, 4 H), 3.8−4.2 (m, 10 H), 4.36 (d, J = 10.4 Hz, 2 H), 5.19 (br s, 2 H); HRMS for $(C_{80}H_{157}O_{16}N_4P_2S_2)^+$, calcd 1556.0511, found 1556.0585.

1,2-Dimyristoyl-1′,2′-dipalmitoyl-*N,N′*-(Dithiopropionyl,1-iminopropyl)bis[*sn*-glycero-3-phosphoethanolamine] ($PG^{14}PE^{16}$). Dithiothreitol (55 mg, 0.36 mmol) was dissolved in 1.5 mL of $CHCl_3$ and the resulting solution purged with argon, and then cooled to 0 °C. To this solution was added, dropwise, a solution that was made from 18.5 mg (0.012 mmol) of $PE^{16}PE^{16}$ in 2.0 mL of $CHCl_3$. The reaction mixture was then stirred for 1 h at 0 °C under an argon atmosphere, and then concentrated under reduced pressure. Purification of the residual oil by column chromatography [silica, $CHCl_3/CH_3OH$ (10/1, v/v) and $CHCl_3/CH_3OH/H_2O$ (13/6/1, v/v/v)] and then preparative TLC [silica, $CHCl_3/CH_3OH/H_2O$ (13/6/1, v/v/v)] afforded 12.6 mg (67%) of the corresponding thiol monomer ($PE^{16}SH$) having R_f 0.65 (Ellman positive). The monomer was then dissolved in a mixture of 3 mL of $CHCl_3/CH_3OH$ (1/2, v/v) and added to a solution of 106.9 mg (0.48 mmol) of 2,2′-dithiodipyridine in 2.0 mL of CH_3OH at room temperature under an argon atmosphere, over the course of 1.5 h. The mixture was then stirred for 12 h and the solvent then removed under reduced pressure. Subsequent column chromatography [silica, $CHCl_3/CH_3OH$ (10/1, v/v) and $CHCl_3/CH_3OH/H_2O$ (13/6/1, v/v/v)] and then preparative thin layer chromatography [silica, $CHCl_3/CH_3OH/H_2O$ (13/6/1, v/v/v)] afforded 11.4 mg (80%) of $PE^{16}SSPyr$: R_f 0.79; ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.85 (t, J = 6.6 Hz, 6 H), 1.22 (s, 48 H), 1.55 (br s, 4 H), 2.25 (q, J = 6.7 Hz, 4 H), 2.91 (br s, 2 H), 3.16 (br s, 2 H), 3.74 (br s, 2 H), 3.97−4.17 (m, 5 H), 4.37 (m, 1 H), 5.20 (m, 1 H), 7.16 (t, J = 6.1 Hz, 1 H), 7.41 (d, J = 8.3 Hz, 1 H), 7.60 (t, J = 7.6 Hz, 1 H), 8.44 (d, J = 3.4 Hz, 1 H), 9.72 (m, 1 H), 10.23 (m, 1 H), 10.78 (m, 1 H); HRMS for $(C_{46}H_{86}O_8N_2P_1S_2)^+$, calcd 889.5563, found 889.5587. To a solution of 14.0 mg (0.016 mmol) of $PE^{16}SSPyr$ in 2.5 mL of $CH_3OH/CHCl_3$ (4/1, v/v) was added a solution of 9.5 mg (0.023 mmol) of $PG^{14}SH$ (prepared using procedures similar to those previously described)[6] in 2.4 mL of $CH_3OH/CHCl_3$ (1/1, v/v) over the course of 2 h. The reaction mixture was stirred for 12 h at room temperature under an argon atmosphere. The solvent was then removed under reduced pressure, and the crude product was purified by preparative TLC [silica, $CHCl_3/CH_3OH/H_2O$ (13/6/1, v/v/v)] to give an 84% yield of $PG^{14}PE^{16}$: R_f 0.44 (silica, $CHCl_3/CH_3OH/H_2O$, 13/6/1); ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.85 (t, J = 6.6 Hz, 12 H), 1.23 (br s, 88 H), 1.56 (br s, 8 H), 2.27 (q, J = 7.1 Hz, 8 H), 2.61 (br s, 2 H), 2.94 (br s, 2 H), 3.01 (br s, 2 H), 3.09 (br s, 2 H), 3.42 (br s, 2 H), 3.59 (br s, 2 H), 3.8−4.2 (m, 10 H), 4.38 (d, J = 9.7 Hz, 2 H), 5.20 (br s, 2 H), 7.5−11.0 (br m, 2 H); HRMS for $(C_{76}H_{145}O_{17}N_3P_2S_2Na_3)^+$, calcd 1566.9183, found 1566.9179.

***N,N′*-(Dithiopropionyl,1-iminopropyl)bis[1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine] ($PG^{14}PE^{14}$)**. Reaction of $PE^{14}SSpyr$ (prepared using procedures similar to those described above for the preparation of $PE^{16}SSPyr$) with $PG^{14}SH$, using procedures similar to those described for the preparation of $PG^{14}PE^{16}$, afforded a 75% yield of $PG^{14}PE^{14}$: R_f 0.47 (silica, $CHCl_3/CH_3OH/H_2O$, 13/6/1); ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.85 (t, J = 6.6 Hz, 12 H), 1.23 (br s, 80 H), 1.56 (br s, 8 H), 2.27 (q, J = 7.1 Hz, 8 H), 2.62 (br s, 2 H), 2.92 (br s, 2 H), 3.02 (br s, 2 H), 3.10 (br s, 2 H), 3.4 (br s, 2 H), 3.59 (br s, 2 H), 3.8−4.2 (m, 10 H), 4.37 (d, J = 10.2 Hz, 2 H), 5.20 (br s, 2 H), 7.7−10.8 (br m, 3 H); HRMS for $(C_{72}H_{137}O_{17}N_4P_2S_2Na_3)^+$, calcd 1510.8558, found 1510.8554.

Preparation of Liposomes and Initiation of the Thiolate−Disulfide Interchange Reaction.[9,12,13] In a typical preparation, 0.45 μmol of $PG^{14}PG^{14}$ and 0.45 μmol of $PE^{14}PE^{14}$ 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 was 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 5.0) 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 5.0) 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 (Spectra/Por Membrane, MWCO 6000−8000) under an argon atmosphere using three 200 mL portions of degassed 10 mM borate buffer (pH 5.0) over the course of 18 h. Typically, the mean diameter of the vesicles formed by such procedures was 300 ± 70 nm, as measured by dynamic light scattering.

The thiolate−disulfide interchange reaction was initiated, after the sample had thermally equilibrated at the desired reaction temperature, by injection of 225 μL of an aqueous solution of 4.8 mM dithiothreitol (1.2 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 40 μL of 0.01 M HCl (final pH 4.0). After removal of water under reduced pressure, the resulting white salt was triturated with 2 mL of $CHCl_3$ and centrifuged and the

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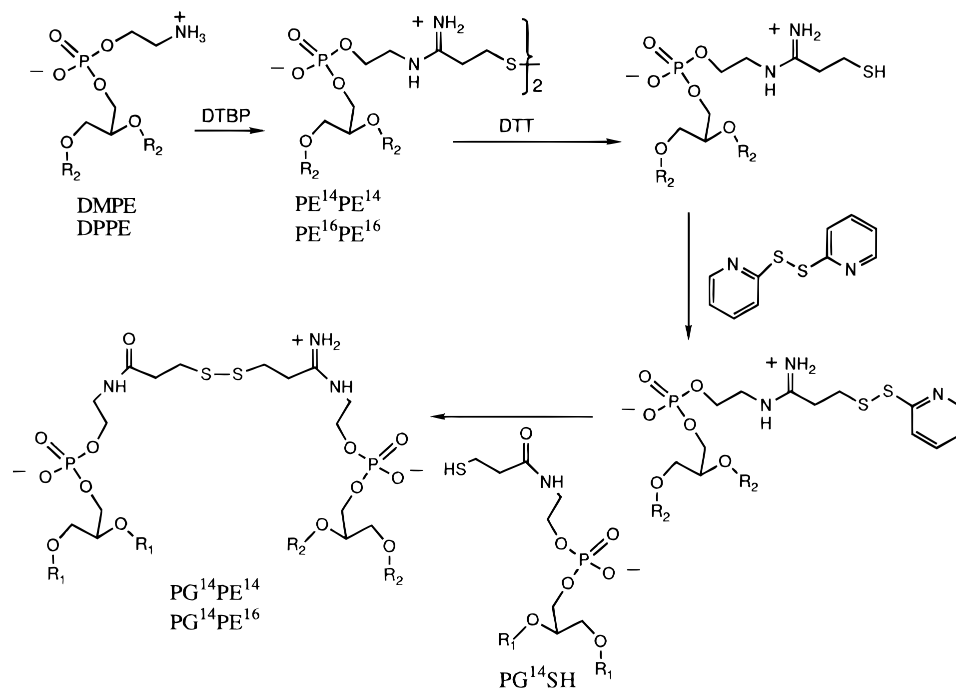
Scheme 1



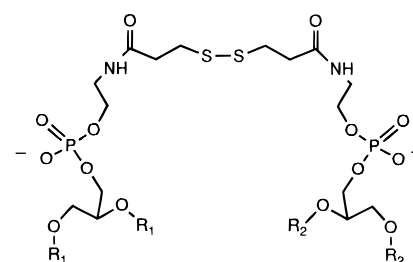
$CHCl_3$ then removed under reduced pressure to yield a clear film. Samples were dissolved in 5 μL of chloroform plus 95 μL of mobile phase (HPLC) prior to injection.

Analysis of Dimer Distributions by High-Performance Liquid Chromatography. Mixtures of lipid dimers were analyzed by HPLC using a Beckman Ultrasphere C18 reversed phase column (4.6 × 250 mm, 5 μm particle size). In general, the premixed mobile phase contained 78% 20 mM tetrabutylammonium acetate (TBA) in denatured ethanol, 11% water, and 11% hexane (v/v/v). 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.). Because of the different molar absorptivities of the PG-like and PE-like monomer units, calibration curves were used prior to each analysis, using known concentrations of each of the three lipid dimers.

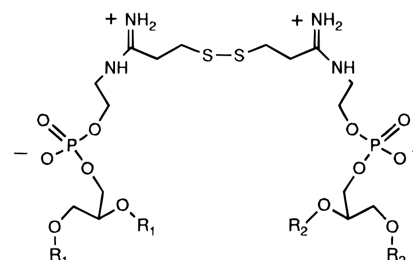
Differential Scanning Calorimetry. All calorimetry measurements were performed using a Microcal MC-2 calorimeter with DA-2 data acquisition and analysis software. Multilamellar vesicles were prepared by dispersing a thin lipid film (1 μmol) in 2.0 mL of 10 mM borate buffer (140 mM NaCl and 2 mM NaN_3, pH 7.4), and their melting behavior was 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. In the case of the PE-like homodimers, vesicle membranes were formed by reverse evaporation methods and a small percentage (10%) of DPPG was included in order to maintain a stable dispersion.

Results

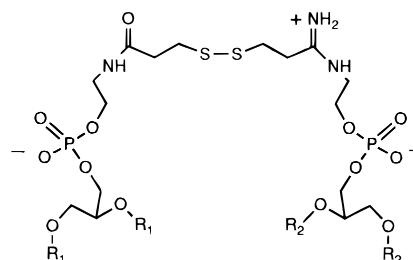
Phospholipid Dimers. Specific phospholipids that were chosen for this investigation were derived from 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE). For convenience, we will refer to PG-like monomers containing myristic and palmitic acids as PG^{14} and PG^{16}, respectively; PE-like analogs are similarly designated as PE^{14} and PE^{16}. In order to probe the mixing behavior of these lipids, four new dimers have been synthesized: $PE^{14}PE^{14}$, $PE^{16}PE^{16}$, $PG^{14}PE^{14}$, and $PG^{14}PE^{16}$. Homodimers $PG^{14}PG^{14}$ and $PG^{16}PG^{16}$ have been described elsewhere.[6]



$PG^{14}PG^{14}$, R_1=R_2=myristoyl
$PG^{16}PG^{16}$, R_1=R_2=palmitoyl



$PE^{14}PE^{14}$, R_1=R_2=myristoyl
$PE^{16}PE^{16}$, R_1=R_2=palmitoyl



$PG^{14}PE^{14}$, R_1=R_2=myristoyl
$PG^{14}PE^{16}$, R_1=myristoyl; R_2=palmitoyl

Synthetic procedures that were used to prepare the requisite dimers are similar to those previously described.[6] In brief, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) and

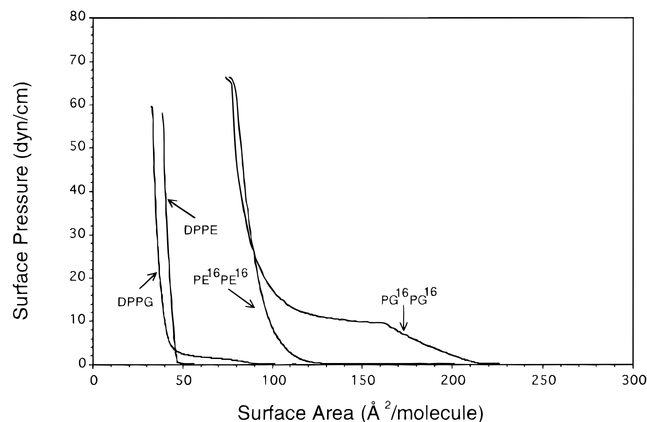


Figure 1. Surface pressure−area isotherms for DPPG, DPPE, $PE^{16}PE^{16}$, and $PG^{16}PG^{16}$ over a pure water subphase at 25 °C.

1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) were first dimerized with dimethyl 3,3′-dithio-bis(propionimidate) dihydrochloride (DTBP) to give PE homodimers (Scheme 1). Subsequent reduction with dithiothreitol (DTT), activation with 2, 2′-dithiodipyridine, and condensation with the thiol form of PG^{14} or PG^{16} afforded the corresponding heterodimers.

Melting Behavior. Examination of $PE^{14}PE^{14}$ and $PE^{16}PE^{16}$ by high sensitivity differential scanning calorimetry (hs-DSC) revealed gel to liquid-crystalline phase transition temperatures (T_m) of 35.2 and 51.3 °C, respectively; heterodimers $PG^{14}PE^{14}$ and $PG^{14}PE^{16}$ exhibited T_m values of 19.4 and 28.1 °C, respectively. It is noteworthy that the PE-like homodimers have T_m values that are significantly higher than those of the PG-like homodimers (the T_m values for $PG^{14}PG^{14}$ and $PG^{16}PG^{16}$ are 22.7 and 41.9 °C, respectively),[6] a feature that is exactly analogous to what has been found for conventional phospholipids (e.g., T_m values for DPPE and DPPG are 65.0 and 41.5 °C, respectively). The higher melting temperature of phosphoethanolamines is presumed to reflect stronger intermolecular interactions that result from hydrogen bonding in the head group region.[12]

Monolayer Properties. Surface pressure−area isotherms that were recorded for $PG^{16}PG^{16}$, $PE^{16}PE^{16}$, DPPG, and DPPE over a pure water subphase are shown in Figure 1. Qualitatively, $PE^{16}PE^{16}$ and $PG^{16}PG^{16}$ resemble DPPE and DPPG, respectively, in terms of their compressibility; the broad phase transition that is associated with $PG^{16}PG^{16}$ and DPPG highlights this distinction. On the basis of their melting properties, head group structures, and monolayer characteristics, $PE^{16}PE^{16}$ and $PG^{16}PG^{16}$ may be viewed as good models for DPPE and DPPG, respectively.

Nearest-Neighbor Recognition Analysis. When fluid bilayers composed of dimers, bearing PG^{14} and PE^{14} monomer units, were chemically equilibrated via thiolate−disulfide interchange, NNR was clearly evident. Thus, the observed heterodimer/PG homodimer ratio was 1.76 ± 0.05 at 60 °C (Table 1). Introduction of a two-methylene unit mismatch between the exchanging monomers (i.e., PG^{14} and PE^{16}) resulted in a significantly higher level of NNR. Thus, the heterodimer/PG homodimer ratio that was observed for membranes composed of PG^{14} and PE^{16} units decreased to 1.53 ± 0.04. In sharp contrast, analogous experiments that were carried out in the presence of 29 mol % cholesterol resulted in a dimer distribution that was statistical (Table 1).

In an effort to determine whether or not the observed NNR reflects the existence of lateral heterogeneity, dilution experiments were performed using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) as a nonexchangeable diluent. Previous

Table 1. Nearest-Neighbor Recognition within Mixed Phospholipid Membranes

lipid components	heterodimer/PG homodimer[a]
PG^{14} + PE^{14}	1.76 ± 0.05
PG^{14} + PE^{16}	1.53 ± 0.04
PG^{14} + PE^{14} + 29 mol % cholesterol[b]	2.00 ±0.06
PG^{14} + PE^{16} + 29 mol % cholesterol[b]	2.00 ± 0.09
PG^{14} + PE^{16} + 50 mol % DPPC[b]	1.41 ± 0.06

[a] Molar ratio of heterodimer to PG homodimer ± 2 standard deviations from the mean. In all cases, equilibrium was reached within ca. 4 h at 60 °C. [b] The mole percent of membrane diluent used is based on total lipid that is present, where each phospholipid dimer is counted as two lipid molecules.

studies have shown that conventional phospholipids, having melting temperatures that lie between those of exchangeable homodimers, can effectively eliminate NNR in the fluid phase.[9,10] For this reason, we chose DPPC (T_m = 41 °C) as a diluent for bilayers derived from PG^{14} and PE^{16} monomers (the T_m values for $PG^{14}PG^{14}$ and $PE^{16}PE^{16}$ are 22.7 and 51.2 °C, respectively). In brief, replacement of 50% of the exchangable phospholipids with DPPC did not enhance the mixing of the monomers; i.e., the heterodimer/homodimer ratio was 1.41 ± 0.06.

Discussion

The fact that the equilibrium dimer distribution for cholesterol-rich bilayers derived from PG^{14}/PE^{14}, and also from PG^{14}/PE^{16}, is statistical provides compelling evidence that the phospholipids are randomly distributed throughout each membrane in the physiologically-relevant fluid phase. In the case of the corresponding cholesterol-free bilayers, where significant NNR has been observed, such recognition may reflect (i) lateral heterogeneity within each monolayer leaflet (i.e., phospholipid segregation), (ii) inherent differences in dimer stability that result from differences in steric constraints, and/or (iii) transmembrane asymmetry, where the inner and outer monolayer leaflets of the bilayer differ in phospholipid composition. Although we cannot rigorously prove the presence of lateral heterogeneity in these systems, our results point strongly in that direction. Previous studies have shown that transmembrane asymmetry can exist in small unilamellar vesicles that are highly curved.[14] For the large unilamellar vesicles that have been used in the present study, however (typical diameters were 3000 ± 700 Å), curvature effects on monolayer composition would not be expected. In addition, the fact that the head group volume for those PE- and PG-like dimers is nearly identical (the only difference is the replacement of a carbonyl by an imine group in each monomer) means that there should be a negligible driving force for transmembrane asymmetry, since the intrinsic curvature of each phospholipid is similar.

More importantly, those vesicles that have been constructed from pure heterodimer must contain an identical molar quantity of PE- and PG-like monomer units in each monolayer leaflet at the start of the exchange reaction. The fact that the half-life for transmembrane exchange of phospholipids is known to be extremely slow (on the order of weeks and months), together with the fact that these NNR experiments require only 4 h to reach equilibrium, provides a compelling argument that transmembrane asymmetry does not contribute to the observed NNR.[15,16] Examination of CPK molecular models further indicates that there is considerable flexibility in the bridging

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(15) For previous studies that have established the slowness of transbilayer exchange, see: (i) Ganong, B. R.; Bell, R. M. *Biochemistry* **1984**, *23*, 4977. (ii) Roseman, M.; Litman, B. J.; Thompson, T. E. *Biochemistry* **1975**, *14*, 4826.

unit that connects two neighboring phospholipids. For this reason, and also based on our previous finding that bilayers composed of dimers derived from PG^{14}/PG^{16}, and also PG^{14}/PG^{18}, are randomly distributed in the fluid phase, it seems highly unlikely that steric contraints contribute, significantly, to the observed NNR.[6] The fact that DPPC does not reduce the level of NNR within equilibrated bilayers derived from PG^{14} plus PE^{16} implies that DPPC is distributed, heterogeneously, throughout these membranes.[8,9]

The apparent difference in the lateral distribution of these phospholipids in the cholesterol-rich versus the cholesterol-free state is noteworthy. Previous ^{2}H NMR studies have shown that the introduction of cholesterol into fluid phospholipid bilayers results in an increase in chain order.[17] In spite of this increase, however, dielectric relaxation and ^{2}H NMR measurements suggest that increases in cholesterol content are accompanied by *increases in motional freedom and looser packing in the head group region*.[18,19] Thus, the apparent loss of lateral heterogeneity upon the introduction of cholesterol can be accounted for in terms of a looser head group region in which the head groups of nearest neighbors are less "recognizable"; i.e., the head groups make a smaller contribution to the overall interaction between nearest neighbors.

(16) In preliminary studies, we have found that the treatment of vesicles prepared from a 1/1 molar mixture of $PG^{14}PG^{14}/PE^{14}PE^{14}$ with phospholipase A_2 for 2 h at 45 °C resulted in ca. 50% digestion of $PG^{14}PG^{14}$ (i.e., the appearance of a plateau in the kinetics of digestion). Dynamic light scattering measurements also showed no significant change in the particle size distribution after digestion. This result is fully consistent with the presence of transmembrane symmetry (TLC, phosphorous analysis). Exposure of $PE^{14}PE^{14}$ to this enzyme, however, appears to result in lipid decomposition beyond that of lysophospholipid formation.

(17) Vist, M. R.; Davis, J. H. *Biochemistry* **1990**, *29*, 451.

(18) Henze, R. *Chem. Phys. Lipids* **1980**, *27*, 165.

(19) Brown, M. F.; Seelig, J. *Biochemistry* **1978**, *17*, 381.

Conclusions

Nearest-neighbor recognition experiments that are reported herein have provided compelling evidence that the head group mismatch between PE- and PG-like phospholipids is not sufficient to induce lateral heterogeneity within cholesterol-rich bilayers in the physiologically-relevant fluid phase. In contrast, results that have been obtained in the absence of cholesterol strongly suggest that such a mismatch can lead to lipid segregation. The fact that the membranes of mammalian cells are rich in cholesterol and those of bacteria are sterol-free, together with the present findings, suggests that head group structure and composition may be more important in defining the two-dimensional organization of bacterial membranes.

Nearest-neighbor recognition methods provide a simple yet powerful approach for probing the lateral organization of phospholipid bilayers in the physiologically-relevant fluid phase. In this regard, the availability of zwitterionic PE-like dimers plus anionic PG-like analogs opens up new avenues for exploration, e.g., defining the influence of electrostatic interactions between negatively charged phospholipids and positively charged peptides on the lateral arrangement of the lipids. Our efforts in this area are continuing.[20]

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(20) A previous report has described a theoretical model for the lateral distribution of two-component lipid bilayers, having heterogeneities that are similar in magnitude to those which appear to be associated with these PE-/PG-like membranes (Freire, E.; Snyder, B. *Biochemistry* **1980**, *19*, 88). Although this model can generate a physical picture of the two-dimensional state, it is empirical in character. We are presently attempting to develop a simpler and nonempirical alternate model. Results of these efforts will be reported in due course.