# Lateral Heterogeneity in Fluid Bilayers Composed of Saturated and Unsaturated Phospholipids

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**Abstract:** Evidence has been obtained for the existence of lateral heterogeneity in fluid bilayers composed of mixtures of saturated and unsaturated phospholipids by use of nearest-neighbor recognition (**NNR**) methods [Vigmond, S. J.; Dewa, T.; Regen, S. L. *J. Am. Chem. Soc*., **1995**, *117*, 7838]. Disulfide-based dimers were synthesized from a homologous series of saturated phosphoethanolamines (PEs) and analogous PEs bearing one or two double bonds and were chemically equilibrated in the bilayer state via thiolate−disulfide interchange reactions. The magnitude of **NNR** that was observed in these systems (i.e., the thermodynamic preference for homodimer formation) was found to correlate with the difference between the gel-to-liquid crystalline phase transition temperatures of the homodimers that were present; the larger the difference, the greater the recognition. Inclusion of 40 mol % of cholesterol had very little influence on the extent of **NNR**. Dilution of two membranes that showed the largest extent of recognition, with a nonexchangable phospholipid (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), resulted in a significant reduction in **NNR**. These results provide compelling evidence for the existence of lateral heterogeneity in the absence of this diluent. The implications of these findings, with respect to the two-dimensional structure of biological membranes, are briefly discussed.

## Introduction

One of the most important issues that remains to be resolved concerning the structure of biological membranes is their two-dimensional organization.[1] In particular, the time-averaged lateral distribution of the lipids and proteins from which they are derived remains to be defined.[2−4] Due to the inherent complexity of biological membranes, most fundamental studies that have focused on bilayer structure have been based on simple model systems. While considerable progress has been made in clarifying the mixing behavior of phospholipids in the gel-fluid coexistence region, their miscibility in the physiologically-relevant fluid phase has been particularly difficult to assess. Previous estimates, for example, of the miscibility of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in the fluid phase range from complete miscibility to complete immiscibility.[5,6]

We have recently introduced a chemical approach for investigating phospholipid mixing.[7,8] In contrast to all other methods that have been developed for investigating lateral organization within lipid bilayers, our technique allows definitive conclusions to be reached in the fluid phase. Moreover, it is one of the most sensitive techniques that is presently available; i.e., it can detect lateral heterogeneity that results from free energy differences between random and nonrandom states as small as ca. 100 cal/mol. In essence, this method involves the chemical equilibration of disulfide-linked phospholipid dimers and analysis of the resulting dimer distribution.[7,8] Specifically, vesicles are first formed from a 1/1 mixture of homodimers (AA and BB) and are then allowed to undergo monomer exchange via thiolate−disulfide interchange. In order to ensure that equilibrium has been reached, a similar exchange reaction is carried out using vesicles that are made from the corresponding heterodimer, AB (Scheme 1). Recovery of the lipids and analysis by HPLC yields the relative stability of the dimers in the bilayer state.

When an equilibrium mixture is found to be statistical (i.e., when the molar ratio of AA/AB/BB is 1/2/1), this finding establishes that the monomer as well as the dimer components are randomly distributed throughout the bilayer.[7] When homodimer formation is thermodynamically favored, the ratio of heterodimer to either of the homodimers is less than 2.0. We refer to this situation as "nearest-neighbor recognition", **NNR**. In such a case, lateral heterogeneity may or may not be present. If the relative stability of the dimers is controlled by both intermolecular and intramolecular forces, and if these forces are of similar magnitude, then **NNR** reflects the presence of lateral heterogeneity. If intramolecular forces dominate, however, then **NNR** reflects a membrane that is composed of a nonstatistical mixture of dimers that are randomly distributed throughout the bilayer. Recently, we have shown that one can distinguish between these two possibilities by carrying out appropriate dilution experiments.[9] In particular, inclusion of a nonexchangable phospholipid, which is capable of promoting mixing between the homodimers and thereby reducing lateral heterogeneity, reduces or eliminates **NNR**.[9]

To date, all of our **NNR** studies have been carried out using saturated phospholipid dimers. Specific homodimers that have been employed (**I**, **II**, and **III**) bear a close resemblance toward

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(6) Melchoir, D. L. *Science* **1986**, *234*, 1577; **1987**, *238*, 550.

(7) Krisovitch, S. M.; Regen, S. L. *J. Am. Chem. Soc*. **1992**, *114*, 9828.

(8) Krisovitch, S. M.; Regen, S. L. *J. Am. Chem. Soc*. **1993**, *115*, 10104.

(9) Vigmond, S. J.; Dewa, T.; Regen, S. L. *J. Am. Chem. Soc*. **1995**, *117*, 7838.

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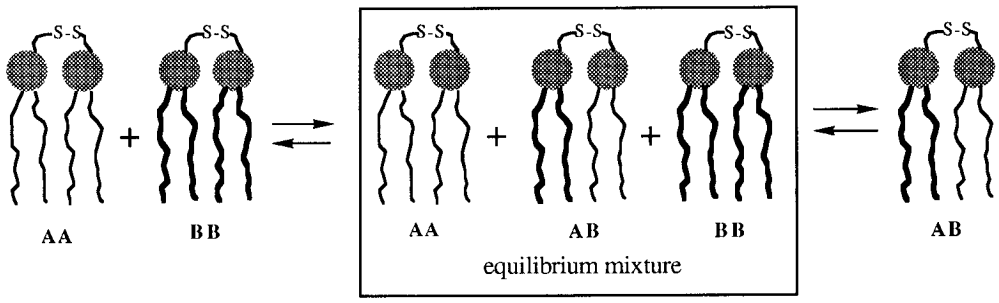
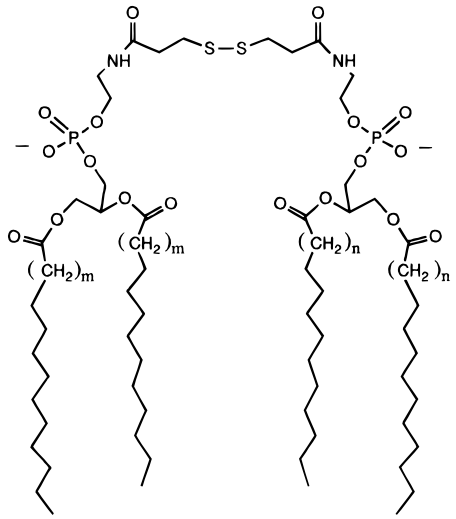
**Scheme 1**




DMPC, DPPC, and DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine) in terms of their acyl chain length and their melting behavior.[7] Since most naturally occurring phospholipids contain one or more double bonds in their acyl chains, the question of whether or not such unsaturation can induce lateral heterogeneity among mixtures of saturated and unsaturated lipids has biological relevance. Although recent studies have shown that phosphoethanolamines that are found in certain strains of bacteria contain *trans* as well as *cis* double bonds, it is the *cis* configuration that has the greatest biological importance; e.g., only *cis* double bonds are present in the lipids that are found in mammalian cell membranes.[10] The primary objective of the work that is described herein was to investigate the mixing behavior of saturated lipids with unsaturated analogs bearing *cis*-double bonds in the fluid phase by use of NNR methods.



**I**, m=3; n=3 (**14-14**)
**II**, m=5; n=5 (**16-16**)
**III**, m=7; n=7 (**18-18**)

## Experimental Section

**General Methods**. All of the general methods that have been used in this study were similar to those previously described.[7] Each of the saturated phospholipid homodimers was prepared using published procedures; the analogous unsaturated homodimers and heterodimers were prepared by similar methods. In all cases, the thiol monomers that were used as synthetic intermediates (and also as initiators for dimer exchange) were purified by preparative TLC prior to use. The phosphoethanolamines (Avanti Polar Lipids, Birmingham, AL) and heterobifunctional coupling agent, *N*-succinimidyl-3-(2-pyridyldithio)-propionate, SPDP (Pierce, Rockford, IL), were used as obtained. The melting temperature of multilayers of **DO-DO** was measured in pure buffer (pH 7.4) using a Perkin Elmer 1020-DSC7 differential scanning calorimeter; multilayers of **PO-PO** were hydrated using a 1/1 (v/v) mixture of buffer and ethylene glycol in order to avoid melting contributions from water.

**1,2-Dimyristoyl-1′-palmitoyl-2′-oleoyl-*N*,*N*′-(dithiodipropionyl)-bis[*sn*-glycero-3-phosphoethanolamine] (14-PO)**. $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$ 0.87 (t, 12H), 1.19−1.42 (br m, 88H), 1.55 (br m, 8H), 1.92−2.20 (m, 4H), 2.29 (virtual q, 8H), 2.62 (t, 4H), 2.99 (t, 4H), 3.48 (br m, 4H), 3.92 (br m, 8H), 4.12 (d of d, 2H), 4.38 (d, 2H), 5.21 (m, 2H), 5.32 (m, 2H), 7.61 (br s, 2H). HRMS for $(C_{78}H_{146}O_{18}N_2P_2S_2Na_3)^+$ calcd: 1593.9180; found: 1593.9148.

**1,2-Distearoyl-1′-palmitoyl-2′-oleoyl-*N*,*N*′-(dithiodipropionyl)bis-[*sn*-glycero-3-phosphoethanolamine] (18-PO)**. $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$ 0.86 (t, 12H), 1.12−1.38 (br m, 100H), 1.56 (br m, 8H), 1.99 (m,4H), 2.28 (virtual q, 8H), 2.62 (t, 4H), 2.99 (t, 4H), 3.43 (br m, 4H), 3.88 (br m, 8H,), 4.13 (d of d, 2H), 4.38 (d, 2H), 5.19 (m, 2H), 5.32 (m, 2H), 7.72 (br s, 2H). HRMS for $(C_{86}H_{162}O_{18}N_2P_2S_2Na_3)^+$ calcd: 1706.0432; found: 1706.0436.

**1,2-Dimyristoyl-1′,2′-dioleyl-*N*,*N*′-(dithiodipropionyl)bis[*sn*-glycero-3-phosphoethanolamine] (14-DO)**. $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$ 0.88 (t, 12H), 1.18−1.38 (br m, 80H), 1.56 (br m, 8H), 1.92−2.06 (m,8H), 2.29 (virtual q, 8H), 2.62 (t, 4H), 2.98 (t, 4H), 3.44 (br m, 4H), 3.89 (br m, 8H), 4.13 (d of d, 2H), 4.38 (d, 2H), 5.19 (m, 2H), 5.31 (m, 4H), 7.70 (br s, 2H). HRMS for $(C_{80}H_{150}O_{18}N_2P_2S_2Na_3)^+$ calcd: 1621.9493; found: 1621.9591.

**1,2-Dipalmitoyl-1′,2′-dioleyl-*N*,*N*′-(dithiodipropionyl)bis[*sn*-glycero-3-phosphoethanolamine] (16-DO)**. $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$ 0.88 (t, 12H), 1.20−1.42 (br m, 88H), 1.57 (br m, 8H), 1.94−2.06 (m,8H), 2.29 (virtual q, 8H), 2.63 (t, 4H), 2.99 (t, 4H), 3.45 (br m, 4H), 3.89 (br m, 8H), 4.14 (d of d, 2H), 4.40 (d, 2H), 5.21 (m, 2H), 5.33 (m, 4H), 7.66 (br s, 2H). HRMS for $(C_{88}H_{156}O_{182}P_2S_2Na_3)^+$ calcd: 1675.9963; found: 1675.9959.

**1,2-Distearoyl-1′,2′-dioleyl-*N*,*N*′-(dithiodipropionyl)bis[*sn*-glycero-3-phosphoethanolamine] (18-DO)**. $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$ 0.86 (t, 12H), 1.10−1.40 (br m, 96H), 1.55 (br m, 8H), 1.99 (m,8H), 2.29 (virtual q, 8H), 2.61 (t, 4H), 2.99 (t, 4H), 3.42 (br m, 4H), 3.89 (br m, 8H), 4.13 (d of d, 2H), 4.39 (d, 2H), 5.19 (m, 2H), 5.31 (m, 4H,), 7.71 (br s, 2H). HRMS for $(C_{88}H_{164}O_{18}N_2P_2S_2Na_3)^+$ calcd: 1732.0589; found: 1732.0587.

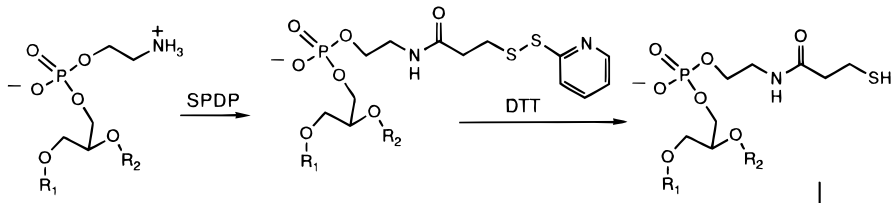
***N*,*N*′-(Dithiodipropionyl)bis[1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine] (PO-PO)**. $^1$H NMR ($CDCl_3$, 500 mHz) $\delta$ 0.88 (t, 12H), 1.20−1.42 (br m, 88H), 1.58 (br m, 8H), 1.93−2.10 (m,8H), 2.30 (virtual q, 8H), 2.63 (t, 4H), 3.00 (t, 4H), 3.46 (br m, 4H), 3.90 (br m, 8H), 4.13 (d of d, 2H), 4.38 (d, 2H), 5.20 (m, 2H), 5.34 (m, 4H), 7.67 (br s, 2H). HRMS for $(C_{84}H_{156}O_{18}N_2P_2S_2Na_3)^+$ calcd: 1675.9963; found: 1675.9991.

***N*,*N*′-(Dithiodipropionyl)bis[1,2-oleoyl-*sn*-glycero-3-phosphoethanolamine] (DO-DO)**. $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$ 0.88 (t, 12H), 1.20−1.42 (br m, 80H), 1.58 (br m, 8H), 1.95−2.06 (m,16H), 2.30 (virtual q, 8H), 2.63 (t, 4H), 3.00 (t, 4H), 3.46 (br m, 4H), 3.91 (br m, 8H), 4.15 (d of d, 2H), 4.39 (d, 2H), 5.21 (m, 2H), 5.34 (m, 8H), 7.66 (br s, 2H). HRMS for $(C_{88}H_{160}O_{18}N_2P_2S_2Na_3)^+$ calcd: 1728.0276; found: 1728.0271.

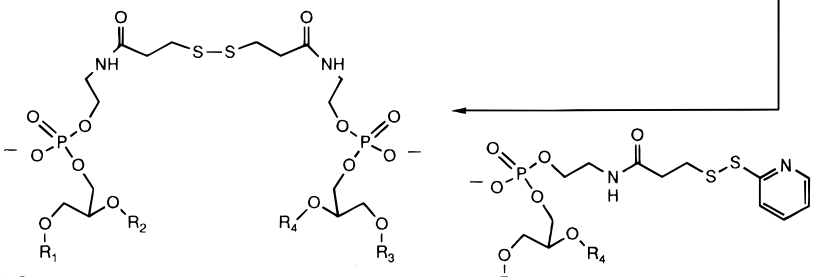
**Preparation of Liposomes and Initiation of Thiolate−Disulfide Interchange Reaction.**[11,12] In a typical preparation, 0.36 $\mu$mol of **14**−**14**, 0.36 $\mu$mol of **PO-PO**, 0.18 $\mu$mol of **14-thiol**, and 0.18 $\mu$mol of **PO-thiol** in chloroform were transferred to a test tube. The chloroform 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

(10) Okuyama, H.; Okajima, N.; Sasaki, S.; Higashi, S.; Murata, N. *Biochim. Biophys. Acta* **1991**, *1084*, 13.

**Scheme 2**




**DMPE,** $R_1$=$R_2$=myristoyl
**DPPE,** $R_1$=$R_2$=palmitoyl
**DSPE,** $R_1$=$R_2$=stearoyl
**POPE,** $R_1$=palmitoyl; $R_2$=oleoyl
**DOPE,** $R_1$=$R_2$=oleoyl



**14-PO**, $R_1$=$R_2$=myristoyl; $R_3$= palmitoyl; $R_4$=oleoyl
**18-PO**, $R_1$=$R_2$=stearoyl; $R_3$= palmitoyl; $R_4$=oleoyl
**14-DO**, $R_1$=$R_2$=myristoyl; $R_3$=$R_4$=oleoyl
**16-DO**, $R_1$=$R_2$=palmitoyl; $R_3$=$R_4$=oleoyl
**18-DO**, $R_1$=$R_2$=stearoyl; $R_3$=$R_4$=oleoyl
**PO-PO**, $R_1$=palmitoyl; $R_2$=oleoyl; $R_3$=palmitoyl; $R_4$=oleoyl
**DO-DO**, $R_1$=$R_2$=$R_3$=$R_4$=oleoyl

was mixed by vortexing. Subsequent addition of 50 $\mu$L 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 5 min, 3.0 mL of a 10 mM borate buffer (140 mM NaCl and 2 mM $NaN_3$, pH 5.0) were 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 150 mL portions of degassed 10 mM borate buffer (pH 5.0) over the course of 18 h.

The thiolate−disulfide interchange reaction was initiated, after the sample had thermally equilibrated at the desired reaction temperature, by increasing the pH to 8.5 with the addition of 50 $\mu$L of 0.15 M NaOH. 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 $CHCl_3$, centrifuged, and the $CHCl_3$ was then removed under reduced pressure to yield a clear film. Samples were dissolved in 5 $\mu$L of chloroform plus 100 $\mu$L 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 $\mu$m particle size). The premixed mobile phase contained 77% 9 mM tetrabutylammonium acetate in denatured ethanol, 13% water, and 10% hexane (v/v/v). The flow rate was 0.9 mL/min, and the column was maintained at 31.2 °C. The retention time for lipids were 5.8 min for **14-14,** 11.1 min for **16-16**, 25.0 min for **18-18**, 11.5 min for **PO-PO**, 13.0 min for **DO-DO,** 7.5 min for **14-PO**, 14.7 min for **18-PO**, 7.6 min for **14-DO**, 11.9 min for **16-DO**, and 15.7 min for **18-DO**. The retention time of cholesterol was 7.0 min. For the **16/DO** system, the separation of the lipid mixture was not complete; in this case, each lipid component was calibrated using a premixed standard. Detection was made by UV (205 nm), and data were collected and processed with a Maxima 820 workstation (Waters).

**Analysis of Double Bond Position After the Dimer Interchange Reaction**. A vesicular dispersion of **PO-PO** dimer, which included 20 mol % **PO-thiol**, was subjected to the standard exchange reaction conditions for 6 h at 60 °C. After the normal workup procedure described above, the sample was dissolved in 1.5 mL of $CH_2Cl_2$ and cooled in a dry ice−acetone bath. Ozone, generated from a Welsbach T-23 Ozonator, was passed through the solution for approximately 10 min, until a perceptible blue color appeared. At that point, the solution was purged with nitrogen for 15 min. Dimethyl sulfide (15 $\mu$L) was then added, and the cold bath was removed after 15 min. The product mixture was stirred overnight at room temperature. After the solvent was evaporated by bubbling argon through the solution, the product mixture was dissolved in 0.4 mL of isopropyl alcohol. An insoluble part was removed by centrifugation, and the solution was directly analyzed by GLC/MS using a Hewlett Packard 5890A gas chromatograph equipped with a 5% phenylmethyl silicone column (Hewlett Packard) and an HP5970 mass selective detector.

## Results and Discussion

**Selection of Phospholipids**. The specific phospholipid dimers that were chosen for this investigation were derivatives of commercially available, saturated and unsaturated phosphoethanolamines; i.e., 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE), 1-palmityoyl-2-oleoyl-*sn*-glycero-3 phosphoethanolamine (POPE), and 1,2-dioleoyl-*sn*-glycero-3 phosphoethanolamine (DOPE). Combinations of the saturated PEs with the monounsaturated PE (POPE), and also with the diunsaturated PE (DOPE), allow one to probe the influence of one versus two double bonds on **NNR** as well as the influence of acyl chain length.

Specific synthetic procedures that were used to prepare unsaturated lipid dimers were similar to those previously reported for the preparation of the saturated analogs.[7] In brief, a given PE was first derivatized with the heterobifunctional coupling agent, *N*-succinimidyl-3-(2-pyridyldithio)propionate, SPDP (Scheme 2). Subsequent deprotection with dithiothreitol (DTT) afforded the corresponding thiol monomer, which was

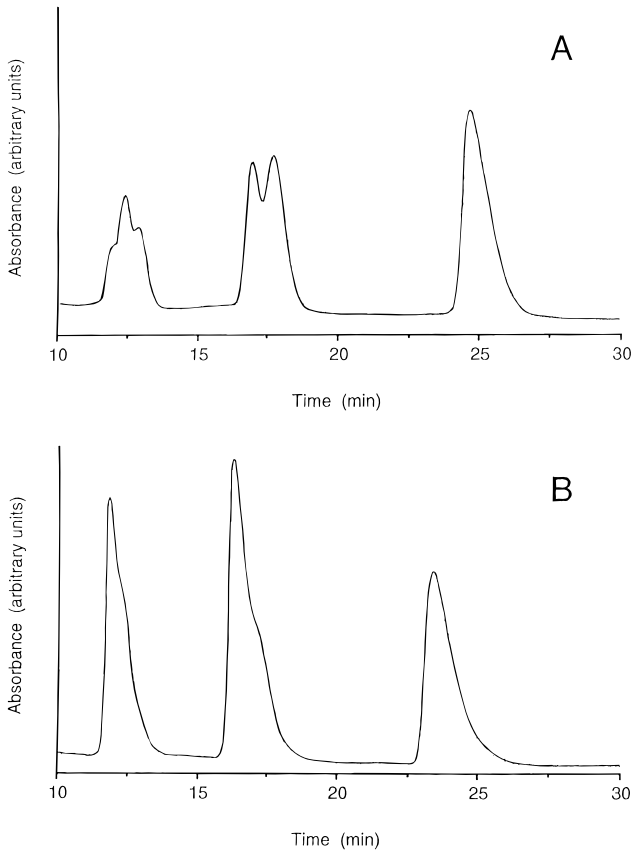



**Figure 1.** HPLC traces of product mixtures formed from **18-18**/**PO-PO** vesicles after 30 min at 60 °C: (A) initiation by use of 1.6 equiv of DTT and (B) initiation by use of 20 mol % **18-thiol**/**PO-thiol** (1/1, mol/mol).

then coupled either with its precursor or with an analogous protected thiol to give the requisite homodimer or heterodimer, respectively. For purposes of convenience, and also in order to draw attention to the number of methylene units that are present in the saturated phospholipids, an abbreviated nomenclature will be used throughout this paper. Thus, homodimers **I**, **II**, and **III** will be referred to as **14-14**, **16-16**, and **18-18**, respectively. Unsaturated homodimers derived from POPE and DOPE will be referred to as **PO-PO** and **DO-DO**, respectively; the corresponding heterodimers are designated as **14-PO**, **18-PO**, **14-DO**, **16-DO**, and **18-DO**.

**Equilibration Experiments**: **Unexpected Cis/Trans Isomerization**. By use of procedures that are described in the Experimental Section, phospholipid dimers were chemcially equilibrated in the bilayer state and then analyzed by HPLC. In all cases, final ratios of heterodimer/homodimer were obtained by averaging the results from vesicles that were initially composed of pure heterodimer with those that were made from a 1/1 molar mixture of the corresponding homodimers.

Initial studies that were carried out with **18-PO** and with **18-18**/**PO-PO**, using DTT as a reducing agent/initiator, gave unexpected results.[7] Specifically, HPLC analysis showed product mixtures with apparent "splitting" of the **18-PO** ("doublet") and the **PO-PO** ("triplet") peaks (Figure 1A). In sharp contrast, the **18-18** component remained as a "singlet". These results strongly suggested to us that the double bond had undergone isomerization; i.e., the doublet could be accounted for by the presence of *cis* and *trans* isomers, and the triplet would represent *cis*/*cis*, *cis*/*trans*, and *trans*/*trans* forms.[13] Control experiments that were carried out in which the DTT-initiation step was omitted yielded single-component starting material (not shown). When vesicles were prepared from pure **PO-PO** and then subjected to the chemical exchange conditions (DTT plus an increase in pH to 8.5), recovery of the dimer and analysis by $^1$H NMR spectroscopy confirmed that *cis*/*trans* isomerization had taken place. Analysis of the product mixture by HPLC showed the expected "triplet". On the basis of the known ability of thiol radicals to isomerize isolated double bonds, we postulate that a small percentage of the thiolate anion of DTT and/or that of the thiol-bearing phospholipid monomer (formed from partial reduction of the dimers) is converted to thiol radical intermediates.[14]

Since the thiol moiety in the head group of each phospholipid monomer is expected to have minimal contact with the hydrocarbon interior of the bilayer, we hypothesized that the use of the monomer, itself, as the initiatior would reduce the extent of isomerization. Consistent with this thinking, we found that inclusion of 20 mol % of a 1/1 mixture of thiol monomers (**18-thiol**/**PO-thiol**) resulted in chemical equilibration of a **18**−**18**/**PO-PO** vesicle membrane with significantly less *cis*/*trans* isomerization (Figure 1B). Analysis of product mixtures at the very early stages of monomer interchange further revealed that most (ca. 90%) of the *cis*/*trans* isomerization had occurred during vesicle formation. The additional isomerization that was observed during monomer exchange is presumed to result from the known ability of the head group to enter the hydrocarbon region of the bilayer via a "flip-flop" mechanism.[15] In order to determine whether or not double bond migration accompanies this *cis*/*trans* isomerization, a sample of **PO-PO** that had been isomerized to ca. 80/20, cis/trans ratio ($^1$H NMR) was subjected to ozonolysis and analysis by GLC/MS. Comparison of the aldehyde product with authentic samples of heptanal, nonanal, and undecanal showed that no migration of the double bond had occurred; i.e., only nonanal was observed. Since we were unable to completely eliminate double bond isomerization under optimal exchange conditions, all of the **NNR** data that are reported in this work include minor contributions (ca. 20%) from *trans* isomers.

**Nearest-Neighbor Recognition**. All **NNR** experiments were carried out at temperatures that are in excess of the gel-to-liquid crystalline phase transition temperature ($T_m$) of the highest melting dimer in each system. Specific $T_m$ values for **14-14**, **16-16**, and **18-18** are 22.7, 41.9, and 54.3 °C, respectively.[7] As expected, the introduction of double bonds significantly lowered the $T_m$ for both unsaturated homodimers **PO-PO** (−7 °C) and **DO-DO** (−22 °C).

All of the equilibrium ratios of heterodimer/homodimer that have been determined for the various combinations of saturated and unsaturated phospholipids are listed in Table 1. In contrast to the **14**/**PO**-based bilayer, which showed only a slight indication of **NNR** (i.e., the heterodimer/homodimer ratio was 1.90 ± 0.07) an analogous membrane that contained a longer saturated phospholipid (i.e., **18**/**PO**-based bilayers) showed significant recognition (dimer ratio of 1.78 ± 0.07). This level of recognition corresponds to a free energy difference of ca. 150 cal/mol, in favor of the nonstatistical distribution.[7] Similar chain length effects were found in doubly unsaturated, **DO**-based membranes; i.e, on going from **14**/**DO** to **16**/**DO** to **18**/

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(12) Liposomes: A Practical Approach; New, R. R. C., Ed.; Oxford University Press: New York, 1989; p 72.

(13) Similar experiments that were carried out with **18-DO** systems showed a "quartet" for the **DO-DO** and a "triplet" for the **18-DO** components.

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**DO**-based systems, a continuous increase in **NNR** was observed from 1.97 ± 0.04 to 1.69 ± 0.05. Inclusion of 40 mol % of cholesterol in all of the systems had very little influence on the extent of **NNR**. This is in sharp contrast to **NNR** measurements that were previously made using only saturated lipid dimers, where cholesterol effects were significant; i.e., for the **14-14**/**18-18** system (60 °C), the presence of 40 mol % cholesterol converted a random mixture of dimers into one in which the heterodimer/homodimer ratio was equal to 1.55.[7] While cholesterol did show a modest influence on **NNR** for the **14-14**/**DO-DO** membrane at 40 °C, its influence at 60 °C was almost negligible. If the effect of cholesterol on **NNR** is a consequence of "moving the bilayer toward a more gel-like state", then the presence of unsaturated dimers having very low melting temperatures would appear to help maintain a more "liquid-like" state.[7]

One general trend that emerges from these results is that *the magnitude of NNR increases as the difference between the $T_m$ of the homodimers increases*. Thus, for the **DO**-bearing membranes, the **18/DO**-based bilayers have the greatest difference in $T_m$ and also the greatest nearest-neighbor recognition. In the case of the **16/DO**-based membranes, the difference in $T_m$ between the homodimers is less, as is the extent of **NNR**; for the **14/DO**- bilayers, which have the smallest difference in $T_m$, no significant recognition could be detected. The same trend is also evident for the **PO**-bearing membranes; i.e., the degree of **NNR** that is observed for **18/PO**-bilayers is greater than that found for the **14**/**PO-**membrane.

Greater differences in $T_m$ between homodimers reflect greater differences in intermolecular forces. If such forces are primarily responsible for the relative stabilities of the dimers, then it is likely that the formation of the higher melting homodimer is what drives **NNR** in the fluid phase, similar to what has been found in the gel-fluid coexistence region.[7] The fact that **NNR** decreases as the chain length difference between the exchanging monomer units increases on going from **18**/**DO** to **16**/**DO** to **14**/**DO** further indicates that the influence of chain length mismatch on **NNR** is relatively unimportant in these systems.

**Evidence for Lateral Heterogeneity**. Does **NNR** in these equilibrating bilayers reflect lateral heterogeneity? In order to address this question, we have carried out dilution experiments for the two systems showing the greatest **NNR** (**18/PO-** and **18/DO**-based membranes).[9] Thus, thiolate−disulfide interchange reactions were carried out in vesicular membranes that contained a 1/1 molar mixture of **18-18**/**PO-PO** (and also pure **18-PO**) where 50% of the exchangable monomer units were replaced by DMPC (a phospholipid having an intermediate $T_m$ of 24.0 °C). The equilibrium mixture of dimers was then found to be close to random (Table 1). Similarly, inclusion of DMPC in equilibrating bilayers of **18-18**/**DO-DO** (and also pure **18-DO**) gave close to a random distribution of dimers. The significant loss of **NNR** in each of these membranes due to the presence of DMPC provides compelling evidence that DMPC promotes mixing between the dimers and that lateral heterogeneity exists in the absence of this diluent.[9]

Finally, it is noteworthy that the general trend that we have observed in these **NNR** experiments bears a resemblance to what has been found for dimer products that were formed within PE-based vesicles.[16] Specifically, the reaction of dimethylsuberimidate with fluid bilayers of DPPE/DOPE (1/1, m/m, 68°C) gave a heterodimer/homodimer ratio of 1.85.[16] In contrast, analogous dimerization reactions that were carried out with 1/1 mixtures of dielaidoylphoethanolamine (DEPE) /DMPE and also with DEPE/dilauroylphoethanolamine (DLPE) yielded random mixtures of dimers. Since the $T_m$ values for DPPE, DMPE, DEPE, DLPE, and DOPE are 65, 49.5, 37.5, 30.5, and −16 °C, respectively, the observation that only the combination of DPPE and DOPE (the pair of PEs having the greatest difference in $T_m$s) affords a nonrandom distribution of dimers is similar to our present findings. Whether or not this similarity is fortuitous, however, remains to be determined. Two uncertainties that preclude definitive conclusions to be reached from these data, with respect to lateral organization, are (i) the interaction between a monoreacted PE (a PE molecule that has been coupled to one "end" of the cross-linking agent) with neighboring PEs may or may not be similar to that of the "native" (precursor) PE and (ii) the dimers may be formed either under thermodynamic or kinetic control.

**Table 1.** Nearest-Neighbor Recognition within Saturated/Unsaturated Phospholipid Membranes

| equilibrating lipid dimers | cholesterol[a] (mol %) | temp (°C) | heterodimer/homodimer[b] |
|---|---|---|---|
| **14-14/14-PO/PO-PO** | 0 | 40 | 1.90 ± 0.07 |
| | 40 | 40 | 1.82 ±0.08 |
| **18-18/18-PO/PO-PO** | 0 | 60 | 1.78 ± 0.07 |
| | 40 | 60 | 1.77 ± 0.04 |
| **14-14/14-DO/DO-DO** | 0 | 40 | 1.96 ± 0.08 |
| | 0 | 60 | 1.97 ± 0.04 |
| | 40 | 40 | 1.79 ± 0.05 |
| | 40 | 60 | 1.87 ± 0.03 |
| **16-16/16-DO/DO-DO** | 0 | 55 | 1.79 ± 0.05 |
| | 40 | 55 | 1.89 ± 0.09 |
| **18-18/18-DO/DO-DO** | 0 | 60 | 1.69 ± 0.05 |
| | 40 | 60 | 1.72 ± 0.05 |
| **18-18/18-PO/PO-PO**[c] + 50 mol % DMPC | 0 | 60 | 1.93 ± 0.04 |
| **18-18/18-DO/DO-DO**[c] + 50 mol % DMPC | 0 | 60 | 1.91 ± 0.08 |

[a] The mol% of cholesterol is based on total lipid that is present, where each phospholipid "counts" as two lipid molecules; the thiol monomer content used was 20 mol % (equal molar mixture of the monomers). [b] Equilibrium ratio of heterodimer/homodimer ± two standard deviations from the mean. In all cases, homodimers were present in equal molar quantities at equilbrium. [c] The thiol monomer content used was 10 mol %.

**Biological Significance**. Although our lipid dimers are negatively charged and natural phosphocholines are zwitterionic and monomeric, the fact that the gel-to-liquid crystalline phase transition temperatures and calorimetric enthalpies and entropies for each type, having the same fatty acid chains, are nearly identical indicates that the intermolecular forces between phospholipid monomers are very similar.[7] In addition, the fact that the surface pressure-area isotherm for **16-16** and DPPC are also very similar (both show a well-defined phase transition at ca. 11 dyn/cm, and the limiting area of **16-16** is ca. twice that of DPPC) lends further support for comparing these two classes of lipids.[17] On the basis of our results, therefore, one would expect that binary mixtures of DMPC and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) should be randomly arranged in the fluid phase but that analogous mixtures of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and DOPC would be laterally heterogeneous. Although phase diagrams have been used, extensively, to probe the mixing behavior of lipids in the gel and in the gel-fluid coexistence regions, we are aware of only one instance in which a phase diagram has indicated the presence of lateral heterogeneity in the fluid phase; i.e., binary mixtures of 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (DEPC) and DPPE.[5,18] In all other cases, the distribution of the lipids

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in the fluid phase is less certain.[19,5] *Our NNR results that are reported herein provide the strongest evidence to date that relatively modest differences in the fatty acid structure of phospholipids can produce lateral heterogeneity in the fluid phase, in the absence of other added components; i.e., sterols, metal ions, proteins, etc*.

Biological membranes contain rich mixtures of saturated and unsaturated phospholipids. Although the lateral distribution of these lipids remains uncertain, the present findings increase the probability that lateral heterogeneity will eventually be found. The need for additional studies that can sort out the relationships that exist between molecular structure and lateral organization is clearly substantial. In this regard, **NNR** studies of the type described herein, should go a long way in helping to bring the fluid mosaic model of biological membranes into sharper focus.

## Conclusions

Nearest-neighbor recognition studies have been carried out using phospholipid dimers derived from saturated (DMPE, DPPE, and DSPE) and unsaturated (POPE and DOPE) phosphoethanolamines. Monomer exchange among the dimers was accompanied by a small extent of isomerization of the *cis*-double bonds. The degree of **NNR** that was observed was found to correlate with the difference between the gel-to-liquid crystalline phase transition temperatures ($T_m$) of the homodimers that were present; the greater the difference, the greater the level of recognition. Phospholipids having two double bonds led to greater **NNR** than those having only a single double bond; a result that can be correlated with the relative $T_m$ values of the lipids. The presence of unsaturation in the acyl chains was found to have a greater effect on **NNR** than their relative lengths. Dilution of two of the membranes having the largest **NNR** with DMPC led to a significant decrease in **NNR**, indicating the presence of lateral heterogeneity in the absence of this diluent.

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