Diluents That Promote Demixing in Fluid Phospholipid Bilayers

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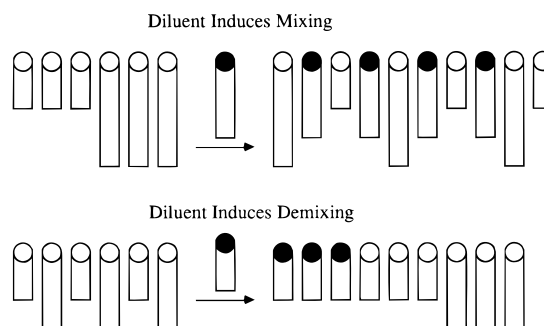
Abstract: The influence of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) on the mixing behavior of phospholipids derived from 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) has been examined in the fluid bilayer state by means of nearest-neighbor recognition methods (Davidson, S. K. M.; Regen, S. L. *Chem. Rev.* **1997**, *97*, 1269). When disulfide-based dimers derived from DMPE and DSPE were allowed to undergo monomer exchange at 60 °C, in the presence of 50 mol % of DPPC, an equilibrium molar ratio of heterodimer to each homodimer was found to be 1.95 ± 0.07; with DMPC as the diluent, a small but significant level of nearest-neighbor recognition was observed (i.e., the dimer ratio was 1.86 ± 0.02). In sharp contrast, the use of DLPC resulted in a very strong demixing effect, which was characterized by a dimer ratio of 0.88 ± 0.04. The mechanistic and practical implications of these findings are briefly discussed.

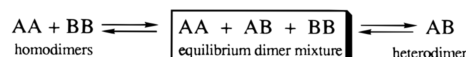
Introduction

In a recent series of papers, we have presented experimental evidence indicating that a membrane diluent can promote the mixing of phospholipids in the fluid bilayer state when its hydrophobicity is intermediate in magnitude.[1−6] In the work that is described herein, we show that a diluent can also promote *demixing* when its hydrophobicity more closely matches one of a pair of phospholipids, i.e., *when the hydrophobic matching is skewed*. We also report unexpectedly-strong demixing effects when the diluent is *shorter* than both phospholipids. These findings have important mechanistic and practical implications, which are briefly discussed.

The technique that we have used to probe phospholipid mixing ("nearest-neighbor recognition", NNR) involves the generation and analysis of equilibrium mixtures of phospholipid dimers.[4] In brief, two phospholipids (A and B) are first converted into exchangeable, disulfide-linked homodimers (AA and BB) and the corresponding heterodimer (AB). Subsequent vesicle formation, using an equimolar mixture of the two homodimers, followed by monomer exchange via thiolate−disulfide interchange leads to an equilibrium mixture. A similar reaction that is carried out with vesicles made from pure heterodimer ensures that an equilibrium point has been reached. When the resulting dimer composition is statistical (i.e., when the molar ratio of heterodimer to each homodimer is 2.0), and when there is no driving force for transmembrane asymmetry (i.e., an uneven distribution of phospholipids between the inner and outer monolayer of the bilayer), this finding establishes that the monomeric components are randomly distributed throughout the membrane. When homodimers are favored (i.e., when NNR is present), lateral heterogeneity may or may not exist. If the intramolecular and intermolecular forces within the membrane are similar in magnitude, then NNR also indicates the presence of lateral heterogeneity.

Chart 1





Experimental Section

General Methods. All of the general methods that have been used in this study were similar to those previously described.[5] 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. Just prior to carrying out a NNR experiment, an appropriate vesicle dispersion was 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 Tris HCl buffer (pH 7.4, 150 mM NaCl, 2.0 mM NaN_3) over the course of 18 h.

Digestion of Large Unilamellar Vesicles by Phospholipase A_2. Chloroform solutions of **1** and **2** (0.6 μmol of each dimer) were added to a 10 mL test tube and the solvent then evaporated under a stream of nitrogen. The resulting thin film of lipid was then dissolved in 400 μL of chloroform and the solution then diluted with 1080 μL of diisopropyl ether. Subsequent addtion of 140 μL of 3.3 mM Tris HCl buffer (pH 7.4, 50 mM NaCl, 0.7 mM NaN_3, and 0.3 mM $CaCl_2$) resulted in an emulsion. After the emulsion was sonicated for 3 min by use of a mild (bath-type) sonicator, the organic phase was removed by gentle evaporation at 60 °C to afford a white gel in the bottom of

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(1) Vigmond, S. J.; Dewa, T.; Regen, S. L. *J. Am. Chem. Soc.* **1995**, *117*, 7838.
(2) Dewa, T.; Vigmond, S. J.; Regen, S. L. *J. Am. Chem. Soc.* **1996**, *118*, 3435.
(3) Dewa, T.; Regen, S. L. *J. Am. Chem. Soc.* **1996**, *118*, 7069.
(4) Davidson, S. K. M.; Regen, S. L. *Chem. Rev.* **1997**, *97*, 1269.
(5) Uragami, M.; Dewa, T.; Inagaki, M.; Hendel, R. A.; Regen, S. L. *J. Am. Chem. Soc.* **1997**, *119*, 3797.
(6) Inagaki, M.; Shibakami, M.; Regen, S. L. *J. Am. Chem. Soc.* **1997**, *119*, 7161.

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the test tube. The gel was then collapsed by vigorous vortex mixing for 5 min, and 1.0 mL of additional buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 2 mM NaN_3, 1 mM $CaCl_2$) was added dropwise with vortex mixing.

A bovine serum albumin solution (30.3 mg, 400 μL of 10 mM Tris buffer) was then added to 0.95 mL of the above vesicle dispersion at 60 °C with stirring. After the mixture was incubated for 15 min, a phospholipase A_2 solution (*Naja naja*, 0.2 unit, 40 μL of 10 mM Tris buffer) was injected to initiate surface hydrolysis. The final concentrations of the enzyme and bovine serum albumin were 6.9×10^{-9} and 3.3×10^{-4} M, respectively. Aliquots (25 μL) were then taken as a function of time, and spotted as a 4-cm line on a silica gel plate (EM Science, 0.25 mm thickness, 5 cm × 14 cm). The samples were eluted with $CHCl_3/CH_3OH/H_2O$, 65/25/4 (v/v/v). The bands that corresponded to **1** ($R_f = 0.34$) and **2** ($R_f = 0.39$), which were visualized by spraying with 2′,7′-dichlorofluorescein, and detecting with UV light at 365 nm, were then removed and analyzed for phosphorus content after combustion. Specifically, the silica gel that contained the desired phospholipid was placed over a strong flame, after addition of 0.4 mL of 7.5% $Mg(NO_3)_2 \cdot 6H_2O$, until the evolution of a colored vapor ceased. The colorless residue was then suspended in 0.5 mL of 0.1 M HCl and heated to 100 °C for 15 min. After the solution was cooled to room temperature, 1.7 mL of a molybdenium reagent (made from 1 part of 10% ascorbic acid in water plus 6 parts of 0.5% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 2 M H_2SO_4) was then added. The mixture was vortex mixed and incubated for 30 min at 45 °C, and its absorbance was immediately measured at 820 nm. Appropriate calibration curves were made by using the starting phospholipids.

Reaction of Large Unilamellar Vesicles with DTNB. Following procedures similar to those described above, large unilamellar vesicles were prepared from **4** (0.6 μmol), **5** (0.6 μmol), and DLPC (1.2 μmol) plus 2 mL of 10 mM Tris HCl buffer (pH 5.0, 150 mM NaCl, 2 mM NaN_3, and 5 mM EDTA). To a disposable polystyrene cuvette was then added 1.1 mL of a 1 mM solution of DTNB (10 mM Tris HCl buffer, pH 7.4, 150 mM NaCl, 2 mM NaN_3 and 5 mM EDTA). After the solution was incubated for 2 min at 30 °C, an aliquot of the vesicle dispersion (100 μL) was added and the absorbance at 412 nm was recorded as a function of time. Similar experiments were carried out in which the buffer solution initially contained 30% ethanol (v/v) (prior to vesicle addition) in order to obtain a value for the total thiol content. Concentrations of the released dye were calculated by use of appropriate calibration curves.

Results and Discussion

Exchangeable Phospholipilds and Diluents. The specific dimers that were used in the present investigation (**1**, **2**, and **3**) have previously been described.[7] In the fluid bilayer state (60 °C), thiolate−disulfide interchange produces a random arrangement of the individual monomer units.[7] To test for demixing, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) were chosen as membrane diluents. In principle, the addition of a diluent that favors association with *one* of a pair of exchangeable phospholipids should promote demixing and NNR. Since DPPC has a chain length mismatch that is the same for monomers of **1** and **2** (two methylenes per alkyl chain), and since the hydrophobicity of a phospholipid increases with increasing numbers of methylenes, DPPC would not be expected to promote demixing if the preference for association between diluent and phospholipid were controlled only by their hydrophobic content.[3] In fact, a *difference in chain length mismatch* that equals zero should favor mixing, which has already been shown to be the case.[1−6] With DMPC, however, a difference in chain length mismatch of four methylene units could lead to demixing. Since the difference in chain length mismatch between DLPC and the exchangeable monomers of **1** and **2** is also four methylene units [i.e., (16 CH_2s−10 CH_2s)−(12 CH_2s−10 CH_2s)], one might expect that DMPC and DLPC would have similar demixing properties.

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

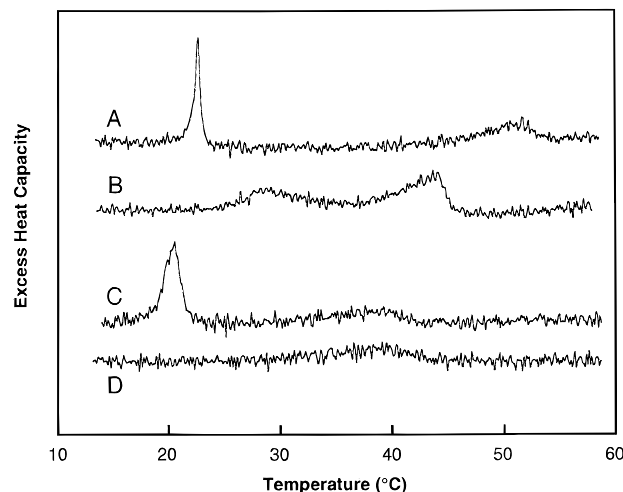
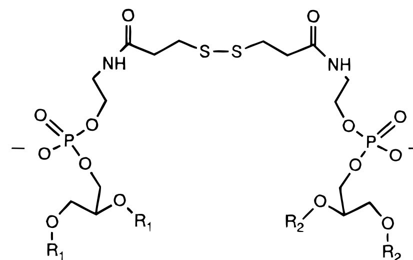


Figure 1. High-sensitivity excess heat capacity profile for multilamellar vesicles made from **1**/**2** (1/1, mol/mol) containing (A) no diluent, (B) DPPC (50 mol %), (C) DMPC (50 mol %), and (D) DLPC (50 mol %).



The Physiologically-Relevant Fluid Phase. To ensure that all NNR experiments were conducted in the fluid phase at 60 °C, the thermotropic phase behavior of an equimolar mixture of **1** and **2**, in the presence of each diluent, was examined by high-sensitivity differential scanning calorimetry (Figure 1). Thus, analysis of multilamellar vesicles made from **1**/**2** (1/1, mol/mol) revealed two distinct endotherms appearing at ca. 23 and 52 °C; introduction of 50 mol % DPPC led to the appearence of two broad endotherms appearing at 29 and 44 °C, respectively. When DMPC was used as the diluent, distinct endotherms were observed at 21 and 37 °C; with DLPC, only one broad endotherm was apparent at 39 °C within the range of 10−60 °C. Since previous studies have shown that the heterodimer, **3**, promotes the mixing of **1** and **2** in the gel-fluid coexistence region, product mixtures that contain this heterodimer must also be in the fluid phase at 60 °C.[7]

Nearest-Neighbor Recognition. Following procedures similar to those previously described, NNR experiments were carried out with bilayers composed of **1**, **2**, and **3** plus DPPC, DMPC, or DLPC at 60 °C.[5,7] In the presence of DPPC, the heterodimer/homodimer ratio that was observed (1.95 ± 0.07) was essentially the same as that found in the absence of diluent, 1.98 ± 0.05 (Table 1); in both cases random distributions were produced. When DMPC was used as a diluent, however, a small but significant level of NNR was detected; i.e., the heterodimer/homodimer ratio was 1.86 ± 0.02. Most strikingly, inclusion of DLPC in the membrane resulted in a very high degree of NNR; i.e., the heterodimer/homodimer ratio was 0.88 ± 0.04,

Table 1. Nearest-Neighbor Recognition within Fluid Phospholipid Membranes[a]

membrane diluent[b]	heterodimer/homodimer[c]
none	1.98 ± 0.05[d]
50 mol % of DPPC	1.95 ± 0.07
50 mol % of DMPC	1.86 ± 0.02
50 mol % of DLPC	0.88 ± 0.04
9 mol % of DLPC	1.21 ± 0.08

[a] All NNR experiments were carried out at 60 °C; chemical equilibrium was generally reached in ca. 3 h. [b] Mole percentage of diluent, where each dimer is counted as 2 mol of phospholipid. [c] Molar ratio of heterodimer to each homodimer ± two standard deviations. [d] See ref 7.

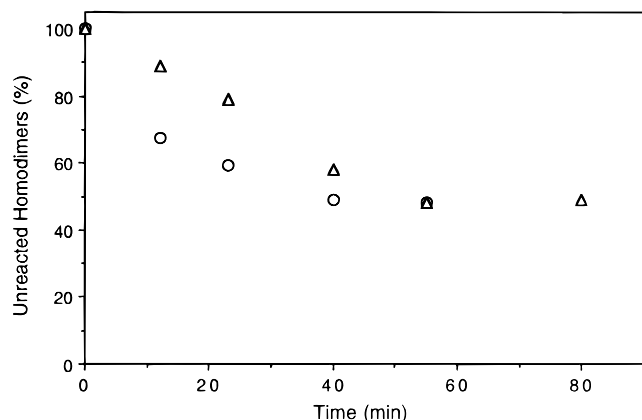


Figure 2. Plot of unreacted (○) **1** and (△) **2** as a function of time for vesicles that have been exposed to phospholipase A_2.

which corresponds to a free energy difference between a random and a nonrandom state of $\Delta G^o = 1.09 \pm 0.06$ kcal/mol. When the DLPC concentration was reduced to 9 mol %, significant demixing was still apparent (Table 1).

Transmembrane Symmetry. Given the fact that large unilamellar vesicles were used in these experiments (10 000 Å diameter, dynamic light scattering, reverse-phase evaporation), the presence of transmembrane asymmetry would not be expected, since curvature effects should be negligible. Confirmation of this assumption was made for vesicles that were made from an equimolar mixture of **1** and **2** by enzymatic degradation and analysis of the outer monolayer leaflet.[8,9] Thus, exposure to phospholipase A_2 and analysis of the dimer content as a function of time (tlc, phosphorus analysis) revealed that exactly 50% of each homodimer underwent rapid hydrolysis (Figure 2). Similar experiments that were carried out in the presence of 50 mol % of DLPC, however, led to the rapid hydrolysis of more than 90% of the dimers (Figure 3). In this case, enzymatic degradation resulted in the complete destruction of the vesicles (dynamic light scattering).[10] In a related series of experiments, "monomer-analogous" vesicles were prepared from an equimolar mixture of **4** and **5**.[11] Subsequent reaction with an excess of 5,5′-dithiobis(2-nitrobenzoic acid), DTNB,

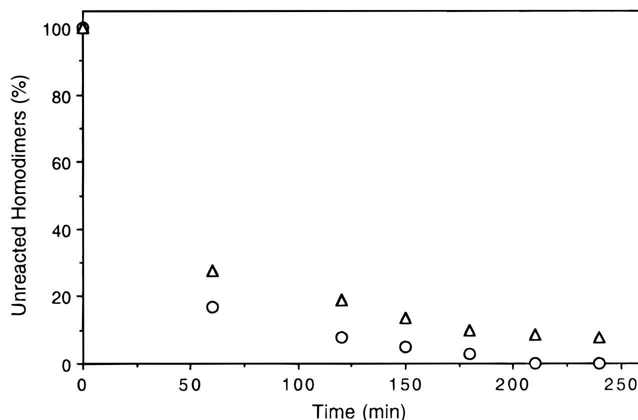


Figure 3. Plot of unreacted (○) **1** and (△) **2** as a function of time for vesicles containing DLPC (50 mol %) that have been exposed to phospholipase A_2.

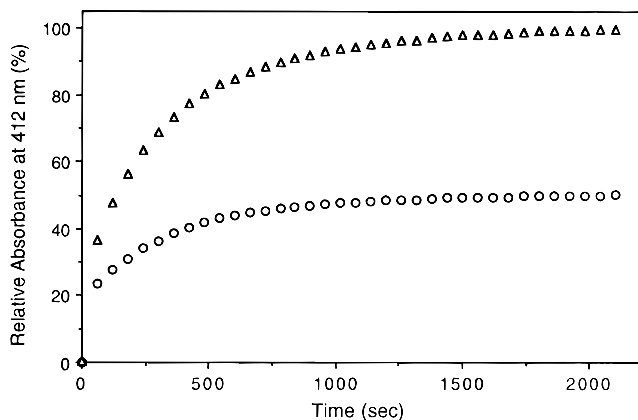


Figure 4. Plot of absorbance at 412 nm as a function of time for the reaction of vesicles made from an equimolar mixture of **4** and **5** with an excess of DTNB in the (○) absence and (△) presence of 30% ethanol.

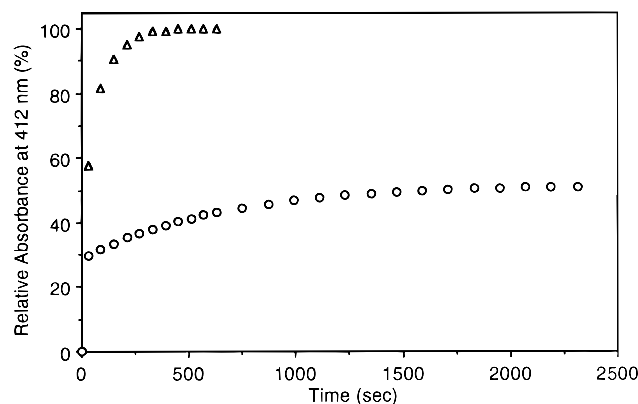


Figure 5. Plot of absorbance at 412 nm as a function of time for the reaction of vesicles made from an equimolar mixture of **4** and **5** plus DLPC (50 mol %) with an excess of DTNB in the (○) absence and (△) presence of 30% ethanol.

and analysis of the 2-mercaptopyridine that was liberated as a function of time (UV−vis), showed that exactly 50% of the thiol-containing monomer (**5**) was present in the outer monolayer, i.e., 50% of **5** underwent reaction (Figure 4).[12] Similar experiments that were carried out with vesicles composed of **4**/**5**/DLPC (1/1/4, mol/mol/mol) gave *identical* results (Figure

(8) Kumar, A.; Gupta, C. M. *Biochemistry* **1985**, *24*, 5157.

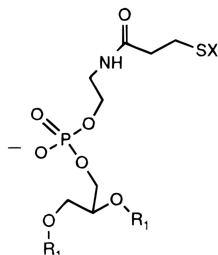
(9) Runquist, E. A.; Helmkamp, G. M., Jr. *Biochim. Biophys. Acta* **1988**, *940*, 10.

(10) An alternate explanation for the rapid hydrolysis of more than 90% of the dimers is that more than 90% of DLPC occupies the inner monolayer leaflet. It should be noted, however, that "very short" phosphocholines are known to favor the *outer* monolayer leaflet of vesicles: Gabriel, N. E.; Roberts, M. F. *Biochemistry* **1987**, *26*, 2432. That the vesicles were completely destroyed upon enzymatic digestion, however, was clearly evident by the absence of light scattering.

(11) Compounds **4** and **5** were prepared by using procedures similar to those previously described.[7] Phospholipid **4** had the expected ^{1}H NMR spectrum; HRMS for $(C_{37}H_{71}NO_9PS)^+$ calcd 736.4587, found 736.4585.

(12) Ganong, B. R.; Bell, R. M. *Biochemistry* **1984**, *23*, 4977.

5). Thus, the presence of DLPC leaves **5** evenly distributed across such bilayers.



4, R_1= $CO(CH_2)_{12}CH_3$; X=CH_3
5, R_1= $CO(CH_2)_{16}CH_3$; X=H

Conclusions

Taken together, the results reported herein provide compelling evidence that a membrane diluent can promote demixing in fluid bilayers when its hydrophobicity more closely matches one of a pair of phospholipids. These results also confirm our earlier interpretations by showing that the hydrophobicity of a diluent can influence phospholipid mixing. The fact that DLPC is a much more potent demixing agent than DPPC, however, implies that a factor other than hydrophobicity is also involved. At the present time, we favor a mechanism of action of DLPC that results from its thinning effect on the bilayer. Specifically, by reducing the average thickness of the membrane, DLPC can force the relatively long phospholipids (i.e., **2**) into a "crumpled" state, where hydrophobic contact is maximized. Thus, we hypothesize that crumpled phospholipids favor other crumpled phospholipids as nearest-neighbors; i.e., **2** is "kicked out" of regions of the membrane that contain more cylindrical-shaped phospholipids (i.e., DLPC and **1**).

From a practical point of view, the feasibility of controlling the two-dimensional structure of fluid bilayers by use of demixing as well as mixing agents raises intriguing possibilities in the area of drug design. In particular, to the extent that the lateral organization of biological membranes controls vital cellular processes (e.g., membrane fusion, cell surface recognition, transport, etc.), one may expect that suitable demixing and mixing agents could be used to modify or destroy appropriate targets, e.g., cancer cells.[13-15] The present findings also suggest a fundamentally new mechanism by which local anesthetics may operate; i.e., their ability to alter the natural functioning of cells may derive from mixing or demixing effects that result from hydrophobic mismatches and/or changes in membrane thickness.[13]

Acknowledgment. We are grateful to the American Cancer Society (Grant No. BE-205) for support of this research.

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(15) Welti, R.; Glaser, M. *Chem. Phys. Lipids* **1994**, *73*, 121.