Nearest-Neighbor Recognition within a Mixed Phospholipid Membrane: Evidence for Lateral Heterogeneity

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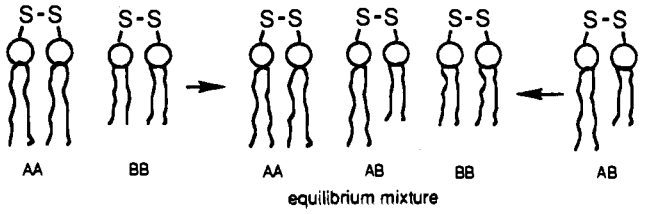
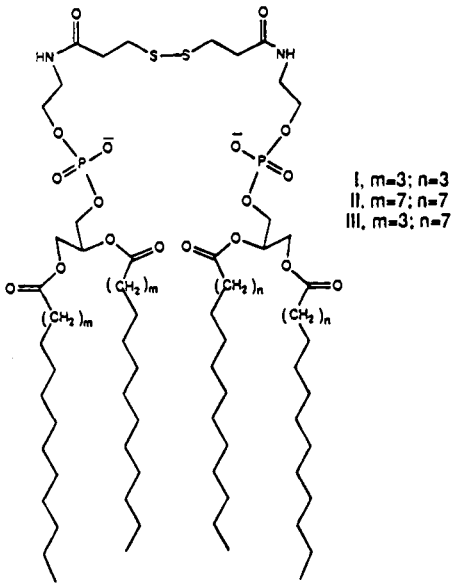
Despite a considerable body of knowledge that currently exists concerning the structure and function of biological membranes, remarkably little is known in terms of their two-dimensional organization.[1−4] Even in the simplest of model systems, the presence or absence of lateral heterogeneity (i.e., a nonrandom arrangement of membrane components) within the physiologically-relevant fluid phase has been difficult to establish. Current 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 to the study of lipid mixing that is based on the exchange and equilibration of disulfide-linked phospholipid dimers.[7,8] A unique feature of this technique is that it can, under certain circumstances, lead to definitive conclusions regarding the arrangement of the lipids in the fluid phase. In brief, vesicular membranes are first prepared from a 1/1 mixture of homodimers (AA and BB) and then allowed to undergo monomer exchange via thiolate−disulfide interchange. In order to ensure that equilibrium has been obtained, a similar exchange reaction is carried out using vesicles made from heterodimer (AB). When a given equilibrium mixture is found to be statistical (i.e., when the molar ratio of AA/AB/BB is 1/2/1), this finding, in and of itself, *proves* that the monomer and dimer components are randomly distributed throughout the bilayer.[7]



While the presence of a statistical mixture leads to definitive conclusions with respect to membrane suprastructure, the formation of a nonstatistical mixture does not. In particular, when homodimers are favored (i.e., when the observed heterodimer/homodimer ratio is less than 2.0), such a situation [which we term "nearest-neighbor recognition" (NNR)] may reflect lateral heterogeneity within the bilayer or it may reflect a membrane that is composed of a nonstatistical mixture of dimers that are randomly distributed throughout the bilayer. The relationship between NNR and lateral heterogeneity depends upon the relative strength of the intermolecular and intramolecular forces that are involved. When these two forces are similar in magnitude, NNR will reflect lateral heterogeneity as well as dimer stability; when the intramolecular forces are dominant, only the relative stability of the dimers is revealed. In this paper *we describe a technique that, for the first time, links NNR in a model membrane to its supramolecular structure.* This method involves the dilution of dimer-based bilayers with conventional phospholipids.

Previous studies have shown that cholesterol induces NNR in vesicular membranes made from phospholipids **I**, **II**, and **III** (i.e., dimers that are composed of "DMPC-like" and "DSPC-like" monomeric units) at temperatures that are in excess of the gel to liquid-crystalline phase transition temperature (T_m) of the highest melting dimer, **II**.[8] Our past assumption has been



that such NNR reflects lateral heterogeneity (i.e., regions that are rich in **I** and regions that are rich in **II**) and that the intermolecular and intramolecular forces are of comparable strength. On the basis of the high miscibility that DPPC is known to have with both DMPC and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) in the *gel−fluid coexistence region* and the fact that **I** and **II** have the same fatty acid composition and nearly identical melting behavior as compared with DMPC and DSPC, respectively, we recently hypothesized that dilution of these dimer-based bilayers with DPPC (a phospholipid of intermediate chain length) could verify our assumption. Specifically, we reasoned that the inclusion of DPPC should eliminate regions of the membrane that are rich in **I** and those that are rich in **II** by creating a more homogeneously mixed bilayer. In addition, the low miscibility that DMPC is known to have with DSPC in the gel−fluid coexistence region led us to hypothesize that DMPC would be less effective than DPPC in promoting the mixing of **I** and **II**.

In order to judge whether or not the difference in head groups between these phospholipid dimers and conventional phosphatidylcholines would prevent mixing, we first examined the influence of DPPC and DMPC on the melting behavior of bilayers made from **I** and **II**. Figure 1 shows high-sensitivity differential scanning calorimetry (hs-DSC) thermograms for a 1/1 mixture of **I**/**II** in the absence and in the presence of equimolar quantities of DPPC and DMPC (based on phosphate

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(10) Previously reported T_m values for **I**, DMPC, DPPC, and **II** are 22.7, 24.0, 41.5, and 55.4 °C, respectively.[8]

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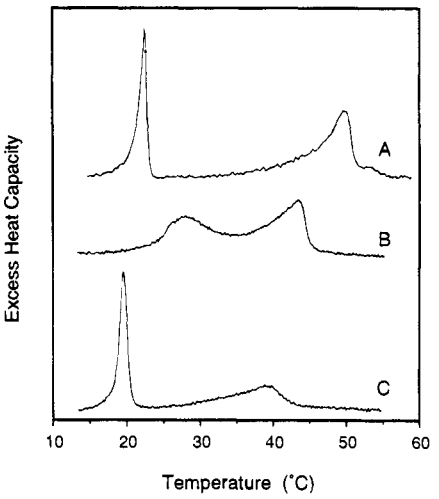

Figure 1. High-sensitivity excess heat capacity profiles of multilamellar vesicles formed from (A) a 1/1 mixture of **I/II**, (B) a 1/1/4 mixture of **I/II**/DPPC, and (C) a 1/1/4 mixture of **I/II**/DMPC; a 30 °C/h scan rate was used. Very similar profiles were obtained from vesicles that were formed via reverse-phase evaporation methods.

content). The merging of the high- and low-temperature endotherms that is induced by the presence of DPPC implies the conversion to a more homogeneously mixed bilayer. Although DMPC also interacts with both types of domains, the fact that *both* endotherms move toward lower temperatures suggests that DMPC is less effective in promoting the mixing of these lipid dimers.

Chemical exchange and equilibration within vesicular bilayers that were made from a 1/1/0.8 molar mixture of **I/II**/cholesterol and ones that were made from a 1/0.4 molar mixture of **III**/cholesterol (20 mol % cholesterol in each case) at 60 °C afforded an equilibrium ratio of heterodimer (**III**)/homodimer (**I** or **II**) equaling 1.55 ± 0.08 (Table 1).[11] In contrast, a similar equilibration reaction that was carried out in which 50% of the exchangeable monomer units were replaced by DPPC (i.e., molar compositions of 0.5/0.5/0.8/2 **I/II**/cholesterol/DPPC and 1/0.8/2 **III**/cholesterol/DPPC) afforded a random distribution of dimers. While a 25 mol % replacement with DPPC proved to be moderately effective in reducing the observed NNR, no significant reduction could be detected when the concentration of DPPC was lowered to 10 and 15 mol %. Similar equilibration experiments that were carried out using 50 mol % DMPC did not significantly reduce the extent of NNR. In related experiments, DPPC was found to be less effective in reducing NNR in equilibrating bilayers of **I**, **II**, and **III**, which contained 40 mol % cholesterol (Table 1).

Table 1. Dilution of Nearest-Neighbor Recognition

cholesterol content[a] (mol %)	membrane diluent	dimer ratio[b]
0		2.07 ± 0.08
20		1.55 ± 0.08
20	50% DPPC	1.97 ± 0.06
20	50% DMPC	1.66 ± 0.06
20	25% DPPC	1.83 ± 0.04
20	25% DMPC	1.63 ± 0.07
20	15% DPPC	1.54 ± 0.02
20	10% DPPC	1.55 ± 0.07
40		1.53 ± 0.06
40	50% DPPC	1.65 ± 0.04

[a] The mole % of cholesterol is based on total lipid that is present, where each phospholipid dimer "counts" as two lipid molecules. [b] Equilibrium ratio of heterodimer (**III**)/homodimer (**I** or **II**) ± 2 standard deviations from the mean. All equilibration experiments were carried out at 60 °C.

The ability of DPPC to effectively reduce NNR in bilayers that contain **I**, **II**, and **III** and cholesterol constitutes compelling evidence that dimers are nonrandomly distributed in the absence of this phosphatidylcholine. If such NNR were dominated by intramolecular forces, then DPPC as well as DMPC would be expected to have a negligible effect on the dimer ratio since both provide a liquid-crystalline microenvironment, similar to that of the dimers. The fact that DMPC is much less effective than DPPC in moving the equilibrium point toward a random value further implies that DMPC is heterogeneously distributed relative to DPPC in these diluted membranes. Thus, *these dilution experiments provide insight into the mixing behavior of biologically-relevant phospholipids as well as the dimers.* One final point that deserves special mention is the finding that cholesterol induces lateral heterogeneity among the phospholipids at temperatures that are in excess of their gel to liquid-crystalline phase transition temperatures. The fact that mammalian cells are rich in cholesterol suggests that such a feature could have important biological consequences in terms of the "proper" and "improper" functioning of cell membranes.

In summary, we have shown that the dilution of a phospholipid membrane that exhibits nearest-neighbor recognition with an appropriate phosphatidylcholine can effectively reduce the extent of recognition and provide compelling evidence for lateral heterogeneity in the absence of this phosphatidylcholine. We have also shown how such dilution experiments significantly expand the scope of the NNR technique by providing insight into the mixing behavior of phospholipids that are of greater biological relevance. Finally, a novel feature of cholesterol has been identified, i.e., its ability to induce lateral heterogeneity within a mixed phospholipid bilayer at temperatures that are in excess of their gel to liquid-crystalline phase transition temperatures. Further studies that make use of this dilution method are currently in progress.

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(11) Dimers were chemically equilibrated via thiolate–disulfide interchange after addition of dithiothreitol. Specific procedures were similar to those previously described.[7] In the present study, vesicles were formed via reverse-phase evaporation methods similar to those described in the literature.[12,13] Typically, lipid films composed of 1.8 μmol of phospholipid (based on phosphate) were dissolved in 0.4 mL of diisopropyl ether plus 0.15 mL of chloroform. After addition of 50 μL of borate buffer, the resulting emulsion was subjected to mild (bath-type) sonication, followed by removal of the organic phase under reduced pressure at 60 °C. Product mixtures were analyzed by high-pressure liquid chromatography using a Beckman Ultrasphere C18 reverse-phase column (4.6 × 250 mm, 5 μm particle size) and an eluting solvent that was composed of water/hexane/9 mM tetrabutylammonium acetate in denatured ethanol (13/10/77, v/v/v). The flow rate and column temperature used were 0.9 mL/min and 31.2 °C, respectively. Samples were dissolved in 5 μL of chloroform plus 100 μL of mobile phase prior to injection. Retention times of **I**, **II**, **III**, and cholesterol were 6, 24, 11, and 7 min, respectively. Detection was made by UV (205 nm), and data were collected and processed with a Maxima 820 workstation (Waters).

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