Nearest-Neighbor Recognition in Phospholipid Membranes

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Contents

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I. Introduction

One of the most significant challenges presently facing chemists and biologists is to define the two-dimensional structure of biological membranes. In particular, the time-averaged, lateral distribution of the lipids and proteins that make up these biological enclosures remains to be clarified.[1] Do lipids organize themselves into nonrandom clusters? If such clusters exist, do they have any functional importance? Are they intimately involved, for example, in basic membrane processes such as fusion, transport, recognition, and catalysis? Do changes in lateral organization accompany the formation of a diseased state; e.g., the malignant transformation of cells? Are lipid clusters in cancer cells unique, and can they serve as specific target sites for chemotherapy? Can differences in the lateral organization between mammalian cell membranes and those of microbes be used as a basis for chemotherapy? Such questions are not only of considerable theoretical interest, but they also have important practical implications. A firm understanding of the two-dimensional structure of biological membranes has the potential for bringing exploitable targets into clear focus, which would assist the rational design of novel classes of therapeutic agents.

Although it is generally assumed that lipids and proteins are nonrandomly arranged within biological membranes, the detection of lateral heterogeneity has proven to be extremely difficult, even for the simplest

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of model systems.[2−4] The major difficulty has been the absence of experimental methods that can provide clear insight into the *physiologically relevant fluid phase*. In this contribution, a new approach to the study of lateral organization is reviewed. In contrast to all other methods that have previously been developed for studying lateral organization, this technique yields *definitive* evidence for randomly arranged phospholipids in the fluid phase. This method can also provide compelling evidence for the presence of lateral heterogeneity in fluid bilayers, as well as a quantitative measure of the thermodynamic driving force that is involved.

Throughout this review article, the term "lateral heterogeneity" is used to describe nonrandomly arranged mixtures of phospholipids. It should be noted that although the term "domain" has been widely used in the literature, no common definition of what a domain actually is at the molecular level has as yet evolved.[3] For this reason, no further mention of "domain" will be made.

II. Background

Biological membranes play a vital role in living cells by serving as selective barriers for transport, and as sites for molecular recognition and catalysis. In essence, biological membranes are composed of a lipid bilayer that contains weakly associated peripheral proteins and strongly associated integral proteins.[1,5] In mammalian cells, the two main classes of lipids are phospholipids and cholesterol. Despite a considerable body of information that has emerged concerning the composition and dynamics of biological membranes, their two-dimensional organization remains to be defined.

As a first step toward solving the two-dimensional structure of biological membranes, considerable attention has focused on simple model systems. In particular, the miscibility of a variety of binary mixtures of phospholipids in the *gel−fluid* coexistence region has been carefully examined by several techniques.[6−14] The primary reason for focusing on the gel−fluid coexistence region has been that clear insight into lateral organization is possible. For example, differential scanning calorimetry, electron paramagnetic resonance spectroscopy, and freeze−fracture electron microscopy can detect "islands" of gel phase that are rich in a higher melting lipid, which are surrounded by a fluid "sea" that is richer in a lower melting component. Unfortunately, such miscibility behavior cannot be extrapolated to the physiologically relevant fluid phase. Although several methods have been devised to probe the lateral organization of fluid bilayers directly, the conclusions that can be drawn from such experiments are not as clear as in the gel−fluid coexistence region. In the following sections, two such methods are briefly reviewed.[4]

A. Detection of Lateral Heterogeneity by Fluorescence Recovery after Photobleaching

Fluorescence recovery after photobleaching (FRAP) is a method that measures the lateral diffusion of fluorescently labeled lipids and proteins. Typically, a lipid or protein molecule, which has been labeled with a fluorescent probe, is introduced into a membrane of interest. Subsequent irradiation of a small region of the membrane (ca. 1 μm in diameter) with a laser beam leads to the photochemical destruction of the fluorophore (photobleaching). When the laser is turned off, the fluorescence intensity within that "spot" returns (fluorescence recovery) due to lateral diffusion of nonbleached molecules from the surrounding medium into the irradiated spot. Such experiments have shown that the diffusion rates of phospholipids within fluid membranes are high. In contrast, the lateral diffusion of most peripheral and integral proteins appears to be somewhat restricted.[15−17] Since FRAP experiments yield lateral diffusion coefficients (D), and the fraction of the probe that is mobile (M), they also provide *qualitative* evidence for the presence lateral heterogeneity.[18] In particular, if there is only a partial recovery of the fluorescence intensity, then the existence of lateral heterogeneity (<1 μm diameter) having relatively immobile molecules can be inferred. Such heterogeneity has, in fact, been identified in the plasma membrane in a variety of cells.[18]

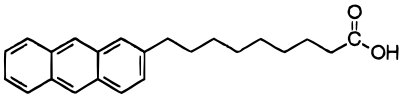
B. Detection of Lateral Heterogeneity by Chemical Cross-Linking

In contrast to FRAP experiments, which may be viewed as a macroscopic approach to the study of lateral heterogeneity, the use of chemical cross-linking methods may be considered as a molecular-level approach.[4] Three closely related cross-linking methods have previously been developed by three independent groups. Early studies by Marinetti and co-workers showed that 1,5-difluoro-2,4-dinitrobenzene can be used to cross-link phosphatidylserine (PS) and phosphoethanolamine (PE) that are present in erythrocyte membranes.[19−21] On the basis of the fact that nonstatistical mixtures of homodimers and heterodimers were formed, these researchers concluded that the PS and PE exist in small, nonrandom clusters. More recently, however, Tocanne has argued that a quantitative interpretation of such data is hazardous since the extent of dimer formation depends upon the reactant concentrations that are used, the reaction time, and also the distance between the two reactive groups of the cross-linking agent.[22]

In a related approach, Welti and Roth showed that binary mixtures of PE's could be cross-linked by use of dimethylsuberimidate.[23] In one such system [i.e., a bilayer composed of a 1/1 molar mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE)], a slight preference for homodimer formation was observed. These results were interpreted as indicating the presence of lateral heterogeneity within the precursor PE-based membrane.

Using a somewhat different approach, Tocanne and co-workers have developed a photochemical cross-linking strategy that employs anthracene-based phospholipids.[24] Here, photoinduced dimerization (formation of 9−9′, 10−10′ covalently bound dimers) results in the "capture" of nearest-neighbors. Specifically, since the dimerization reaction proceeds on a

nanosecond time scale, and since the time that is required for a lipid to exchange its lattice position with another lipid is ~100 ns, photodimerization corresponds to an integration in time of a series of instantaneous "pictures" of the lipid distribution within the membrane.[22] Using phosphatidylglycerols (PG's) and dimmanosyldiacylglycerols (DMDG's), bearing 9-(2-anthryl)nonanoic acid (**ANno**), these investigators demonstrated that photodimerization within the bacterium, *Micrococcus luteus*, leads to a nonrandom distribution of dimers. The primary conclusion that was drawn from this study was that these two lipids tend to self-aggregate within the bacterium. When similar photodimerization reactions were carried out in liposomal membranes that were devoid of proteins, however, only random distribution of dimers were observed. On the basis of these two findings, these researchers concluded that *membrane proteins induce lateral heterogeneity among these two classes of lipids.*



ANno

III. Nearest-Neighbor Recognition Analysis

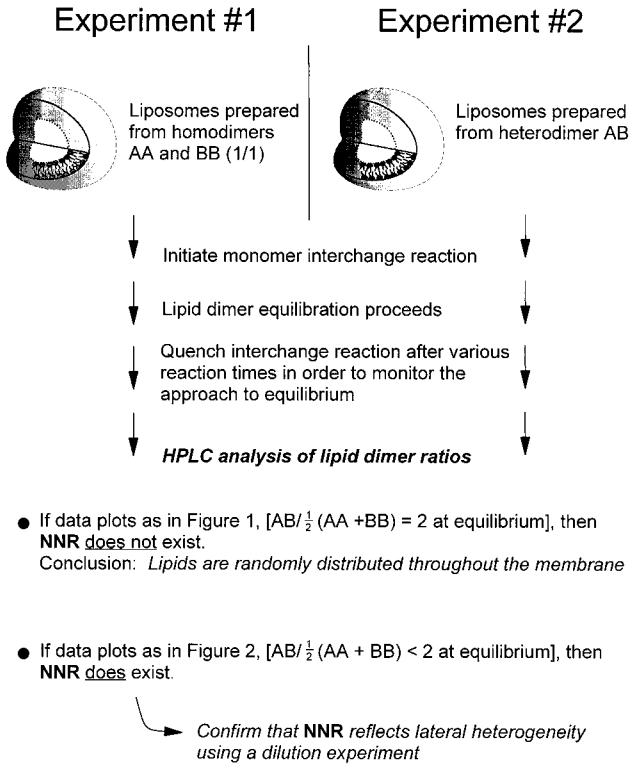
A. General Principles

In simplest terms, nearest-neighbor recognition involves *the chemical equilibration and analysis of phospholipid homodimers and heterodimers through the interchange of their monomeric components.* A "homodimer" refers to a molecule composed of two *identical* phospholipid units (monomers) that are covalently bonded through their head groups (AA and BB in Scheme 1). A 'heterodimer' is composed of two *different* covalently bonded phospholipid units (AB in Scheme 1). In a typical NNR experiment, two phospholipid molecules of interest (A and B) are first converted into these exchangeable homodimers and heterodimers (AA, BB, and AB). Phospholipid vesicles are assembled from an equimolar mixture of the homodimers (AA and BB, experiment 1), and separately, from pure heterodimer (AB, experiment 2).[25−27] A chemical reaction is then initiated in each of these vesicle systems that results in the generation of a small amount of reactive lipid monomer. This, in turn, begins the process of lipid monomer interchange by the breaking and re-forming of a particular covalent bond located in the head group region of the lipid dimers. The thermodynamically preferred equilibrium state of AA, AB, and BB has been reached when the product mixture in experiment 1 equals that in experiment 2.

When equilibrium dimer distributions are found to be statistical [i.e., when the molar ratio, R = AB/[$^1/_2$(AA + BB)] is 2.0], this finding establishes that (i) the phospholipid dimers as well as the individual monomer units are randomly distributed throughout

Scheme 1

Scheme 2. How NMR Analysis Works



the membrane, and (ii) there is no thermodynamic preference for one phospholipid to be a nearest-neighbor of another. When homodimers are favored, however, (i.e., when R is less than 2.0), *such a preference is defined as nearest-neighbor recognition* (NNR). Here, the dimers may be either randomly or nonrandomly arranged throughout the bilayer. In the former case, dimer stability is dominated by intramolecular interactions between the monomer units. In the latter case, intramolecular and intermolecular interactions contribute similarly to dimer stability, and NNR reflects the presence of lateral heterogeneity; i.e., *lateral segregation of the phospholipids.* 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.[28] Specifically, a laterally heterogeneous state is indicated by the reduction or elimination of NNR. In the limiting case, where $R = 0$ and where the introduction of a diluent promotes heterodimer formation, complete segregation of the phospholipids is indicated. Scheme 2 summarizes the essence of the NNR method. Finally, it should be noted that one very different type of NNR is also theoretically possible; i.e., when heterodimer formation is favored ($R > 2.0$). For convenience, we refer to this special type of recognition as *hetero*-NNR. To date, however, such recognition has not been observed. For this reason, and because *hetero*-NNR does not provide insight into the segregation properties of phospholipids (the primary objective of all NNR studies), no further consideration will be given to it in this review.

B. Relationship between NNR and Chemical Cross-Linking Approaches

Nearest-neighbor recognition bears a resemblance to the chemical cross-linking methods described above since the conclusions that are reached are based upon dimer distributions. In contrast to all of these methods, however, nearest-neighbor recognition *directly measures the thermodynamic preference for a phospholipid to become a nearest-neighbor of another in the bilayer state.*

Data that have been obtained from the cross-linking methods of Marinetti and Welti can only be interpreted if it is assumed that the interaction between half-reacted PE's with neighboring lipids is similar to the interaction of the "native" (precursor) PE with these same neighboring lipids.[19−21,23] How reasonable such an assumption is, however, is not clear. Similarly, interpretations of photodimer distributions that are produced from anthracene-based lipids (the Tocanne approach) also requires that certain assumptions be made. Specifically, one must assume that the dimerization constants for each of the three photocoupling reactions are identical, since *product mixtures are formed under kinetic control.* In addition, one must also assume that the photodimerization reaction occurs exclusively within the same membrane leaflet (head-to-head photodimers).[24] Although Tocanne has argued that cross-linkages between two lipids located in opposite leaflets (head-to-tail photodimers) are unlikely, since it would force the two lipids to overlap by six carbon atoms, there is certainly precedence for interdigitation in lipid bilayers.[29,30] Whether or not extensive interdigitation can occur in these systems, which would allow for head-to-tail dimer formation, remains to be established. Also, how different polar head groups may affect photodimerization constants between anthracene-based lipids remains to be defined.

In sharp contrast, for those NNR experiments that are based on lipid dimers having exchangeable groups located within the head group region, the formation of intermediates states, the reaction kinetics, and the formation of head-to-tail dimers are irrelevant issues. Although intermediates states are formed throughout the equilibration process, the NNR method is based only on the final equilibrium product mixture; i.e., *products are formed under thermodynamic control.* Since the placement of exchangeable groups in the head group region ensures that only head-to-head coupling is possible, coupling across the bilayer does not have to be considered. Although most phospholipids that occur in nature are monomeric (the exception being cardiolipin), and while the NNR method is based on the use of exchangeable phospholipid dimers, it should be noted that NNR analysis probes the mixing behavior of the *individual monomer components.* It is also noteworthy that NNR analysis can, in certain cases, yield insight into the *relative mixing behavior of natural phospholipids with exchangeable phospholipid monomers,* when they are used as membrane diluents for confirming lateral heterogeneity (*vide infra*).

C. Disulfide-Based Phospholipid Dimers

1. Design Rationale

All NNR experiments that have been carried out to date have employed *disulfide groups* as exchangeable bridging units. The disulfide moiety was specifically chosen for NNR studies because of its ability to undergo chemical exchange via thiolate−disulfide interchange reactions.[31,32] Thus deprotonation of thiols to form thiolate anions, which then can undergo facile S_N2 reactions with disulfides, produces a new disulfide molecule and a new thiolate anion (Scheme 3). For NNR experiments, the thiolate−disulfide interchange reaction is confined to the surface of the bilayer. Thiol-bearing phospholipid monomers that are required for this interchange reaction are either initially present in the membrane, or they can be generated by partial reduction of the lipid dimers by treatment with dithiothreitol. Since the extent of deprotonation can be adjusted by the pH of the dispersion, the kinetics of the thiolate−disulfide interchange can be conveniently controlled.

An illustration of a hypothetical disulfide-based lipid dimer equilibration is shown in Scheme 4. By quenching the interchange reaction at various time periods, the approach to equilibrium can be monitored starting from pure AB and also from a 1:1 molar mixture of AA and BB. Experiments that produce a statistical mixture of dimers result in a plot of the type that is shown in Figure 1. The upper curve in this stylized representation shows the progress of the equilibration reaction starting with vesicles prepared from the heterodimer, AB. The lower curve shows the progress of the reaction

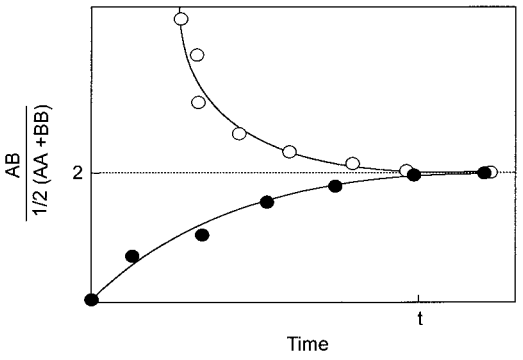
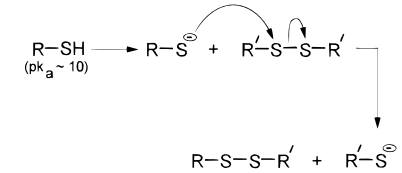
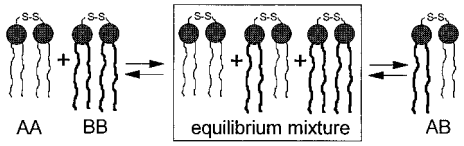


Figure 1. Stylized illustration of an equilibration experiment producing a statistical mixture of lipid dimers, starting from heterodimer (○) and a 1/1 mixture of homodimers (●).

Scheme 3



Scheme 4



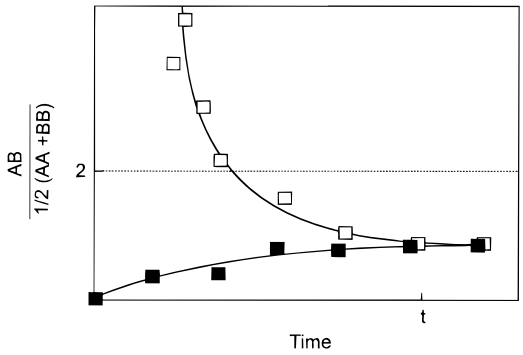


Figure 2. Stylized illustration of an equilibration experiment producing a nonstatistical mixture of lipid dimers, starting from heterodimer (□) and a 1/1 mixture of homodimers (■).

starting with vesicles composed of a 1:1 molar mixture of the homodimers AA and BB. After a given amount of time (*t*), both vesicles contain the same dimer composition. When the final molar ratio of AA/AB/BB is 1/2/1, a random distribution of lipids is indicated and NNR is absent. Equilibration experiments that favor homodimer formation indicate the presence of NNR (Figure 2).

2. Phospholipid Synthesis

The synthetic strategy that has been used to form exchangeable phospholipid dimers for NNR studies is outlined in Scheme 5. In brief, a PE of interest is first derivatized with the heterobifunctional coupling agent, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Subsequent deprotection with dithiothreitol (DTT) affords the corresponding thiol monomer, which is then coupled either with its precursor or with an analogous protected thiol to give a homodimer or a heterodimer, respectively.[33]

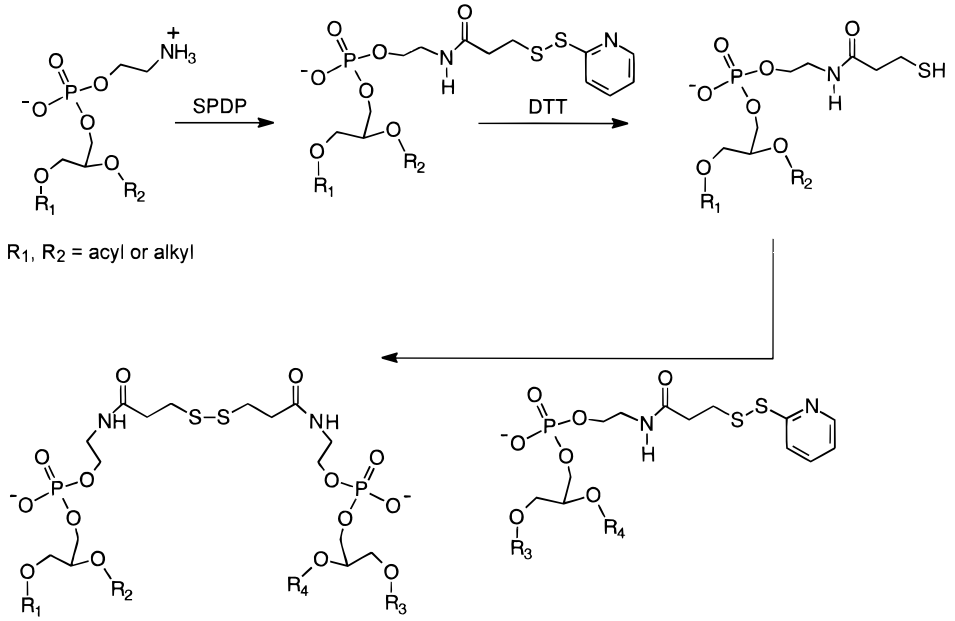
3. Melting Behavior of Disulfide-Based Phospholipid Dimers

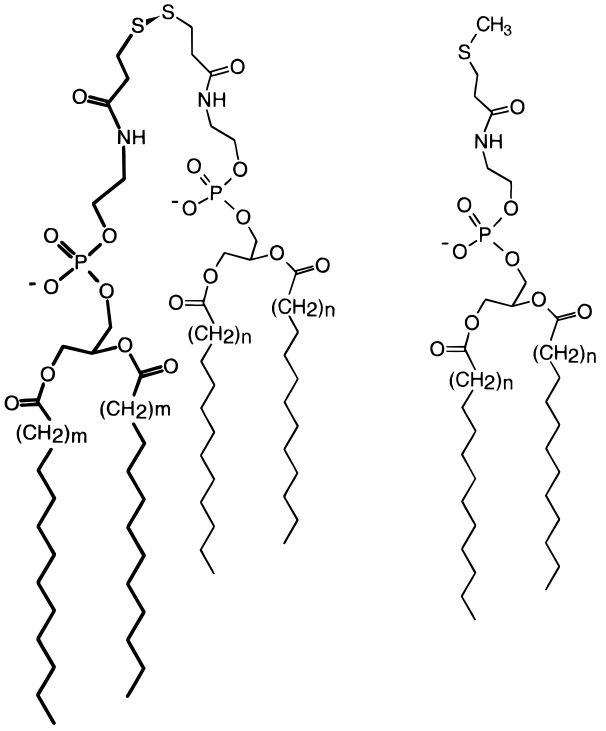
The temperature at which a phospholipid bilayer is half-converted from a gel into a fluid phase is, by definition, its characteristic gel to liquid crystalline phase transition temperature (T_m). Such temperatures are commonly measured by high-sensitivity differential scanning calorimetry (*hs*-DSC). At temperatures below the onset of such melting, the alkyl chains are in an ordered, all-anti configuration. When melting does occur, gauche conformations are introduced into the alkyl chains, and they become disordered and fluid-like in character.[34,35]

Figure 3 shows the structures of three phospholipid dimers whose monomer units resemble 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), as well as analogous phosphatidylglycerols, in terms of their fatty acid chains. For purposes of convenience, these dimers will be referred to as **14-14**, **16-16**, and **18-18**, respectively, where the number refers to the total number of carbon atoms in each saturated fatty acid. Measurement of the gel to liquid crystalline phase transition temperatures of each of these dimers, and also the corresponding calorimetric enthalpies and entropies per mole of phospholipid monomer, show a strong similarity to those of the corresponding phosphatidylcholines and phosphatidylgylcerols (Table 1).[36] In addition, the heterodimers, **14-16** and **14-18**, exhibit gel−fluid transition temperatures and enthalpies that are averaged values relative to the two corresponding homodimers. These observations imply that dimerization does not significantly alter the packing properties of the exchangeable monomers; a conclusion that is further supported by the fact that the melting characteristics of a monomeric analog (**16s**) is nearly identical to that of **16-16**.

On the basis of their melting behavior and their acidic head groups, **14-14**, **16-16**, and **18-18** may be viewed as excellent models for DMPG, DPPG, and DSPG, respectively. On the basis of their melting behavior alone, they would also appear to serve as good models for the analogous phosphocholines, even though the latter are zwitterionic.

Scheme 5





Homodimers

14-14, m=3; n=3
16-16, m=5; n=5
18-18, m=7; n=7

16s, n=5

Heterodimers

14-16, m=3; n=5
14-18, m=3; n=7

Figure 3. Chemical structures of saturated phospholipid dimers and a monomer analog.

Table 1. Gel to Liquid Crystalline Main Phase-Transition Properties for Phospholipid Dimers and Their Corresponding Phosphatidylcholines[57] and Phosphatidylglycerols[58]

phospholipid	T_m (°C)	$\Delta T_{1/2}$ (°C)[a]	ΔH (kcal/mol)	ΔS [cal/(K mol)]
DMPC	24.0		6.5	21.9
DMPG	23.7		6.9	23.3
14-14	22.7	0.5	14.7	49.7
DPPC	41.5	0.3	8.7	27.7
DPPG	41.5		8.9	28.3
16-16	41.9	0.4	18.7	59.4
16s	39.9	0.5	9.3	29.7
DSPC	54.3		10.4	33.3
DSPG	54.5		10.5	32.1
18-18	55.4	0.4	21.7	66.1
14-18	33.9	1.3	18.7	60.9
14-16	31.2	0.5	16.7	54.9

[a] Width at half-maximum excess specific heat.

With the exception of **14-18**, the peak widths at half-maximum excess specific heat ($\Delta T_{1/2}$) for all of the phospholipid dimers are small, and similar to those of conventional monomeric phospholipids. The unusually broad endotherm for **14-18** suggests that the chain melting process is less cooperative than for that of the homodimers, and that the effective size of the molecular unit, over which the motion of the molecules undergoing the phase transition is transmitted, is relatively small. On the basis of the observed $\Delta T_{1/2}$, and the measured calorimetric enthalpy of the transition (ΔH), the estimated aggregate size is ca. 27 molecules, where the aggregate size (or cooperativity unit, CU) is calculated from the relationship, $CU = \Delta H_{VH}/\Delta H$, and ΔH_{VH} is the van't Hoff enthalpy that is defined by the equation, $\Delta H_{VH} = 6.9\ (Tm^2/\Delta T_{1/2})$.[34,37] The cooperativity units that have been determined for **14-14**, **16-16**, and **18-18** are approximately three times larger than this value. The relatively low degree of cooperativity for **14-18** is a likely consequence of the mismatch in chain length of the two lipid components.

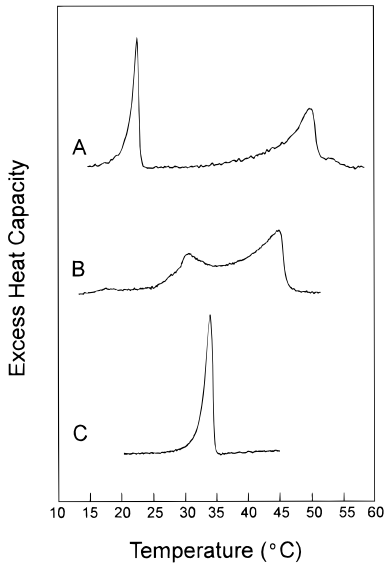


Figure 4. High-sensitivity excess heat capacity profile of (A) **14-14**/**18-18**, 1/1; (B) DMPC/DSPC, 1/1; (C) **14-18**.

Examination of a 1/1 molar mixture of **14-14** and **18-18** by *hs*-DSC reveals two distinct and broadened endotherms. This result indicates that these lipids phase separate in the gel−fluid coexistence region, where gel-like domains that are rich in **18-18** exist in a fluid "sea" that is rich in **14-14** (Figure 4A). The same general features can also be seen with a 1:1 molar mixture of DMPC and DSPC; in this case, however the extent of phase separation is considerably less (Figure 4B). Apparently, *homodimerization enhances the immiscibility of the two different monomer units*. A similar trend is also evident for phospholipids that differ by two methylenes per alkyl chain; e.g., compare the endotherms produced from a 1/1 molar mixture of **14-14** and **16-16** with that of a 1/1 mixture of DMPC/DPPC (Figure 5, parts A and B).

In sharp contrast, comparison of the melting behavior of bilayers composed of pure heterodimers (**14-18**, and also **14-16**) with that of membranes made from equimolar amounts of analogous phosphocholines (DMPC/DSPC and DMPC/DPPC, respectively) shows that *heterodimerization enhances the miscibility of the two different monomer units*. (Figures 4C and 5C). Thus, by "tying together" two phospholipids, which would otherwise be free to become randomly arranged in a bilayer, a more "uniform" membrane is created. In essence, a bilayer that is composed of a pure heterodimer "demands" a *uniform* (as opposed to random) distribution of the exchangeable monomers throughout the membrane; i.e., for every ~180 Å^2 within the membrane (an area that

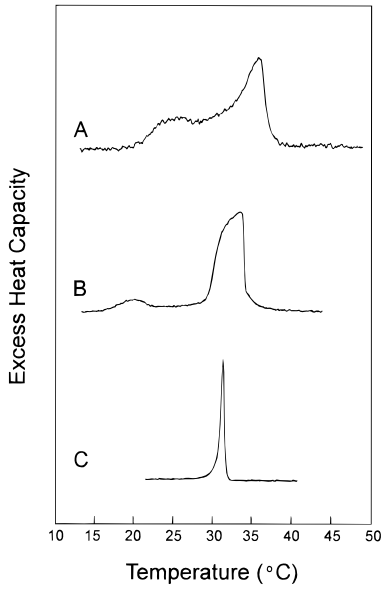


Figure 5. High-sensitivity excess heat capacity profile of (A) **14-14**/**16-16**, 1/1; (B) DMPC/DPPC, 1/1; (C) **14-16**.

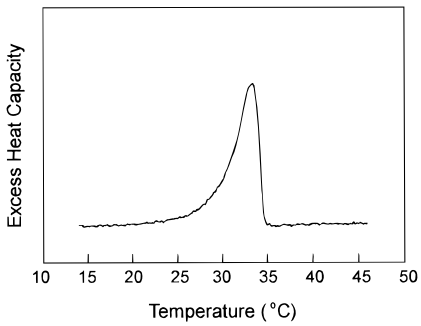


Figure 6. High-sensitivity excess heat capacity profile of **14-14**/**14-16**/**16-16** (1/2/1 molar ratio).

corresponds to approximately two lipid dimers), exactly two molecules of each monomer must be present. For this same reason, the endotherms that are observed for these heterodimers are much narrower than those of 1/1 molar mixtures of the corresponding phosphocholines (compare with Figures 4B and 5B).

Examination of a 1/2/1 molar mixture of **14-14**/**14-16**/**16-16** by *hs*-DSC further shows how the segregation effects that result from homodimerization are counterbalanced by the mixing effects of heterodimerization (Figure 6). Thus, in contrast to a 1/1 mixture of **14-14**/**16-16**, the resulting endotherm bears a striking similarity to that of a 1/1 molar mixture of DMPC and DPPC.

IV. Applications of Nearest-Neighbor Recognition

A. Chain Length Mismatch

In a previous report, which was based on a novel quick-freeze differential scanning calorimetry technique, it was concluded that DMPC and DPPC are completely immiscible in the fluid phase.[38] Such a conclusion, however, is exactly opposite to what one would predict on the basis of phase diagrams that have been constructed for these two lipids, where complete miscibility would be expected.[6,11] Thus, this question of whether or not a difference of only two methylene units per alkyl chain is sufficient to induce complete immiscibility between two phospholipids is open to debate. Since, **14-14** and **16-16** are good models of DMPC and DPPC, respectively, NNR experiments involving these dimers should provide insight into the influence of chain-length mismatch on lateral heterogeneity. An NNR analysis of bilayers composed of **14-14** and **18-18** (lipids differing by four methylenes per alkyl chain) also bears directly on the issue of chain-length mismatch.

In brief, NNR experiments that have been carried out with **14-14**/**16-16**, and also with **14-14**/**18-18**, at 60 °C (a temperature that maintains the fluid phase in each system) have shown that *a statistical mixtures of lipid dimers are produced in both cases*;[33] i.e., no NNR was observed. Thus, equilibrating phospholipid monomers that differ by as much as four methylene groups per alkyl chain are randomly arranged in the fluid state. Although the quick-freeze DSC results that have been reported are intriguing, on the basis of these NNR results, and also the phase diagram for DMPC/DPPC, it appears highly probable that these phosphocholines are *completely miscible in the fluid phase*.[33]

1. Cholesterol Effects

Despite numerous studies on the effects of cholesterol in phospholipid membranes,[9,39−42] its precise role in defining the lateral organization within the fluid phase is poorly understood. When high concentrations of cholesterol (∼20 mol %) are included in model membranes at temperatures that are in excess of the phospholipid's gel to liquid-crystalline phase transition temperature (i.e., conditions that simulate cholesterol-rich mammalian membranes), the bilayer moves from a liquid crystalline (L_α) phase to a more condensed "fluid β" phase, where the alkyl chains become more fully extended.[43]

In order to probe the effects that cholesterol has on chain length mismatch, NNR experiments have been carried out using bilayers composed of **14-14**/**14-16**/**16-16**, and also **14-14**/**14-18**/**18-18** phospholipid dimers at temperatures that are in excess of the T_m for the highest melting dimer.[44,45]

Results that have been obtained for **14-14**, **14-16**, and **16-16**, in the presence of varying concentrations of cholesterol (9−29 mol %) indicate that *this sterol has no measurable effect upon the lateral organization of the phospholipids*.[44,45] In all cases, a random mixture of dimers was obtained. In sharp contrast, for equilibrated bilayers composed of **14-14**, **14-18**, and **18-18**, the presence of 17 mol % cholesterol induced significant NNR.[28,44,45] For example, bilayers that were made from a 1/1/0.8 molar mixture of **14-14**/**18-18**/cholesterol and also from ones that were made from a 1/0.4 molar mixture of **14-18**/cholesterol (17 mol % cholesterol in each case) at 60 °C yielded an equilibrium heterodimer (**14-18**)/homodimer (**14-14**) ratio of 1.55 ± 0.08.[28] This level of NNR corresponds to a thermodynamic preference for homodimer formation of $\Delta G = \sim 170$ cal/mol. Apparently, the sterol's effect on NNR is a sensitive function of the difference in chain length among the equilibrating monomers.

2. Evidence for Lateral Heterogeneity

In order to clarify whether or not NNR in membranes composed **14-14**, **14-18**, **18-18**, and cholesterol reflects lateral heterogeneity, analogous membranes were prepared in which ~50% of the phospholipids were replaced by DPPC. Since DPPC has a fatty acid chain length and a melting temperature that is intermediate between that of **14-14** and **18-18**, one might expect that it could act as a mixing agent and reduce or eliminate NNR, if lateral heterogeneity was present. Since DMPC has a T_m and fatty acid chain length that are the same as that of **14-14**, one would also expect that it would be a relatively poor mixing agent. Consistent with these hypotheses, inclusion of DPPC was found to completely eliminate NNR in the **14-14**/**14-18**/**18-18**/cholesterol-based membranes; dilution with DMPC, however, had little effect on the extent of NNR.[28] These experimental results provide compelling evidence that, in the absence of DPPC, these equilibrating lipids are nonrandomly arranged and that lateral heterogeneity is present. The fact that DMPC is much less effective than DPPC in eliminating lateral heterogeneity further indicates that *DMPC is heterogeneously distributed within these membranes relative to DPPC*.

3. Nearest-Neighbor Recognition in the Gel–Fluid Coexistence Region

Although NNR studies in the physiologically relevant fluid phase has the greatest biological relevance, analogous experiments that can be performed in the gel−fluid coexistence region are useful since they provide further evidence that such equilibration measurements can detect lateral heterogeneity. For example, significant NNR is expected in the gel−fluid region for those systems that mimic DMPC/DSPC bilayers, but not those that mimic DMPC/DPPC membranes, since only the former shows a clear phase separation by *hs*-DSC. Consistent with this prediction, the equilibrium dimer ratio of **14-18**/**14-14** at 33 °C was 0.76 ± 0.06/1; in contrast, a 2/1 equilibrium dimer ratio was observed for **14-16**/**14-14** at that same temperature.

B. Mixed Saturated and Unsaturated Systems

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 in a mixture of saturated and unsaturated lipids has biological relevance. Although recent studies have shown that certain strains of bacteria have phosphoethanolamines with both *cis* and *trans* double bonds, it is the *cis* configuration that predominates in nature.[46] For example, only *cis* double bonds are present in the lipids found in mammalian cells.[46] For this reason, the mixing behavior of saturated lipids with unsaturated analogs bearing *cis* double bonds have been investigated in fluid-phase membranes.[47]

Specific saturated lipid dimers that have been used in NNR analysis of the mixing behavior of saturated and unsaturated phospholipids were **14-14**, **16-16**, and **18-18**; unsaturated homodimers that were employed were derived from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). For convenience, these unsaturated dimers are designated as **PO-PO** and **DO-DO**, respectively; the corresponding heterodimers that were required for the NNR experiments are referred to as **14-PO**, **18-PO**, **14-DO**, **16-DO**, and **18-DO**.

Table 2. Nearest-Neighbor Recognition within Saturated/Unsaturated Phospholipid Membranes

equilibrating lipid dimers	cholesterol[a] (mol %)	T (°C)	heterodime/ homodimer[b]
14-14/14-PO/PO-PO	0	40	1.90 ± 0.07
	29	40	1.82 ± 0.08
18-18/18-PO/PO-PO	0	60	1.78 ± 0.07
	29	60	1.77 ± 0.04
14-14/14-DO/DO-DO	0	60	1.97 ± 0.04
	29	60	1.87 ± 0.03
16-16/16-DO/DO-DO	0	55	1.79 ± 0.05
	29	55	1.89 ± 0.09
18-18/18-DO/DO-DO	0	60	1.69 ± 0.05
	29	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 equilibrium. [c] The thiol monomer content used was 10 mol %.

Table 2 lists the equilibrium ratios of heterodimer/homodimer that have been determined for the various combinations of saturated and unsaturated phospholipids.[47] The first two entries show the results obtained with **PO**-based bilayers. Only a slight indication of NNR was found in the **14**/**PO** system; with analogous bilayers prepared from a longer saturated phospholipid (**18**/**PO** system) significant recognition was observed. Similar chain length effects were found in the doubly unsaturated, **DO**-based membranes. For example, progression from the **14**/**DO** to **16**/**DO** to the **18**/**DO** systems resulted in a continuous increase in NNR from 1.97 ± 0.04 to 1.69 ± 0.05. Addition of 29 mol % of cholesterol to each of these membranes had little influence on the extent of NNR. This is in sharp contrast to the results obtained from fluid phase bilayers prepared from the saturated lipid dimers **14-14**/**14-18**/**18-18** where the addition of high levels of cholesterol induced significant NNR.[44,45] Apparently, the presence of unsaturated dimers, having very low melting temperatures, helps to maintain a more "liquid-like" state and minimizes van der Waals forces between the lipids.

The magnitude of NNR that has been observed in these systems has been found to correlate with the difference between the gel to liquid crystalline phase transition temperatures of the homodimers; i.e., the larger the difference, the greater the recognition. For example, the **18**/**DO** system has the greatest difference in T_m values and also the greatest level of NNR. With **16**/**DO**-based membranes, the difference in T_m between the homodimers is less, as is the extent of NNR. In the case of the **14**/**DO** system, which has

the smallest difference in T_m, no significant recognition could be detected. The observation that NNR decreases as the chain length difference between the exchanging monomer units increases indicates that the influence of chain length mismatch on NNR is relatively unimportant in these systems.

Evidence that NNR in these systems reflects lateral heterogeneity was obtained using the membrane dilution technique described above. Here, the thiolate−disulfide interchange reactions were carried out in membranes containing a 1/1 molar mixture of **18**-18/**PO-PO** (and also pure **18-PO**) in which 50% of the exchangeable monomer units were replaced by DMPC. This lipid was chosen because it has an intermediate phase-transition temperature of 24 °C, and was expected to be an effective mixing agent. Resulting equilibrium distribution were found to be very close to random (Table 2). Similar dilution studies that were conducted in the **18**/**DO** bilayer system also support the existence of lateral heterogeneity. These NNR recognition results provide the strongest evidence to date that relatively modest differences in the fatty acid structure of the phospholipids can produce lateral heterogeneity in the fluid phase, in the absence of other added components, such as sterols, metal ions, or proteins.[48,49]

Finally, it is interesting to note that the correlation that has been observed between the difference in T_m values among the exchanging lipids and the magnitude of NNR bears a striking resemblance to what has been found in Welti's cross-linking experiments.[23] Specifically, the reaction of dimethylsuberimidate with fluid bilayers composed of 1/1 molar mixtures of DPPE/DOPE yielded a heterodimer/homodimer ratio of 1.85. In contrast, analogous dimerization reactions that were carried out with 1/1 molar mixtures of dielaidoylphosphoethanolamine (DEPE)/DMPE and also with DEPE/dilauroylethanolamine (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 PE's having the greatest difference in T_m's) affords a nonrandom distribution of dimers is exactly analogous to what has been observed in NNR studies.

C. Ester/Ether Mismatch

In very recent studies, the mixing behavior of ester- and ether-based phospholipids has been examined in the fluid phase.[50] While most phospholipids in biomembranes are of the diacyl-type, significant quantities of lipids that contain ether linkages, i.e., plasmalogens, are also present. The role that these ether lipids play with respect to membrane function, and how they are laterally organized in the bilayer, are two important questions that have yet to be clarified.[51] In previous work, it has been shown that gel-phase bilayers composed of diacylphospholipids behave quite differently than their ether-based analogues. For example, whereas DPPC forms the usual bilayer structure in the gel phase, the ether analogue, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC) adopts chain-interdigitated "monolayers".[52] Thus, the seemingly minor replacement of two ester carbonyl groups with two methylene units can have a profound effect on the organizational properties of the phospholipid. The effect of these structural differences on lipid organization in the physiologically relevant fluid phase, however, has remained uncertain.

Table 3. Nearest-Neighbor Recognition within Ether/Ester Phospholipid Membranes

equilibrating lipid dimers	cholesterol[a] (mol %)	T (°C)	heterodimer/homodimer[b]
14-14/14-DH/DH-DH	0	60	1.84 ± 0.04
	29	60	1.88 ± 0.02
16-16/16-DH/DH-DH	0	60	1.98 ± 0.01
	29	60	1.98 ± 0.01
18-18/18-DH/DH-DH	0	60	2.03 ± 0.03
	29	60	1.95 ± 0.06
14-14/14-DH/DH-DH + 50 mol % DPPC	0	60	2.03 ± 0.02

[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 equilibrium.

Nearest-neighbor recognition studies that have been carried out have addressed three specific questions concerning ester/ether miscibility: (i) Can the replacement of both ester carbonyl groups of a phospholipid with two methylene units provide a driving force for lateral heterogeneity? (ii) Can an ester/ether mismatch combine, synergistically, with a chain length mismatch to produce lateral heterogeneity? (iii) Can the presence of cholesterol significantly affect the mixing behavior of ester- and ether-based phospholipids? The specific experiments that were carried out in order to address these questions were based on the lipid diacyl dimers **14-14**, **16-16**, and **18-18** and an ether-based analogue of **16-16**; i.e., a dimer derived from 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE), which has been designated as **DH-DH**. The corresponding heterodimers that were employed have been referred to as **14-DH**, **16-DH**, and **18-DH**. The first question posed above was addressed by examining equilibrated bilayers composed of **16-16**/**16-DH**/**DH-DH**, where the ester carbonyls were simply replaced by methylene groups. The second question was addressed by use of membranes composed of **14-14**/**14-DH**/**DH-DH** and **18-18**/**18-DH**/**DH-DH**. Finally, the third question was examined by carrying out NNR experiments in the absence and presence of cholesterol.

The T_m values that were measured for the **DH**-bearing dimers were: 46.2 °C (**DH-DH**), 32.3 °C (**14-DH**), 44.2 °C (**16-DH**), and 50.7 °C (**18-DH**). In each case, the peak width at half-maximum specific heat capacity was 0.5 °C. The T_m value that was measured for the ether phospholipid dimer, **DH-DH**, was significantly higher than that of its ester analog, **16-16**; a result that is analogous to the corresponding phosphocholines; i.e., whereas the T_m of 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC) is 43.7 °C, the T_m for DPPC is 41.5 °C.

Nearest-neighbor recognition experiments that were carried out with **16-16**/**16-DH**/**DH-DH** resulted in a statistical mixture of dimers (Table 3). Clearly, the

replacement of two carbonyl groups with two methylene units, in and of itself, is not sufficient in order to provide a driving force for creating lateral heterogeneity. However, in equilibrated bilayers composed of **14-14/14-DH/DH-DH**, NNR was observed; i.e., the observed ratio of heterodimer/homodimer was 1.84 ± 0.04. The elimination of this recognition by the addition of DPPC (having an intermediate T_m) also provides compelling evidence for the presence of lateral heterogeneity.[28] Interestingly, when the longer saturated diacyl dimer (**18-18**) was examined in combination with **DH-DH**, NNR was not observed. It should be noted that while the two exchanging monomer units in each of these systems differ by two carbons, *the difference in the number of methylene groups is not the same*; i.e., **14**, **DH**, and **18** monomers contain 12, 15, and 16 methylene groups, respectively. Therefore, converting the ester carbonyl group into a methylene group "moves" the hydrophobicity of the hydrocarbon chains of **DH** closer to that of the **18** monomer. This smaller difference in hydrophobicity between the exchanging monomers is apparently insufficient for nearest-neighbor recognition.

The fact that NNR is observed in the **14**/**DH**-based membranes, but not in **14**/**18**-based systems is intriguing. Since **14** and **DH** have a smaller difference in hydrophobicity than **14** and **18** (three vs a four methylene group difference), the observation of NNR in only the former system indicates that the absence of carbonyl groups in **DH** plays a key role in creating lateral heterogeneity. Since an ester/ether mismatch, by itself, is not sufficient to produce lateral heterogeneity, and since saturated diacylphospholipids that differ by four methylene groups distribute themselves randomly in the fluid phase, the ester/ether mismatch in bilayers of **14-14/14-DH/DH-DH** must be combining, synergistically, with the three-methylene unit difference to produce a heterogenous state.[50]

Finally, examination of the effects of cholesterol on NNR in each of these systems indicates that its presence does not enhance the extent of recognition (Table 3). Apparently, the condensing effect of this sterol cannot be detected through either the ester/ether or the hydrophobic mismatches that exist within these mixed bilayers.

V. Biological Relevance and Prospectus

Nearest-neighbor recognition measurements provide an experimental basis for sorting out the relationship that exists between the molecular structure of phospholipids and their tendency to cluster in the physiologically relevant fluid phase. Studies that have been carried out to date, for example, have demonstrated that chain length mismatch, membrane compactness, presence of unsaturated chains, and ether vs acyl linkages all contribute to lateral organization in the bilayer state. The tendency of ether-linked lipids to cluster in membranes that contain both ester- and ether-based phospholipids is particularly noteworthy. This fact, together with the fact that unusually high concentrations of ether lipids are known to exist in a variety of human brain tumors,[53−56] suggests that such clustering may contribute to the malignant state.

The finding that relatively modest differences in lipid composition and structure can create lateral heterogeneity in the fluid phase further suggests that other and more pronounced differences among phospholipids should contribute to an even greater extent. It seems highly likely, for example, that differences in head group charge (e.g., negatively charged versus zwitterionic), and the presence of hydrogen-bonding elements within and beneath the head group region (e.g., phosphoethanolamines, and sphingomyelins, respectively), should play an important role in defining the organizational state of a fluid membrane. In order to test this hypothesis, however, new exchangeable phospholipid dimers will have to be synthesized that more closely mimic the natural phosphocholines, phosphoethanolamines, sphingomyelins, etc. Such lipids would also allow one to begin to examine the effects that positively charged peptides (e.g., polymixin B), and divalent metal ions (e.g., Ca^{2+}) have on the lateral organization of membranes composed of zwitterionic and negatively charged lipids. The effects that integral and peripheral proteins have on NNR in each of these systems would also be revealing. Finally, it should be noted that although NNR studies have been confined to relatively simple model systems, similar experiments should also be possible in much more complex assemblies (e.g., reconstituted membranes) provided that the analytical chemistry remains tractable. The feasibility of analyzing for lipid dimers, produced in erythrocyte membranes and the bacterium, *Micrococcus luteus*, using the cross-linking methods of Marinetti and Tocanne, provides ample cause for optimism that analogous NNR studies should also be possible.

VI. Conclusions

Nearest-neighbor recognition methods provide a unique opportunity for probing the thermodynamic preference for one lipid to become a nearest-neighbor of another in the bilayer state. Studies that have been carried out to date have demonstrated that lateral heterogeneity can exist in the fluid phase and have begun to unravel some of the variables that control lateral organization.

The need for further studies that can sort out the relationships that exist between molecular structure and lateral organization within the physiologically relevant fluid phase is 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.

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VIII. References

(1) Gennis, R. B. *Biomembranes: Molecular Structure and Function;* Springer-Verlag: New York, 1989.
(2) For a recent collection of articles on this subject, see: (i) *Comments Mol. Cell. Biophys.* **1992**, *8*, 1. (ii) *Mol. Membr. Biol.*

1995, *12*, 1. For reviews of lipid mixing in monolayer membranes, see: (i) McConnell, H. M. *Annu. Rev. Phys. Chem.* **1991**, *42*, 171. (ii) Mohwald, H. Phospholipid Monolayers. In *Phospholipids Handbook;* Cevc, G., Ed.; Dekker: New York, 1993; pp 579−602.
(3) Welti, R.; Glaser, M. *Chem. Phys. Lipids* **1994**, *73*, 121.
(4) Tocanne, J.-F.; Cezanne, L.; Lopez, A.; Piknova, B.; Schram, V.; Tournier, J.-F.; Welby, M. *Chem. Phys. Lipids* **1994**, *73*, 139.
(5) Singer, S. J.; Nicolson, G. L. *Science* **1972**, *175*, 720.
(6) Mabrey, S.; Sturtevant, J. M. *Proc. Natl. Acad. Sci. USA* **1976**, *73*, 3862.
(7) Bach, D. Calorimetric Studies of Model and Natural Biomembranes. In *Biomembrane Structure and Function*; Chapman, D., Ed.; Verlag Chemie: Basel, 1984; pp 1−41.
(8) Ali, S.; Lin, H.-N.; Bittman, R.; Huang, C.-H. *Biochemistry* **1989**, *28*, 522.
(9) Lentz, B. R.; Barrow, D. A.; Hoechli, M. *Biochemistry* **1980**, *19*, 1943.
(10) Shimshick, E. J.; McConnell, H. M. *Biochemistry* **1973**, *12*, 2351.
(11) Luna, E. J.; McConnell, H. M. *Biochim. Biophys. Acta* **1978**, *509*, 462.
(12) Hui, S. W. *Biophys. J.* **1981**, *34*, 383.
(13) Copeland, B. R.; McConnell, H. M. *Biochim. Biophys. Acta* **1980**, *599*, 95.
(14) Verkleij, A. J.; Ververgaert, P. H. J.; De Kruyff, B.; Van Deenen, L. L. M. *Biochim. Biophys. Acta* **1974**, *373*, 495.
(15) Etemadi, A. H. *Biochim. Biophys. Acta* **1980**, *604*, 347.
(16) Benga, G.; Holmes, R. P. *Prog. Biophys. Mol. Biol.* **1984**, *43*, 195.
(17) Cherry, R. J. *Biochmim. Biophys. Acta* **1979**, *559*, 289.
(18) Tocanne, J. F.; Cezanne, L. D.; Lopez, A.; Tournier, J. F. *FEBS Lett.* **1989**, *257*, 10.
(19) Marinetti, G. V. *Biochim. Biophys. Acta* **1977**, *465*, 198.
(20) Marinetti, G. V., Love, R. *J. Membr. Biol.* **1976**, *30*, 213.
(21) Marinetti, G. V.; Crain, R. C. *J. Supramol. Struct.* **1978**, *8*, 191.
(22) Tocanne, J-F. *Comments Mol. Cell. Biophys.* **1992**, *8*, 53.
(23) Roth, M. R.; Welti, R. *Biochim. Biophys. Acta* **1991**, *1063*, 242.
(24) DeBony, J.; Lopez, A.; Gilleron, M.; Welby, M.; Laneelle, G.; Rousseau, B.; Beaucourt, J. P.; Tocanne, J. F. *Biochemistry* **1989**, *28*, 3728.
(25) Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R. *Biochim. Biophys. Acta* **1985**, *812*, 55.
(26) Szoka, F.; Papahadjopoulos, D. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4194.
(27) *Liposomes: A Practical Approach*; New, R. R. C., Ed.; Oxford University Press: New York, 1989; p 72.
(28) Vigmond, S. J.; Dewa, T.; Regen, S. L. *J. Am. Chem. Soc.* **1995**, *117*, 7838.
(29) Ruocco, M. J.; Siminovitch, D. J.; Griffin, R. G. *Biochemistry* **1985**, *24*, 2406.
(30) Boggs, J. M.; Wang, H.-Y.; Rangaraj, G.; Tummler, B. *Biochim. Biophys. Acta* **1989**, *985*, 199.
(31) Houk, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, *109*, 6825.
(32) Singh, R.; Whitesides, G. M. *J. Am. Chem. Soc.* **1990**, *112*, 6304.
(33) Krisovitch, S. M.; Regen, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 9828.
(34) Mabrey-Gaud, S. In *Liposomes: From Physical Structure To Therapeutic Applications;* Knight, C. G., Ed.; Elsevier/North Holland Biomedical Press: New York, 1981; Chapter 5.
(35) (a) For a review, see: Marsh, D. *Chem. Phys. Lipids* **1991**, *57*, 109. (b) Trauble, H.; Haynes, D. H. *Chem. Phys. Lipids* **1971**, *7*, 324. (c) Oldfield, E.; Chapman, D. *FEBS Lett.* **1972**, *21*, 302.
(36) For a general discussion of the thermotropic behavior of phospholipid analogues, see: Silvius, J. R. *Chem. Phys. Lipids* **1991**, *57*, 241.
(37) Mabrey, S.; Sturtevant, J. M. *Methods Memb. Biol.* **1978**, *9*, 237.
(38) Melchior, D. L. *Science* **1986**, *234*, 1577; **1987**, *238*, 550.
(39) Presti, F. T. In *Membrane Fluidity in Biology;* Aloia, R. C., Boggs, J. M., Eds.; Academic Press: New York, 1985; Vol. 4, pp 97−146.
(40) Yeagle, P. L. *Biochim. Biophys. Acta* **1985**, *822*, 267.
(41) Finnean, J. B. *Chem. Phys. Lipids* **1990**, *54*, 147.
(42) (a) Mabrey, S.; Mateo, P. L.; Sturtevant, J. M. *Biochemistry* **1978**, *17*, 2464. (b) Melchior, D. L.; Scavitto, F. J.; Steim, J. M. *Biochemistry* **1980**, *19*, 4828. (c) Subcznski, W. K.; Antholine, W. E.; Hyde, J. S.; Kusimi, A. *Biochemistry* **1990**, *29*, 7936. (d) Sankaram, M. B.; Thompson, T. E. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8686. (e) Almeida, P. F.; Vaz, W. L. C.; Thompson, T. E. *Biophys. J.* **1993**, *64*, 399. (f) Spink, C. H.; Manley, S.; Breed, M. *Biochim. Biophys. Acta* **1996**, *1279*, 190.
(43) Vist, M. R.; Davis, J. H. *Biochemistry* **1990**, *29*, 451.
(44) Krisovitch, S. M.; Regen, S. L. *J. Am. Chem. Soc.* **1993**, *115*, 1198.
(45) Davidson, S. M. K.; Liu, Y.; Regen, S. L. *J. Am. Chem. Soc.* **1993**, *115*, 10104.
(46) Okuyama, H.; Okajima, N.; Sasaki, S.; Higashi, S.; Murata, N. *Biochim. Biophys. Acta* **1991**, *1084*, 13.
(47) Dewa, T.; Vigmond, S. J.; Regen, S. L. *J. Am. Chem. Soc.* **1996**, *118*, 3435.
(48) Wu, H.-W.; McConnell, H. M. *Biochemistry* **1975**, *14*, 847.
(49) Silvius, J. R. *Biochim. Biophys. Acta* **1986**, *857*, 217.
(50) Dewa, T.; Regen, S. L. *J. Am. Chem. Soc.* **1996**, *118*, *7069*.
(51) Paltauf, F. *Chem. Phys. Lipids* **1994**, *74*, 101.
(52) (a) Kim, J. T.; Matti, J.; Shipley, G. G. *Biochemistry* **1987**, *26*, 6592. (b) Laggner, P.; Lohner, K.; Degovics, G.; Muller, K.; Schuster, A. *Chem. Phys. Lipids* **1987**, *44*, 31. (c) Ruocco, M. J.; Siminovitch, D. J.; Griffin, R. G. *Biochemistry* **1985**, *24*, 2406.
(53) Albert, D. H.; Andersen, E. C. *Lipids* **1977**, *12*, 188.
(54) Paltauf, F. In *Ether Lipids: Biochemical and Biomedical Aspects*; Mangold H. K., Paltauf, F., Eds.; Academic Press: New York, 1983; p 314.
(55) Marinetti, G. V.; Crain, R. C. *J. Supramol. Struct.* **1978**, *8*, 191.
(56) Satoh, O.; Umeda, M.; Imai, H.; Tunoo, H.; Inoue, K. *J. Lipid Res.* **1990**, *31*, 1293.
(57) Blume, A. *Biochemistry* **1983**, *22*, 5436.
(58) Findlay, E. J.; Barton, P. G. *Biochemistry* **1978**, *17*, 2400.

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