Push and Pull Forces in Lipid Raft Formation: The Push Can Be as Important as the Pull

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Ⓢ *Supporting Information*

ABSTRACT: Nearest-neighbor recognition measurements have been made using exchangeable mimics of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine in the liquid-ordered (l_o) and liquid-disordered (l_d) states. In the l_d phase, the net interaction between these two lipids is repulsive. In the l_o phase, their interactions are neither attractive nor repulsive. These results, together with previous nearest-neighbor measurements, imply that the overall driving force for lipid domain formation in bilayers composed of high-melting lipids, low-melting lipids, and cholesterol, corresponds to a strong pull (attraction) between the high-melting lipids and cholesterol, a significant push (repulsion) between the low-melting and high-melting lipids, and a significant push between the low-melting lipids and cholesterol. In a broader context, these results provide strong support for the notion that repulsive forces play a major role in the formation of lipid rafts.

The lipid raft hypothesis, expressed in simplest terms, assumes that cholesterol combines with high-melting sphingolipids to form transient "rafts" that float in a "sea" of phospholipids.[1−6] Despite growing acceptance of this hypothesis, many key questions remain unanswered. In particular, the average size, size distribution, and lifetimes of such domains remain to be defined.[7−9] At a more fundamental level, the forces that drive lipid domain formation have not been firmly established.[10] To date, virtually all of the attention has focused on attractive interactions between cholesterol and sphingolipids or high-melting mimics such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC).[11−13] Remarkably little attention has been paid to the role that low-melting phospholipids may play in lipid raft formation. This is especially surprising in view of the abundance of phospholipids in cell membranes. In one previous study, fluorescence resonance energy transfer measurements that were combined with Monte Carlo simulations yielded inferential evidence for the existence of *repulsive* interactions between 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphochline (POPC) and porcine brain sphingomyelin and with cholesterol.[14]

Our own efforts in this area have focused, sharply, on obtaining *direct, quantitative insight into nearest-neighbor interactions* in model systems using the nearest-neighbor recognition (NNR) method.[15] In brief, NNR measurements quantify the thermodynamic tendency of exchangeable lipids to become nearest-neighbors of one another. Typically, two lipids of interest (**A** and **B**) are converted into exchangeable homodimers **AA** and **BB** and the corresponding heterodimer, **AB**, via the introduction of a disulfide bond. By allowing the lipid monomers to undergo exchange via thiolate−disulfide interchange, an equilibrium mixture is produced that yields an equilibrium constant, *K* (Figure 1).[16] Random mixing is then

$$ AA + BB \underset{}{\overset{K}{\rightleftharpoons}} 2\ AB $$

$$ K = \frac{[AB]^2}{[AA]\,[BB]} $$
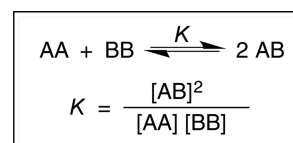
Figure 1. Homodimers **AA** and **BB** in equilibrium with the heterodimer **AB**.

indicated by an equilibrium constant equaling 4.0. In contrast, favored homoassociations are indicated by values of *K* that are less than 4.0, and favored heteroassociations are indicated by *K* > 4.0. The nearest-neighbor interaction free energy between **A** and **B** (i.e., the net interaction between **A** and **B**) is then given by $\omega_{AB} = -1/2RT \ln(K/4)$. Since the heterodimer is statistically favored over each homodimer by a factor of 2, the value of *K* must be divided by 4. In addition, a factor of 1/2 is included in this equation to take into account the fact that two dimers are involved in this equilibrium.

In our NNR studies, we have made extensive use of exchangeable lipids, **1**, **2**, and **3**, for probing nearest-neighbor interactions in lipid bilayers (Chart 1). As discussed elsewhere, in detail, despite the negative charge and the presence of a disulfide linkage in **1** and **3**, these lipids have proven to be excellent mimics of DPPC and the cyclopropyl derivative of POPC, 1-palmitoyl-2-dihydrosterculoyl-*sn*-glycero-3-phosphocholine (PDSPC), respectively.[15] Also, despite the presence of a disulfide linkage in **2**, this exchangeable sterol has proven to be an excellent mimic of cholesterol.

Previous NNR measurements that were made at 45 °C have shown that the net interaction between **1** and **2** is strongly attractive in the liquid-ordered (l_o) phase but is neither attractive nor repulsive in the l_d phase.[17] In sharp contrast, the net interaction between **2** and **3** is significantly repulsive in the l_d phase but is neither attractive nor repulsive in the l_o phase.[18] A diagram that summarizes these net interactions is shown in Figure 2. What has been sorely missing are direct measure-

Chart 1

Scheme 1
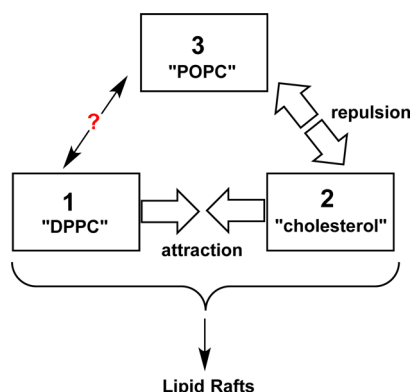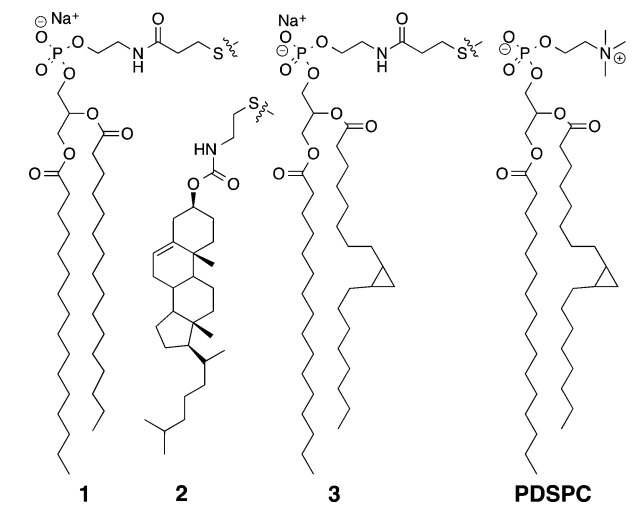
Figure 2. Diagram illustrating (i) strong attractive interactions between **1** and **2**, (ii) significant repulsive interactions between **2** and **3**, and (iii) unknown interactions between **1** and **3** in the l_o and l_d phases.

ments that quantify the interactions between **1** and **3** in the l_o and l_d phases. To complete this diagram, we have now measured these interactions.

With this objective in mind, we first synthesized the requisite heterodimer {**1-3**} using the sequence of reactions that is outlined in Scheme 1. Thus, PDSPC was converted into the corresponding phosphoethanolamine, **4**, by treatment with phospholipase D in the presence of ethanolamine. Subsequent condensation with 3-(2-pyridyldithio)propionic acid (using 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]-pyridinium 3-oxide hexafluorophosphate as the condensing agent) to give **5**, followed by reduction with threo-1,4-dimercapto-2,3-butanediol (DTT) and coupling with N-[3-(2-pyridyldithio)propionyl]-1,2-dipalmitoyl-sn-glycero-3-phospho-ethanolamine, **6**, afforded {**1-3**}. The syntheses of the corresponding homodimers {**1-1**} and {**3-3**} have previously been described.[17,18]

Using established procedures, large unilamellar liposomes (200 nm, extrusion) were prepared from PDSPC as the host membrane along with 2.5 mol % of {**1-3**}. At 45 °C, these membranes are fully in the l_d phase.[18] Thiolate−disulfide interchange was then initiated by addition of DTT at pH 7.4. Measurement of the formation of the homodimers {**1-1**} and {**3-3**} and the depletion of the {**1-3**} as a function of time afforded an equilibrium concentration of dimers that was
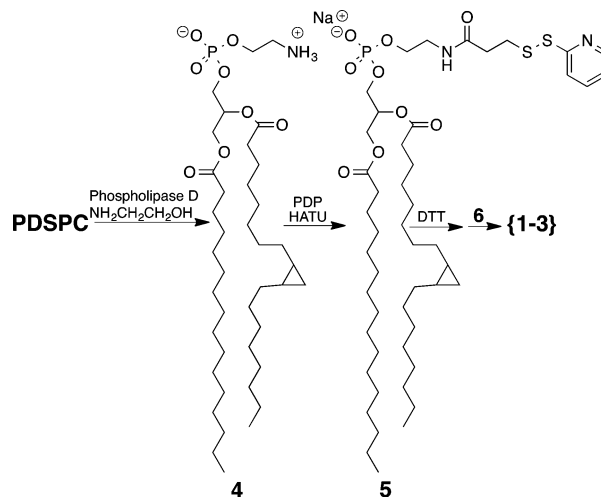
governed by $K = 2.5 \pm 0.23$. This corresponds to a net push of $\omega_{AB} = +150 \pm 30$ cal/mol. Dynamic light scattering measurements confirmed that there was no significant change in the particle size at the end of the exchange reaction.

Similar NNR measurements were also made in the l_o phase using host membranes made from DPPC and cholesterol. Specifically, liposomes were prepared from a mixture of DPPC, cholesterol, and{**1-3**} using the molar ratio of 57.5/37.5/2.5.[17,19] In contrast to the results obtained in the l_d phase, the mixing of **1** with **3** was found to be random, that is, $K = 4.0 \pm 0.24$ and $\omega_{AB} = 0 \pm 19$ cal/mol. Here also the stability of the liposomal size under these reaction conditions was confirmed by dynamic light scattering measurements.

To put these findings into perspective, they have been included in Table 1, along with values previously measured for

Table 1. Nearest-Neighbor Interactions in the l_o and l_d States[a]

1 + 2[b]	l_o	9.2 ± 0.19	−260 ± 6.3
1 + 2[b]	l_d	3.9 ± 0.25	+ 12 ± 19
2 + 3[c]	l_o	4.0 ± 0.10	0.0 ± 7.9
2 + 3[c]	l_d	2.4 ± 0.25	+160 ± 30
1 + 3	l_o	4.0 ± 0.24	0.0 ± 19
1 + 3	l_d	2.5 ± 0.23	+150 ± 30

[a]All measurements were made at 45 °C. [b]Data taken from ref 17. [c]Data taken from ref 18.

the interactions between **1** and **2** and between **2** and **3**. These data show, quite clearly, that the two repulsive interactions between **1** and **3** and between **2** and **3** in the l_d phase are essentially the same in magnitude but less than the attractive interaction between **1** and **2** in the l_o phase. Taken together, these results imply that the total driving force for forming lipid domains in membranes made from cholesterol, DPPC, and POPC consists of one strong pull between DPPC and cholesterol, one significant push between POPC and DPPC, and one significant push between POPC and cholesterol.

Our use of a POPC mimic in the present work was motivated by the fact that POPC is the major unsaturated lipid found in eukaryotic membranes. It should be noted, however, that many phospholipids that are also present in eukaryotic membranes contain multiple kinks, such as multiple cis double bonds. The fact that low-melting, unsaturated phospholipids can stabilize

ordered domains and that this stabilization tends to increase with increasing numbers of *cis* double bonds raises an intriguing possibility.[20−22] In particular, the push exerted by a "polykinked" phospholipid on cholesterol and on high-melting lipids could be even stronger than the pull exerted by cholesterol on sphingolipids. In other words, it is conceivable that pushing outweighs pulling in cell membranes and that repulsive interactions may be the dominant force that drives lipid raft formation. Studies aimed at examining such a possibility are currently in progress.

Finally, we wish to point out one major advantage of NNR measurements over commonly used fluorescence microscopy for investigating lipid domain formation. Specifically, NNR measurements can be used in combination with Monte Carlo simulations to quantify submicroscopic domains.[23] In contrast, fluorescence microscopy requires visualization and is not applicable to those membranes that do not exhibit l_o−l_d macroscopic phase separation, such as DPPC/POPC/cholesterol.[24−26]

In summary, nearest-neighbor recognition measurements have been made in model systems to gain insight into the push−pull properties of a low-melting phospholipid interacting with a high-melting lipid in the liquid-disordered and liquid-ordered states. Our results indicate that such interactions are significantly repulsive in the liquid-disordered phase but are neither attractive nor repulsive in the liquid-ordered state. When combined with previously reported NNR measurements, these findings provide strong support for the notion that repulsive forces play a major role in the formation of lipid rafts.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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