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Transbilayer Complementarity of Phospholipids. A Look beyond the Fluid Mosaic Model

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Received May 26, 2004; E-mail: slr0@lehigh.edu

Recent efforts to refine the fluid mosaic model of biological membranes have focused on lipid−lipid interactions *within the lipid monolayers*.[1,2] Such studies have led to the discovery of transient clusters composed of high-melting lipids and cholesterol, commonly referred to as "lipid rafts".[3−11] In contrast, lipid−lipid interactions *across the bilayer* have received much less attention, and the possibility of transbilayer clustering has remained largely hypothetical.[3] Here, we provide experimental evidence for favored association of long and short phospholipids across a fluid bilayer using a variation of the nearest-neighbor recognition (NNR) method.[12]

As discussed elsewhere, NNR measurements take molecular-level snapshots of membrane organization by detecting and quantifying the tendency of specific lipids to become nearest neighbors.[10−12] Experimentally, equilibrium mixtures of exchangeable lipid dimers (i.e., dimers having monomer units that can undergo exchange with neighboring lipid monomers) are generated via thiolate−disulfide interchange reactions. The interchange of monomers **A** and **B** among **AA**, **BB**, and **AB** is governed by the equilibrium constant $K = [AB]^2/([AA][BB])$, which characterizes their mixing behavior (Chart 1). Thus, $K = 4.0$ when **A** and **B** mix ideally, $K < 4$ when homophospholipid associations are favored, and $K > 4$ when heterophospholipid associations are favored.

To test for transbilayer complementarity (i.e., a preference for the pairing of a long phospholipid with a short phospholipid across the bilayer), we have carried out NNR experiments where either a part of **A** was substituted by a nonexchangeable (i.e., inert) monomer **A′** or its dimer **A′A′**, or part of **B** by **B′B′** or **B′**. Thus, if **A** and **B** are favored membrane-spanning pairs (Chart 2), then the inclusion of **B′B′** in the membrane should promote the formation of **AA** in the adjoining monolayer leaflet by acting as a template (Chart 3). In essence, NNR measures lateral interactions within each monolayer as well as transbilayer interactions, directly. In the same way, introduction of **A′A′** should selectively stabilize **BB** in an adjoining monolayer. In principle, both **A′A′** and **B′B′** should generate the same stabilization and, hence, identical $K < 4$ values. If, on the other hand, the nonexchangeable dimers merely promoted the formation of homodimers of the same chain length in the same monolayer by some intramonolayer force, then the shorter dimer (**A′A′**) would provide less energy than the longer dimer (**B′B′**) and,
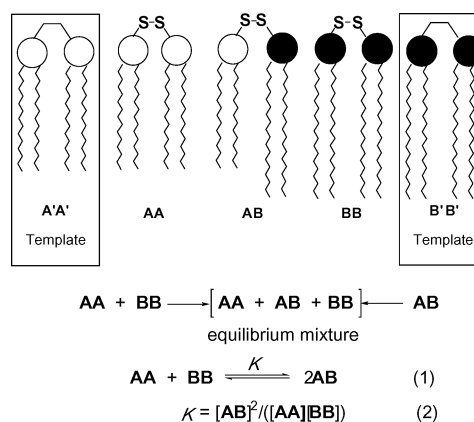
Chart 1

$$AA + BB \longrightarrow [AA + AB + BB] \longleftarrow AB$$

equilibrium mixture

$$AA + BB \underset{}{\overset{K}{\rightleftharpoons}} 2AB \qquad (1)$$

$$K = [AB]^2/([AA][BB]) \qquad (2)$$

Chart 2

complementary
membrane-spanning pairs

randomly-arranged
membrane-spanning pairs

Chart 3

template
stabilized
dimer
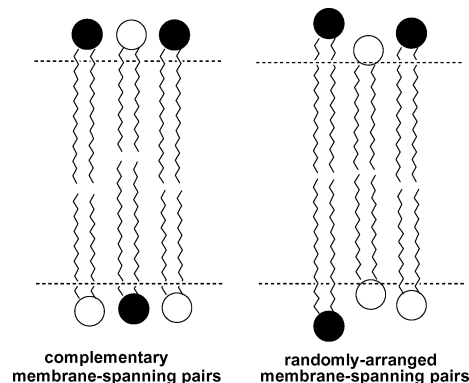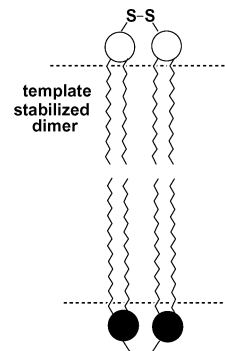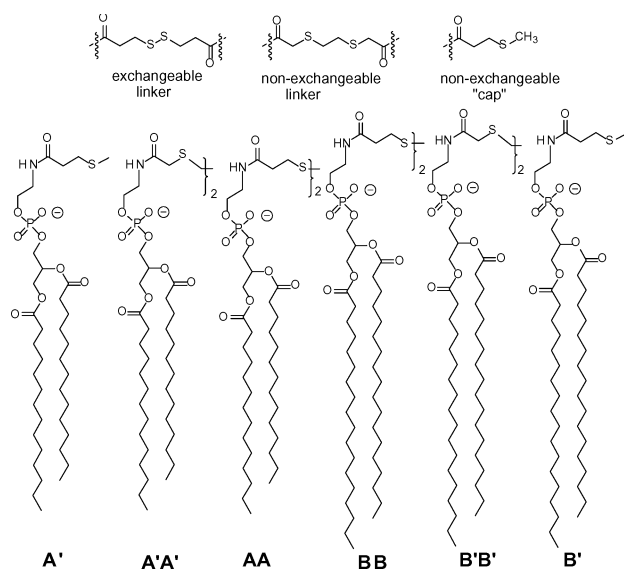
hence, its K value would be larger. In contrast, substitution of **A** by **A′** or **B** by **B′** should not change the dimer equilibria in the adjoining monolayer as long as these inert monomers mix ideally with the other lipids.

The nonexchangeable dimers **A′A′** and **B′B′** that were used in this study were synthesized from DMPE, DSPE, and a nonex-

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Chart 4

Table 1. Template Effects on Dimer Equilibria

additive[a]					
type	mol %	**AA**[b] mol %	**AB**[b] mol %	**BB**[b] mol %	K^c
none	0.00	50.0	00.0	50.0	3.99 ± 0.03
		00.0	100	00.0	
A′A′	25.0	25.0	00.0	50.0	2.82 ± 0.15
		00.0	50.0	25.0	
B′B′	25.0	50.0	00.0	25.0	2.76 ± 0.20
		00.0	50.0	25.0	
A′A′	37.5	12.5	00.0	50.0	2.05 ± 0.16
		00.0	25.0	37.5	
B′B′	37.5	50.0	00.0	12.5	2.06 ± 0.06
		37.5	25.0	00.0	
A′	40.0	20.0	00.0	40.0	4.16 ± 0.03
		00.0	40.0	20.0	
B′	40.0	40.0	00.0	20.0	4.11 ± 0.16
		20.0	40.0	00.0	

[a] Nonexchangeable dimer or monomer. [b] Starting compositions of exchangeable dimers. [c] Equilibrium constants calculated from eq 2 (±1 SD) are averages from both sets of experiments; equilibrium was reached in all cases within 3 h at 60 °C.

changeable linker, [2,2′-(ethylenedithio)diacetic acid (Fluka)] using methods similar to those employed for the preparation of **AA** and **BB** (Chart 4).[13] Nonexchangeable monomers **A′** and **B′** were synthesized by direct condensation of DMPE and DSPE with a nonexchangeable "cap" [3-(methylthio)propionic acid, Lancaster] using methods similar to those previously described.[13] Analysis of **A′A′** and **B′B′** by high-sensitivity differential scanning calorimetry revealed gel to liquid-crystalline-phase transition temperatures (T_m) of 21.1 and 55.6 °C, respectively, which are similar to those of **AA** ($T_m = 22.7$ °C) and **BB** ($T_m = 55.4$ °C); **AB** has a T_m of 33.9 °C. The T_m values for **A′** and **B′** are 19.4 and 54.0 °C, respectively.

Using experimental protocols outlined elsewhere, large unilamellar vesicles were prepared by reverse-phase methods from the dimer and template compositions that are listed in Table 1.[14] To preserve the thickness of the bilayer, **A** and **B** were always substituted by a mole-of-phosphorus-equivalent amount of nonexchangeable phospholipid. Nearest-neighbor recognition measurements were then carried out by promoting thiolate−disulfide interchange reactions. To ensure that equilibrium had been reached, product mixtures were obtained from vesicles that were formed from homodimers, as well as from a mixture of heterodimer plus homodimer. Equilibrium constants that are reported in Table 1 were calculated from eq 2 (±1 SD) and are averages of both sets of experiments.[13]

In the absence of nonexchangeable lipids, **A** and **B** mixed ideally, as reflected by a K value of 3.99 ± 0.03. Also, when **AA** was replaced with the nonexchangeable monomer **A′** or **BB** by **B′**, the mixing of **A** and **B** remained essentially ideal. In contrast, when 50% of **AA** was replaced by **A′A′** (i.e., when 25 mol % **A′A′** was used), K was lowered to 2.82 ± 0.15, and when 50% of **BB** was replaced by **B′B′**, K was 2.76 ± 0.20. Increasing the concentration of either nonexchangeable dimer to 37.5 mol % further increased the preference for homodimer formation, still to exactly the same extent (Table 1).

The variant of the NNR method presented above provides a means for probing one of the most subtle, but yet important, structural aspects of lipid membranes: their transbilayer organization. Taken together, our results support the existence of transbilayer complementarity in fluid phospholipid membranes. Such interleaflet communication, whereby phospholipids in one monolayer interact with specific phospholipids in the adjoining monolayer, is likely to have an influence on a variety of cellular events, including signal transduction and membrane fusion.[2] Also, one may now expect that structural or compositional modifications of one leaflet of a cell membrane will bring about a compensating modification in the adjoining leaflet.

Supporting Information Available: Procedures for the synthesis of **A′A′**, **B′B′**, **A′**, and **B′** and for carrying out NNR reactions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA046892A

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