

A Quantitative Assessment of the Influence of Permanent Kinks on the Mixing Behavior of Phospholipids in Cholesterol-Rich Bilayers

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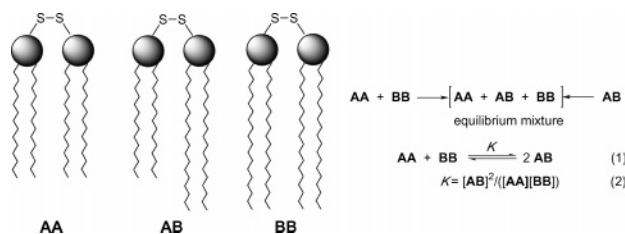
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The biological consequences of consuming *trans*-fatty acids is of considerable current interest.^{1,2} At the membrane level, the effects of replacing *cis*- with *trans*-double bonds in phospholipids (i.e., the removal of “permanent kinks”) on bilayer organization remain poorly understood.² In this report, we provide a quantitative assessment of the influence of permanent kinks on the mixing behavior of phospholipids in cholesterol-rich bilayers under condensing and fluidizing conditions. The implications of our findings, with respect to the “*trans*-fatty acid debate”, are briefly discussed.

In this work, we investigated phospholipid mixing by use of the nearest-neighbor recognition (NNR) method (Chart 1).³ As dis-

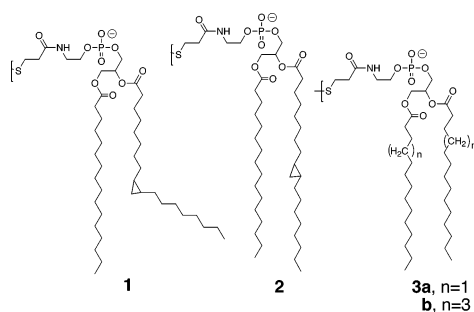
Chart 1



cussed elsewhere, NNR measurements take molecular-level snapshots of membrane organization by detecting and quantifying the tendency of specific lipids to become nearest neighbors.³ Experimentally, equilibrium mixtures of exchangeable lipid dimers are generated via thiolate-disulfide interchange reactions. The interchange of monomers **A** and **B** among **AA**, **BB**, and **AB**, is then 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 homo-phospholipid associations are favored, and $K > 4$ when hetero-phospholipid associations are favored.

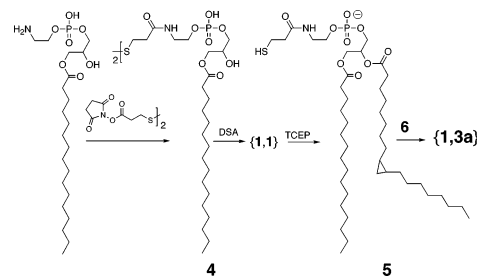
Four specific lipids that were chosen for this study were **1**, **2**, **3a**, and **3b** (Chart 2). Our use of a *cis*-cyclopropyl moiety to create

Chart 2



a permanent kink in **1** was based on three considerations. First, previous NNR experiments have shown that double bonds are configurationally unstable during thiolate-disulfide exchange

Scheme 1



reactions.^{3f} Apparently, *cis*–*trans* isomerization takes place via the reversible addition of adventitious thiyl radicals.⁴ Because we could not find literature precedent for thiyl radical addition to cyclopropanes, we hypothesized that the cyclopropyl moiety would be stable during an NNR experiment. Second, the melting properties of phospholipids containing *cis*-cyclopropyl groups are similar to ones having *cis*-double bonds. For example, the phosphocholine analogue of **1** has a T_m of -10°C , which is similar to that of its unsaturated analogue, i.e., 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, $T_m = -5^\circ\text{C}$).⁵ Third, the mixing behavior of **1** can be directly compared with its *trans*-analogue (i.e., **2**), since both have identical compositions but differ in that **1** has a permanent kink and **2** does not.⁶ Given the fact that mammalian membranes are rich in phospholipids having T_m values that are lower than physiological 37°C and sphingolipids having T_m values higher than 37°C , we sought to measure the mixing of **1** and **2** with **3a** ($T_m = 22.7^\circ\text{C}$) and **3b** ($T_m = 41.9^\circ\text{C}$) in cholesterol-containing bilayers at an intermediate temperature (30°C), that is, where the sterol has a condensing effect on **3a** and a fluidizing effect on **3b**.^{7,8}

Scheme 1 outlines the synthetic approach that was used to prepare a homodimer of **1** and a heterodimer made from **1** and **3a**, i.e., **{1,1}** and **{1,3a}**, respectively. In brief, acylation of two molecules of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine with dithiobis[succinimidyl propionate] afforded an exchangeable lysodimer **4**. Subsequent acylation with the anhydride of dihydrostercylic acid (DSA) afforded **{1,1}**. Reduction of **{1,1}** with tris(2-carboxyethyl)phosphine to give the thiol monomer (**5**), followed by reaction with 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanol (2'-pyridyldithio)propionamide (**6**) yielded **{1,3a}**. Dimers **{2,3a}** and **{2,2}** were prepared using similar methods; homodimers **{3a,3a}** and **{3b,3b}** were prepared using established procedures.^{3d}

Surface pressure–area isotherms that were recorded for **{1,1}** and **{2,2}** over a pure water subphase were very similar (not shown). Both lipids gave a limiting area of ca. $160 \text{ \AA}^2/\text{molecule}$ or $80 \text{ \AA}^2/\text{phospholipid monomer}$, which is close to that found for POPC. Examination of the melting behavior of a multilamellar dispersion of **{2,2}** by high-sensitivity differential scanning calorimetry revealed an endotherm at 21°C . In the case of **{1,1}**, no endotherm could be detected from 10 to 60°C . Based on the T_m of POPC and its cyclopropyl analogue, and the similar melting behavior of 1,2-

Table 1. Phospholipid Mixing Defined by NNR Measurements

PL ^a	Ch ^b mol %	K ^c
1 + 3a	0	4.00 ± 0.02
2 + 3a	0	4.01 ± 0.01
1 + 3a	30	3.36 ± 0.04
2 + 3a	30	3.04 ± 0.04
1 + 3b	30	3.58 ± 0.03
2 + 3b	30	2.87 ± 0.01

^a PL = exchangeable phospholipids. ^b Cholesterol content, where each phospholipid dimer is counted as two lipids. ^c Equilibrium constants calculated from eq 2 (±1 SD) are averages from two sets of experiments (i.e., using homodimers and also heterodimers as the starting material). Equilibrium was reached in all cases within 7 h at 30 °C.

dimyristoyl-*sn*-glycero-3-phosphocholine and {**3a,3a**}, we estimate the T_m of {**1,1**} to be ca. −10 °C.⁹ Thus, based on the melting behavior of these phospholipids, cholesterol is expected to have a condensing effect on bilayers derived from **1 + 3a** and **2 + 3a** at 30 °C and a condensing and fluidizing effect on bilayers made from **1 + 3b** and **2 + 3b**.

Using procedures similar to those previously described, large unilamellar vesicles were prepared from an equimolar mixture of {**1,1**} and {**3a,3a**} by reverse phase methods. The lipids were then subjected to thiolate-disulfide interchange at 30 °C. To ensure that product mixtures were thermodynamically controlled, NNR experiments were also carried out in vesicles made from the corresponding heterodimer, {**1,3a**}. An equilibrium constant, K , was then calculated from the dimer compositions from both sets of experiments. As shown in Table 1, the mixing of **1** and **3a** was ideal. To confirm the absence of *cis*–*trans* isomerization, the lipids were recovered and analyzed by ¹H NMR spectroscopy. Comparison of the cyclopropyl region with that of pure {**1,1**} and {**2,2**} established that the *cis*-stereochemistry was fully retained. The addition of 30 mol % cholesterol to these membranes resulted in a modest preference for homo-phospholipid association. Analogous experiments with **2** and **3a** gave very similar results, except the preference for homo-phospholipid association was slightly greater (Table 1). At 30 °C, the effect of the kink in this system corresponds to a ΔG° of ca. 30 cal/mol. Similar results were obtained for the mixing of **3b** with **1** versus **2**, except the difference was approximately twice as great, corresponding to a ΔG° of ca. 65 cal/mol of kinked phospholipid.

Previous studies from our laboratories have shown that cholesterol induces homo-phospholipid association by favoring pairs of like-phospholipids as nearest neighbors.^{3b,g} Based on these earlier findings, the lower K values found for bilayers containing **2** relative to **1** can be accounted for in terms of increased hydrophobic interactions between cholesterol and pairs of **2**; that is, removal of the permanent kink allows these lipids to become more intimately associated. Similarly, the fact that **2** and **3b** mix poorly, relative to **2** and **3a**, is a likely consequence of stronger hydrophobic interactions between pairs of **3b** plus cholesterol relative to pairs of **3a** plus cholesterol, resulting from the longer acyl chains of **3b**. The present findings clearly show that kinks have a significant influence on phospholipid mixing, especially under fluidizing conditions.

Mammalian membranes are presumed to have regions that are rich in low-melting, kinked phospholipids (liquid-disordered phase) and ones that are rich in high-melting sphingolipids plus cholesterol (liquid-ordered phase).⁸ The latter are thought to play an important role in signal transduction and membrane trafficking.¹⁰ The present findings imply that the replacement of *cis*- with *trans*-double bonds should shift the balance between liquid-disordered and liquid-ordered regions in favor of the latter. To the extent that normal cellular function depends on a “proper” balance between these two regions, the introduction of *trans*-fatty acids may well have significant biological consequences.

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Supporting Information Available: Procedures for the synthesis of {**1,1**}, {**1,3a**}, {**1,3b**}, {**2,2**}, {**2,3a**}, and {**2,3b**}; measurement of NNR; plus surface pressure–area isotherms for {**1,1**} {**3,3**}, and POPC; and ¹H NMR spectra of the cyclopropyl region after an NNR experiment (6 pages, print/PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (6) Due to the presence of two stereogenic centers in their cyclopropyl moiety, phospholipids **1** and **2** are mixtures of stereoisomers.
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- (9) An analogue of **2**, bearing a palmitoyl group at the *sn*-1 position and a stearoyl group at the *sn*-2 position (i.e., **3c**), has a T_m of 49 °C [Sugahara, M.; Urugami, M.; Tokutake, N.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 2697]. Thus, the T_m values 49, 21, and −10 °C for **3c**, **2**, and **1**, respectively, show the same trend found for 1,2-distearoyl-*sn*-glycero-3-phosphocholine (T_m = 55 °C), 1,2-di-laidoyl-*sn*-glycero-3-phosphocholine (T_m = 10 °C) [Higashino, Y.; Ohki, K. *J. Biochem.* **2001**, *130*, 393], and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (T_m = −20 °C) [Ulrich, A. S.; Sami, M.; Watts, A. *Biochim. Biophys. Acta* **1994**, *1191*, 225]. Thus, **2** is a reasonable mimic of a *trans*-olefinic phospholipid by having a melting temperature that lies between that of a *cis*-olefinic (**1**) and a saturated phospholipid analogue (**3c**).
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