Is the Linkage Region of Sphingolipids Responsible for Lipid Raft Formation?

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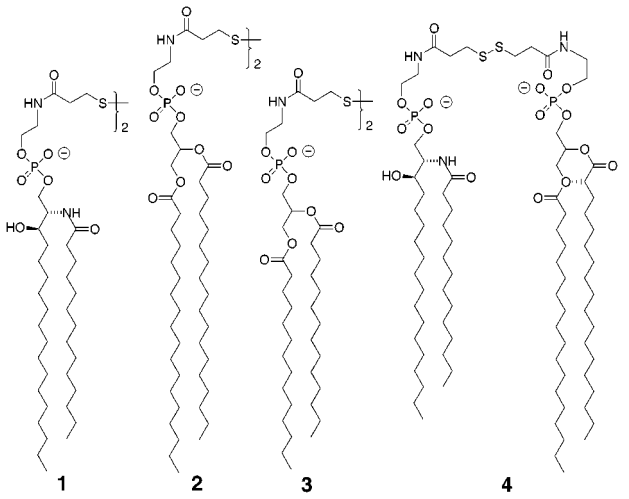
The hypothesis that sphingolipids (glycosphingolipids and sphingomyelins) combine with cholesterol to form clusters in biological membranes is of considerable current interest. In particular, there is a growing body of evidence that suggests that such clusters (commonly referred to as "lipid rafts") are formed in a "sea" of glycerolipids, and that these rafts may play a key role in processes such as membrane trafficking and signal transduction.[1−10] If this hypothesis is correct, then understanding those factors that are responsible for lipid raft formation is of great importance.

In this paper, we report the synthesis of an exchangeable sphingolipid dimer (**1**) and show that its monomer units mix, nonideally, with those of a longer chain glycerolipid (**2**) in cholesterol-rich fluid bilayers. We further show that such sphingolipid−glycerolipid mixing is closer to ideal than analogous glycerolipid−glycerolipid mixing found with monomers of **2** and **3**, i.e., *heterolipid associations are favored*. Our principal results, which are reported herein, provide the first quantitative insight into how the linkage region of a sphingolipid and a glycerolipid influences their mixing behavior in the physiologically relevant, fluid bilayer state.

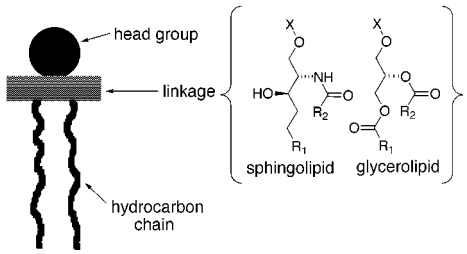


1 **2** **3** **4**

To gain insight into the mixing properties of sphingolipids and glycerolipids, we have begun to examine the miscibility of exchangeable mimics by use of the nearest-neighbor recognition (NNR) method.[11] As described elsewhere, this chemical technique, which probes nearest-neighbor interactions by measuring equilibrium dimer distributions, provides quantitative insight into lipid mixing. Thus, when equilibrium mixtures of dimers are found to be statistical, such a finding establishes that the lipids are ideally mixed. When homodimers are found to be in excess (i.e., NNR is observed), and when this excess can be reduced or eliminated by the presence of a nonexchangeable lipid that functions as a mixing agent, nonideal mixing is indicated.[12]

The purpose of the work described herein was to probe the effects of one structural feature, which distinguishes all sphingolipids from glycerolipids, on lipid miscibility, i.e., the linkage region—that portion of the lipid that connects the headgroup to the hydrocarbon chains. Chart 1 highlights the major differences that exist between the linkage region of glycerolipids and sphingolipids. It should be noted that many but not all naturally occurring sphingolipids also contain a *trans* double bond in this region. In principle, the presence of amide and hydroxyl groups could promote self-clustering and raft formation via intermolecular hydrogen bonding.[13,14] Such bonding should be significant, especially if this region of the membrane were hydrophobic in character, i.e., penetration of water beneath the headgroup was minimal.

Chart 1



In the present study, we sought an exchangeable sphingolipid that could be compared with an exchangeable glycerolipid having the *same chain length*. Since we have previously shown that monomers of **2** and **3** are nonideally miscible in cholesterol-rich bilayers, homodimer **1** and the corresponding heterodimer, **4**, were viewed as attractive synthetic targets. In particular, by using equilibrated bilayers made from **2** and **3** as a frame of reference, insight into sphingolipid−glycerolipid mixing should be possible by quantifying the relative mixing behavior of the monomers of **1** and **2**. Specifically, a higher degree of nearest-neighbor recognition for bilayers derived from **1** and **2** would indicate that the linkage region favors segregation of the two different lipids; a lower degree of recognition would reflect a preference for sphingolipid−glycerolipid association.

Acylation of D-*erythro*-sphinganine (Avanti Polar Lipids) with *N*-succinimidyl tetradecanoate afforded **5**. Subsequent benzoylation and silylation of the primary and secondary hydroxyls, respectively, to give **6**, followed by debenzoylation, and introduction of a *t*-Boc-protected phosphoethanolamine moiety (via the reaction sequence shown in Scheme 1) afforded **7**. Deprotection of the secondary hydroxyl group and the amino moiety, followed by acylation of the latter with *N*-[*O*-1,2,3-benzotriazin-4(3H)one-yl]-3-(2-pyridyldithio)propionate [BPDP] yielded **8**.[15] Finally, reductive cleavage of the activated disulfide and reaction with its precursor (**8**) afforded **1**. Heterodimer, **4**, was obtained by reacting **8** with 1 equiv of the thiol monomer of **2**.

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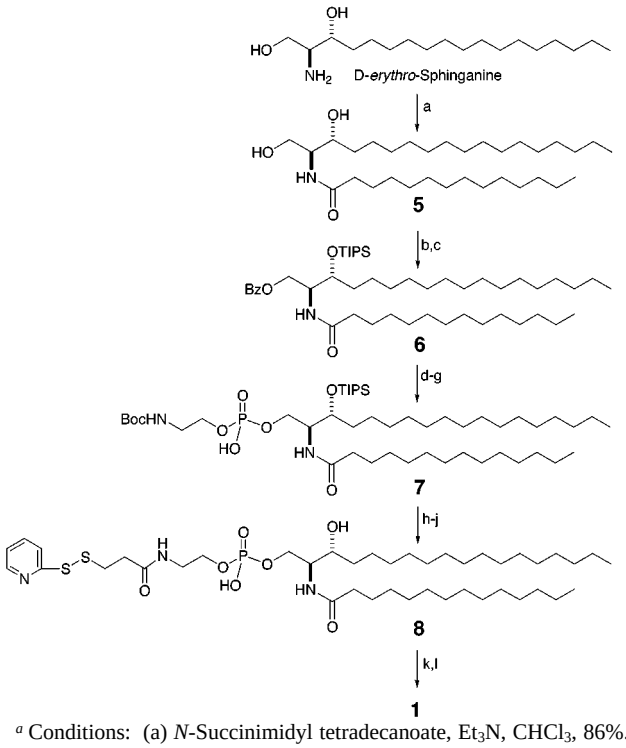
Scheme 1[a]



[a] Conditions: (a) *N*-Succinimidyl tetradecanoate, Et_3N, $CHCl_3$, 86%. (b) Benzoyl chloride, DMPA, pyridine, 60%. (c) TIPSOTf, 2,6-lutidine, CH_2Cl_2, 77%. (d) $NaOCH_3$ (0.5 equiv), CH_3OH, 79%. (e) PCl_3, imidazole, Et_3N, toluene, 0 °C. (f) *N*-(*tert*-Butoxycarbonyl)ethanolamine, pivaloyl chloride, pyridine. (g) I_2, H_2O, 62%. (h) HF/pyridine, CH_3CN/CH_2Cl_2. (i) TFA, CH_2Cl_2. (j) BPDP, Et_3N, $CHCl_3$, 56%. (k) DTT, $CHCl_3$, 0 °C. (l) **8**, $CHCl_3$.

Examination of a multilamellar dispersion of **1** in "borate buffer" (10 mM borate, pH 7.4, 140 mM NaCl, 2 mM NaN_3) via high-sensitivity differential scanning calorimetry revealed a melting temperature (T_m) of 25.5 °C and an enthalpy of 12.2 kcal/mol (Figure 1A). This melting behavior is very similar to that previously reported for the glycerophospholipid analogue, **3**, where the values of T_m and ΔH were 22.7 °C and 14.7 kcal/mol, respectively; **2** melts at 54 °C.[16,17] Monolayers of **1** that were spread at the air/water interface showed a "lift-off" area of 1.94 $nm^2 \cdot molecule^{-1}$ and a limiting area 1.65 ± 0.03 $nm^2 \cdot molecule^{-1}$. The lift-off and limiting areas for **3** were 2.05 and 1.58 ± 0.03 $nm^2 \cdot molecule^{-1}$, respectively. Thus, the melting behavior and monolayer properties of **1** and **3** are similar in character.

Large unilamellar vesicles were formed from 1/1 molar mixtures of homodimers (**1**/**2** or **2**/**3**) plus 29 mol % cholesterol via reverse phase evaporation methods in borate buffer.[18] To promote monomer exchange via thiolate−disulfide interchange, 20 mol % of the corresponding thiol monomers were also included in the membranes. Equilibrium was reached in all cases within 10 h. To confirm that true equilibrium values were obtained, product mixtures were also generated from vesicles that were made from the corresponding heterodimer. Specific experimental procedures that were used for synthesizing the lipids, forming vesicles, initiating the thiolate−disulfide interchange reaction, and analyzing dimer distributions by HPLC were similar to those previously described.[12,16]

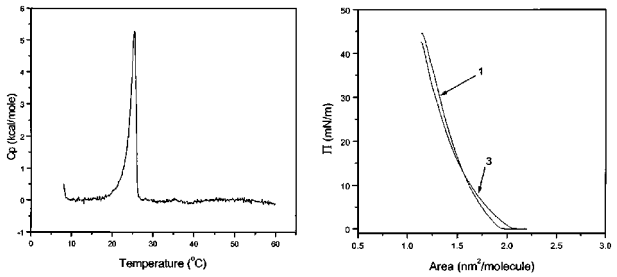


Figure 1. (A) High-sensitivity excess heat capacity profile of **1**. (B) Surface pressure−area isotherm for **1** and **3** over 1.0 M NaCl (used to prevent solubilization) at 25 °C, compressed at 24 $Å^2$/min/molecule.

Table 1. Equilibrium Heterodimer/Homodimer Ratios[a]

entry	exchangeable monomers[b]	DPPC (mol %)	heterodimer[c] homodimer
1	*14*/*18*	0	1.55 ± 0.02
2			(1.55 ± 0.08)[12a]
3	*14*/*18*	50	2.07 ± 0.07
4			(1.97 ± 0.06)[12a]
5	14^{SL}/*18*	0	1.71 ± 0.01
6	14^{SL}/*18*	50	2.02 ± 0.07

[a] All thiolate−disulfide interchange reactions were carried out at 60 °C to maintain a fluid phase. In each case, 29 mol % cholesterol plus 20 mol % of the corresponding thiol monomers were included in the membrane. [b] Monomers of **1** (14^{SL}) and **2** (*18*). [c] Convergent molar ratio of heterodimer to **2**; error values represent one standard deviation.

A summary of our principal findings is shown in Table 1. Miscibility measurements that were carried out with the exchangeable glycerolipids **2** and **3** in the presence, and in the absence, of a mixing agent (i.e., 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, DPPC) gave results that were in excellent agreement with those previously reported (entries 1−4).[12a] Here, nonideal mixing is indicated by the elimination of the nearest-neighbor recognition when DPPC is included in the membrane. Analogous experiments that were carried out with **1** and **2** showed, qualitatively, similar behavior, except that the degree of nearest-neighbor recognition was lower in the absence of DPPC; i.e., the dimer distribution was closer to the statistical value of 2.0, reflecting random mixing (entries 5 and 6). Taken together, these results indicate that *the affinity of sphingolipids toward glycerolipids is greater than the affinity of sphingolipids toward themselves*. This preference for heteroassociation corresponds to the difference in free energy of mixing of ca. 130 cal/mol at 60 °C (calculated from the equilibrium constants for the two different systems). The exact reason why heterolipid association is favored is not clear at present.

On the basis of these model studies, we conclude that it is highly unlikely that the difference in the linkage region of natural sphingolipids and glycerolipids, by itself, provides a driving force for lipid raft formation when both lipids are in the fluid phase. That the self-association of these amide-bearing lipids is, in fact, disfavored strongly suggests that the linkage region is "wet" and that a hydrophilic microenvironment exists, which is not conducive for intermolecular hydrogen bonding between neighboring sphingolipids.

The extent to which other factors may contribute to raft formation (e.g., the inclusion of transmembrane proteins, differences in headgroup structure and charge, etc.) remains to be established. Systematic studies aimed at evaluating the influence of such factors via the nearest-neighbor recognition method are currently in progress.

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Supporting Information Available: Procedures for the synthesis of **1**, **4**, **5**, **6**, **7**, and **8** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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