

Dynamics of lipids in synthetic membranes

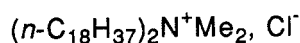
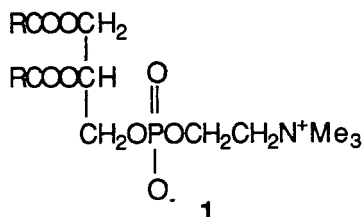
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Abstract: Bilayer coliposomes were created from blends of head-group functionalized and non-functionalized synthetic lipids. Functionalized lipids in the outer leaflets of the bilayers were then chemically differentiated from the (unchanged) functionalized lipids of the inner leaflets, thus permitting study of the dynamics of the ensuing reequilibration that occurred via thermally activated transbilayer lipid migration ("flip-flop"). Our particular focus was the relation between lipid molecular structure and flip-flop dynamics within the bilayer membranes. The structural factors investigated included chain length, unsaturation, cyclopropanation, alkylation, and "stiffening". The effects of head group type (ammonium ion or phosphocholine) and overall lipid character (monopolar or bolaamphiphilic) were also examined. The results highlight factors that simultaneously allow both the membrane fluidity and maintenance of leaflet differentiation that are essential to proper biological function.

Synthetic bilayer lipid vesicles or liposomes are of interest in their own right and as models for biological membranes (ref. 1,2). These closed, hollow spheres or ellipsoids create aqueous reaction loci of different chemical or microenvironmental properties separated by hydrocarbon barriers. Thus the permeation of reagents and ions across these barriers can be controlled, and their reactivity modulated. Moreover, one can chemically differentiate the exoliposomal and endoliposomal surfaces, furthering the analogy to biological membranes, where transverse asymmetry between the exo and endo leaflets of the bilayer is common and functionally significant (ref. 1-5). With differentiated bilayer surfaces, we can study the dynamics of individual lipid molecules as they undergo reequilibration, and we can relate the dynamics to the lipid's molecular structure.

The most common biological lipids are phosphoglycerides, e.g. 1. Kunitake (ref. 6) and



2

Fendler (ref. 7) showed that double chain quaternary ammonium ions (2) could also form liposomes, so that one could model bilayer membranes with lipids of the simplest structure.

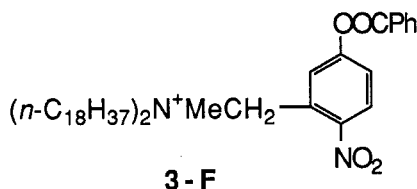
Moreover, we could envision a rational sequence of lipid molecular structures that gradually increased in complexity, incrementally bridged the gulf between **2** and **1**, and traced the relation between lipid structure and lipid dynamics at the aggregate or liposomal level.

SURFACE DIFFERENTIATION OF LIPOSOMES

The generation of surface differentiated liposomes is a crucial element in the modeling of biological membranes (ref. 8-10). Our methodology chemically differentiates exo from endo liposomal head groups of functionalized lipids by reactions that preferentially occur at the bilayer's exoliposomal surface.

For example, 1:9 coliposomes of functional (F) (**3-F**) and non-functional (NF) (**2**) lipids were created by sonication at pH 3.9 (ref. 11). These liposomes had a gel to liquid crystalline phase transition temperature (T_c) of 35.5°C, where the phase change is associated with conversion of the all *s*-trans, staggered conformer, well-packed lipid chains of the relatively rigid, non-leaky, lower temperature gel phase liposomes to the looser, leakier, and "fluid" liquid-crystalline liposomes which feature gauche conformers or "kinks" in their chains (ref. 1-3).

The **3-F/2** coliposomes were surface differentiated by brief exposure to glutathione at an external pH of 7.9, thus rapidly cleaving the exoliposomal *p*-nitrophenyl benzoate residues (**G**) of **3-F** to the corresponding *p*-nitrophenylate moieties (**G'**). This process was monitored at 400 nm and occurred with a "fast" rate constant, $k_f = 0.11 \text{ s}^{-1}$.



Subsequent, slower ($k_s = 7.5 \times 10^{-4} \text{ s}^{-1}$) endoliposomal cleavage of **G** was rate limited by trans-membrane H^+/OH^- (actually concurrent counterion) permeation driven by the imposed pH 7.9/3.9 gradient (ref. 12). Counterion (Na^+ or K^+) permeation was slowed by the liposomal bilayer's cationic lipids; the pH gradient rapidly collapsed if valinomycin was added to "ferry" the metal cations (ref. 13).

The biphasic absorption changes attending the exo/endo cleavage of the **3-F/2** coliposomes are illustrated in Figure 1. After the initial, exoliposomal cleavage (~1 min), the exo surfaces bear ArO^- (**G'**) residues at pH 8, while the endo liposomal membranes still carry intact ester groups at pH 3.9. We then monitor endo to exo transverse bilayer migration ("flip-flop") of the functional lipids by the following protocol (ref. 11). (1) After exoliposomal cleavage by RS^- at pH 8, the external pH is reduced to 3.9 with HCl, quenching further esterolysis or H^+/OH^- permeation. (2) The now surface-differentiated liposomes are incubated at a preselected temperature (e.g. $\geq T_c$) for a variable time period. (3) The liposomes are cooled to 25°C. (4) The pH is readjusted to 7.9 with NaOH and esterolysis is monitored at 400 nm. Figure 2 illustrates typical results.

The key observation is the appearance of a new fast cleavage induced by the incubation. In the absence of the heating step, we observe only the normal biphasic behavior shown in Figure 1; the dotted line in Figure 2 shows a control experiment in which heating is omitted. In contrast, the heating process (solid line) bifurcates the original, slow, endoliposomal

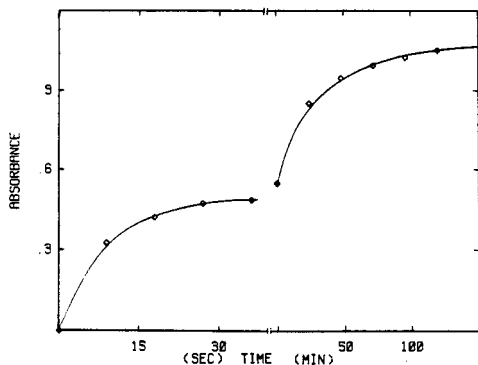


Figure 1. Absorbance at 400 nm vs. time for the appearance of *p*-nitrophenylate from coliposomal **3-F/2** at an external pH of 7.9

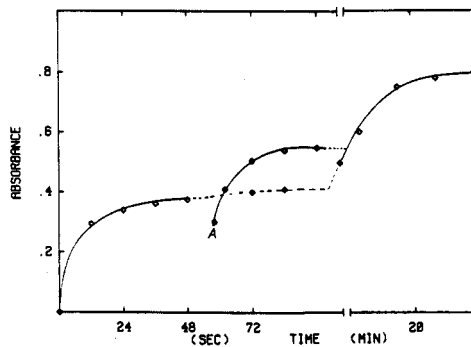


Figure 2. Same as Figure 1, but with the imposition of the flip-flop protocol described in the text.

cleavage into 2 parts, a new fast phase and a residual slow phase. The new k_f represents 15% of cleavage after 1 min at 40°C, 20% after 2 min, and 28% after 6 or 12 min of heating. These results represent those intact **3-F** lipids that flipped from endo to exo locations during incubation. After 6 min, the original k_S is replaced by equal contributions of k_f and k_S ; the endoliposomal **3-F** lipids have been equilibrated between endo and exo sites.

Note that the new fast cleavage begins below the end absorbance of the initial fast cleavage (Fig. 2, point A), corresponding to cleaved (**G'**) exo **3-F** lipids that "flopped" to endo sites during incubation. Their **G'** residues protonated to *p*-nitrophenols under the pH 3.9 endoliposomal conditions and lost absorbance at 400 nm.

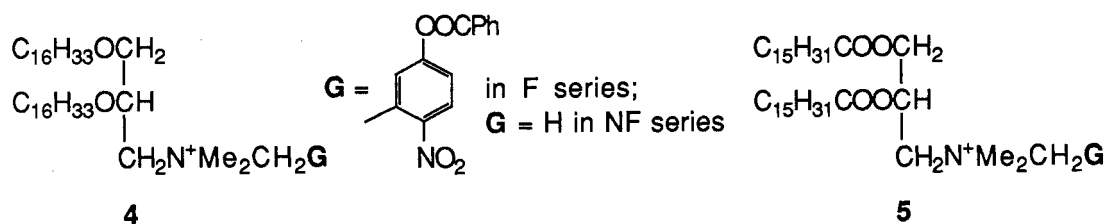
The half-time ($t_{1/2}$) for reequilibration of the surface-differentiated **3-F/2** coliposomes is only ~2 min at 40°C, whereas $t_{1/2}$ for dipalmitoylphosphatidylcholine (1, R=C₁₅H₃₁) liposomes is ~6.5 h at 30°C (ref. 14). The disparity, reflecting the simpler molecular structure of **3-F** as well as its cationic head groups, will be discussed below.

LIPID ARCHITECTURE AND DYNAMICS

Chain length and backbone

With the surface differentiation and flip-flop protocols we can determine how variations in the molecular structures of functional lipids affect their dynamic behavior within liposomal membranes. A simple application examines the dependence on chain length for homologues of **3-F** and **2**.

With R= η -C₁₆H₃₃, η -C₁₈H₃₇, or η -C₂₀H₄₁, 1:10 **F/NF** coliposomes had I_c 's of 26, 39 or 48°C. The corresponding surface-differentiated liposomes exhibited flip-flop dynamics determined by their I_c 's; i.e., by their gel to liquid crystalline transitions (ref. 15). The C₁₆ coliposomes (surface differentiated at 15°C) reequilibrated in ~1 min at 25°C; the C₁₈ coliposomes were stable to flip-flop for >1 h at 25°C (below I_c) but reequilibrated in ~1 min at 38-40°C. Finally, the C₂₀ coliposomes were stable at 40°C, but rapidly reequilibrated at 50°C (ref. 15). Liposomes constructed of simple ammonium ions thus display flip-flop that is governed by chain packing: below I_c the well-packed gel phase lipids resist flip-flop, above I_c the more fluid, liquid crystalline lipids rapidly reequilibrate between the endo and exoliposomal leaflets.

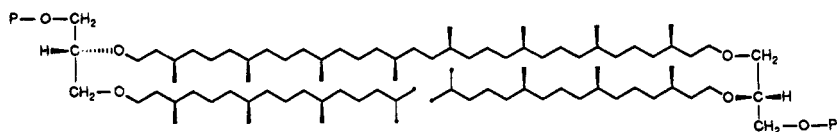


Greater molecular complexity confers additional stability. Lipids **4** and **5** feature 3-carbon pseudoglycerol backbones with ether (**4**) or ester (**5**) linkages to 16-carbon chains at C₁ and C₂; C₃ carries head groups analogous to those of **2** (NF, G = N⁺Me₃) or **3** (F, G = *p*-nitrophenyl benzoate) (ref. 16). The T_c's of 1:7 F/NF coliposomes are 37°C (**4**) and 44°C (**5**). Surface differentiated coliposomes of **4** reequilibrate with t_{1/2} ~5 min at 40°C and 1 min at 45°C, whereas the ester coliposomes of **5** display t_{1/2} ~5 min at 55°C and ~1 min at 65°C. Both **4** and **5**, in contrast to **3**, display some resistance to flip-flop above their T_c's, probably reflecting better chain packing from their vicinal double long chain pseudoglycerol architecture.

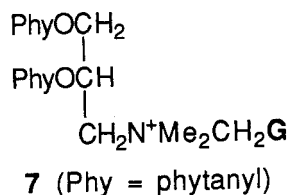
Phytanyl lipids and bolaamphiphiles

Unusual lipids (**6**) were isolated from thermophilic archaebacteria that thrive at high (50-80°C) temperatures (ref. 17). These phytanyl isoprenoid lipids feature ether linkages to glycerol backbones with phosphoethanolamine head groups. They are "double-headed" or bolaamphiphiles, capable when extended of forming a monolayer liposomal membrane (note the 32-carbon main chain of **6**).

A simple model for **6** is the 1,2-diphantanyl pseudoglycerolammonium lipid, **7** (ref. 18), but compared to 1:10 F/NF coliposomes of straight chain ether lipid **4**, phytanyl coliposomes of **7**



6

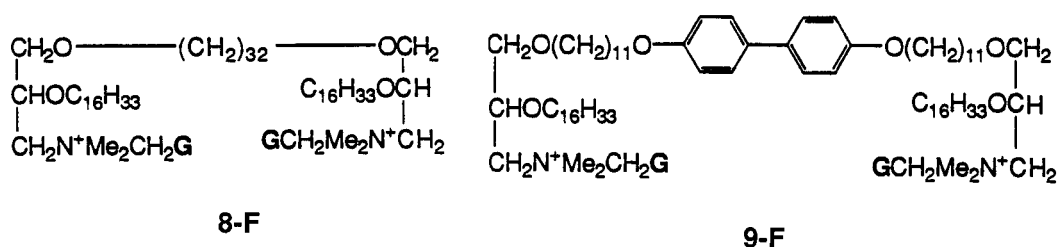


are very floppy. The T_c of **7** is <10°C, and the phytanyl coliposomes (surface-differentiated at 5°C) reequilibrate with t_{1/2} <1 min at 12°C. Chain branching and interchain Me/Me interactions must reduce the stability of coliposomal **7** relative to that of **4** (t_{1/2} >10 min at 25°C) (ref. 17-19).

A better model for **6** is the desmethylated, pseudoglycerol functional bolaamphiphile **8-F** (ref. 20). Coliposomes of 1:14 **8-F** in nonfunctional host **4**, when surface-differentiated, reveal a 70:30 exoliposomal (k_f=0.14 s⁻¹) to endliposomal (k_s=5.8 x 10⁻⁴ s⁻¹) kinetic partition of the

8-F esterolysis. This excludes coliposomal arrangements in which the **8-F** lipids assume only extended, bilayer bridging conformations that extend across both leaflets of the bilayer. Such a situation would demand a 50:50 exo/endo kinetic partition. The observations are consistent with at least 40% of the **8-F** lipids adopting U-plan, bent conformations that concentrate their head groups in the outer bilayer leaflet, where, relative to the endo leaflet, greater surface area is available due to the gentler curvature. Similar U-plan bolaamphiphile conformations are seen in air/water monolayers (ref. 17).

After surface differentiation of the **8-F/4-NF** coliposomes, endoliposomal **8-F** head groups undergo facile flip-flop ($t_{1/2} \sim 5$ min at 25°C) at a rate similar to that of the host lipid molecules ($t_{1/2} \sim 5$ min at 40°C for **4-F/4-NF** coliposomes) (ref. 20). Although **8-F** fails to manifest exclusive bilayer bridging and resistance to flip-flop, these desirable properties can be evoked if we "stiffen" the main chain with a biphenyl unit; cf. **9-F**. Now U-plan conformations are inhibited and the bolaamphiphile extends across the bilayer. Exoliposomal/endoliposomal surface differentiation exhibits a 50:50 partition, with very slow flip-flop of the extended **9-F** lipids (ref. 21); we observe only $\sim 11\%$ reequilibration after 1 h at 50°C, reflecting the difficulty



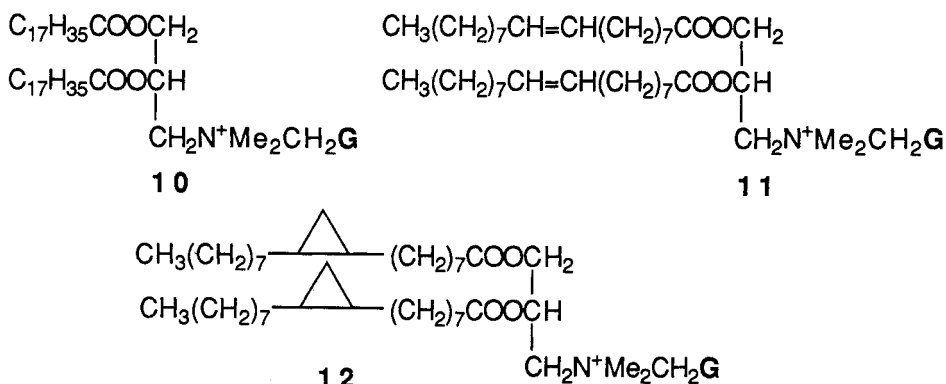
in bending biphenyl-stiffened **9-F** within the bilayer. Thus extended **9-F** lipids maintain their bilayer-bridging arrangement at temperatures that greatly exceed the T_C of the host-bilayer. This should allow appropriate analogues to serve as reporter probes during the interconversions of gel and liquid crystalline host bilayers.

Unsaturated and cyclopropanated lipids

A carbon-carbon double bond in a lipid's hydrocarbon chain creates a permanent "kink" that disturbs chain packing, lowers the T_C , and fosters fluidity within the liposome (ref. 1-3). Unsaturated lipids figure prominently in biological membranes, so we prepared the bis- C_{18} pseudoglycerol model lipids **10** (stearoyl) and **11** (*cis*-**11**, oleoyl; *trans*-**11**, elaidoyl) (ref. 22).

The contrast in T_C 's of their liposomes is instructive; liposomes of saturated **10** have $T_C = 59$ °C, whereas those of *trans*-**11** and *cis*-**11** display depressed T_C 's of 14°C and <10°C. The unsaturation-induced fluidity is expressed in the liposomal dynamics where 1:10 F/NF surface differentiated coliposomes of **10** stoutly resist flip-flop below their T_C ($t_{1/2} > 30$ min at 40°C), while coliposomes of *cis*- or *trans*-**11** undergo rapid flip-flop with $t_{1/2} \sim 3$ or 8 min, respectively, at 15°C (ref. 22). The greater floppiness of the *cis*-**11** liposomes reflects the greater disturbance to chain packing created by a *cis* vs. a *trans* double bond (ref. 1-3).

The suppressed T_C and decreased resistance to flip-flop is consistent with previous reports of looser chain packing in liposomes constructed from unsaturated lipids (ref. 23), but our cationic lipids appear more susceptible to flip-flop at ambient temperature than related, zwitterionic phospholipids; dioleoylphosphatidylcholine liposomes are reported to have a $t_{1/2}$ of several days at 30°C (ref. 24). The difference must be related to the differing head groups (see below).

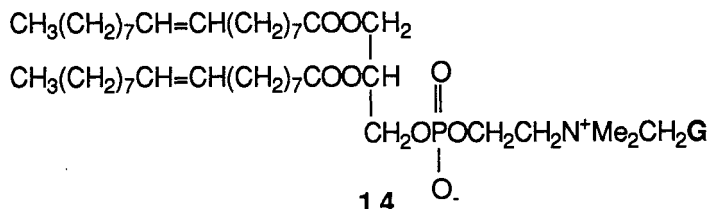
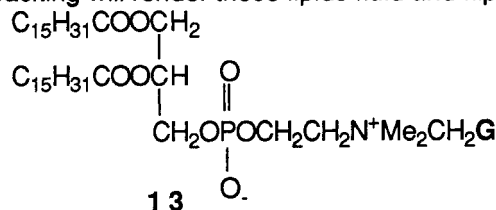


Certain bacteria and protozoa possess phospholipids bearing cyclopropanated chains derived from dihydrosterculic acid. These afford bilayers that are less fluid and more ordered at ambient temperatures than bilayers composed of analogous unsaturated lipids (ref. 25). The cyclopropyl group induces a low T_C (like C=C), but it also provides a barrier to the transmission of segmental motion along the chain, imposing greater ordering relative to the unsaturated chain. High local ordering and long motional correlation times might fit the cyclopropyl lipids for a biological regulatory role in the dynamic processes that affect membrane stability.

We prepared the bis-cyclopropanated pseudoglycerol ammonium ion lipid, **12**, for comparison to bis-oleoyl lipid *cis*-**11** (ref. 26, 22). The small (450 Å) 1:10 F/NF coliposomes of dihydrosterculoyl **12** displayed a low T_C (<10°C), similar to that of oleoyl liposomes of **11**, but the surface differentiated cyclopropanated liposomes reequilibrated more slowly: $t_{1/2} \sim 16$ min at 25°C, compared to $t_{1/2} < 1$ min for the analogous *cis*-**11** liposomes at the same temperature (ref. 26). The origins of this disparity can be traced to the lipids' conformational states. Fourier transform IR revealed fewer disruptive double gauche conformational sites in the acyl chains of **12** than in those of *cis*-**11**. This can be associated with better chain packing, increased chain order, and greater resistance to flip-flop in liposomal **12**. Though dynamically less stable than such saturated analogues as **5** or **10**, cyclopropanated liposomes of **12** are indeed more stable than their unsaturated analogues.

Saturated and unsaturated phospholipids

Pseudoglycerol ammonium ion liposomes are relatively unstable toward flip-flop; above their T_C , the fluid, liquid crystalline liposomes are subject to facile trans-bilayer lipid migration. The cationic head groups of these lipids are mutually repulsive, so that their positional stability within the bilayer must depend entirely upon the packing of their long chains. Any factor that degrades close packing will render these lipids fluid and flippable.



Biological membranes contain unsaturated lipids and are generally fluid, as required for facile passage of essential molecular traffic. Yet these membranes retain surface differentiation, including a transverse asymmetry of lipid distribution (ref. 1-5). Their maintenance of bilayer fluidity and surface differentiation must be a function of their head groups, which are often zwitterionic (e.g., **1**), and can be mutually attractive, in contrast to the cationic head groups of synthetic ammonium lipids like **5**.

To test these ideas, we compared coliposomes constructed from saturated and unsaturated ammonium ion lipids **5**(**10**) and **11** with saturated and unsaturated phosphatidylcholines **13** and **14** (ref. 13). Coliposomes were prepared from 1:7 or 1:10 F/NF lipid blends, were small ($d = 310\text{-}420 \text{ \AA}$), and unilamellar. The T_C 's of the saturated liposomes are similar (**5**, 44°C and **13**, 40°C), but the surface differentiated phospholipids are more resistant to flip-flop than the ammonium ion lipids: approximate $t_{1/2}$ values at 60°C are 16 min for **13** and 2 min for **5** (ref. 13). [Note that these reequilibrations are conducted above the T_C 's; at 25°C the reequilibration of either liposome would require hours.]

The enhanced stability of the phospholipids becomes dramatically clear when chain unsaturation is introduced. As we have seen (ref. 22), liposomes of unsaturated pseudoglycerylammonium ions (e.g., *cis*-**9**) feature low T_C 's, and are fluid and floppy: $t_{1/2} \sim 3$ min at 15°C . In contrast, no diminution in resistance to flip-flop attends unsaturation in phospholipids. As expected, the T_C drops sharply with unsaturation (40°C for dipalmitoyl liposomal **13** vs. $<0^\circ\text{C}$ for dioleoyl **14**), insuring bilayer fluidity at ambient or physiological temperatures. However, stability toward reequilibration of the surface differentiated, unsaturated phospholipids is actually enhanced: $t_{1/2} \sim 6$ min at 65°C for **13**, but $t_{1/2} \sim 20$ min at 65°C for **14** (ref. 13).

Thus, bilayers constructed of unsaturated, zwitterionic phosphatidylcholines retain their resistance to flip-flop far above their T_C . Indeed, an Arrhenius study yields an activation energy of ~ 24 kcal/mol for the reequilibration of liposomal **14**, affording $t_{1/2} \sim 24$ h at 30°C by extrapolation. This is in order of magnitude agreement with McConnell's value of $t_{1/2} \sim 2.6\text{-}7$ h at 30°C for a spin-labeled dipalmitoylphosphatidylcholine probe in egg lecithin liposomes (ref. 14). It seems possible that \pm/\pm attractive electrostatic interactions may oppose flip-flop with **14** or **13**, even when their bilayers are in the fluid state (ref. 27).

In dynamic terms, coliposomes containing functional lipids **13** or **14** are good models for biologically relevant bilayer membranes. Our investigations can now turn toward the effects of additives such as cholesterol, peptides and proteins, or sphingomyelin on membrane permeability and stability. Most recently, we have shown that mechanical stress created by thermally responsive polymers bound to the exteriors of differentiated liposomes of **13** can "catalyze" endo to exoliposomal flip-flop, presumably by generating defects or "holes" in the exoliposomal leaflets (ref. 28).

Acknowledgments

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