

Highly impermeable vesicles composed of conformationally restricted phosphatidylethanolamine

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Vesicles composed of a phosphatidylethanolamine derivative with a cyclopropyl-containing interfacial region are twenty-seven times less permeable than vesicles composed of a closely related analogue.

One of the broad aims of liposome science is to identify the supramolecular factors that control bilayer membrane self-assembly.¹ A more specific technological objective, with numerous practical applications, is the development of highly stable, non-leaky vesicles.² To achieve these goals, it is important to fully understand the structural features that determine if a polar lipid can form a stable, impermeable membrane. The classic experimental approach is to conduct structure–property studies. For example, the various lipid structures that permit thermophilic bacteria to function under extreme conditions have inspired chemists to prepare and investigate compounds such as double-headed amphiphiles (bolaamphiphiles)³ and single-headed amphiphiles with macrocyclic non-polar tails.⁴ In other cases, membranes with high mechanical stability have been produced using polar lipids with fluorocarbon tails,⁵ or three hydrocarbon tails.⁶

The structure of a typical glycerophospholipid can be divided into three parts, a highly polar phosphate diester head group, a moderately-polar interfacial region containing the glycerol carboxylic esters, and the non-polar tails. Numerous studies have shown how membrane permeability changes with phospholipid head group and tail structure, but there are very few published reports on the importance of the interfacial structure.⁷ In this contribution, we describe a dramatic supramolecular effect produced by a very subtle structural change in the interfacial region of a phospholipid. Specifically, we compare the membrane permeabilities of vesicles composed of the nearly-identical phosphoethanolamine derivatives **1** or **2**. The only difference between cyclopropyl-containing **1** and analogue **2** is the substitution of two C–H bonds for a C–C bond in the interfacial region of the molecule. In general, phosphoethanolamines are known for their propensity to form non-bilayer assemblies such as inverse hexagonal phases.⁸ For example, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine, **3**, which can be considered as a close glycerol analogue of **1** and **2**, undergoes a transition from bilayer to inverse hexagonal phase at 43 °C. Despite this precedence we find that dispersions of phosphoethanolamines **1** or **2** readily form encapsulating vesicles in high or low ionic strength buffer at neutral pH. Even more unexpectedly, the vesicle permeabilities are quite different, vesicles composed of **2** are much more leaky than vesicles composed of constrained analogue **1**.

Compounds **1** and **2** were prepared *via* the corresponding alcohols **4** and **5**.[†] Both **1** and **2** readily form unilamellar vesicles upon aqueous dispersion at neutral pH followed by repeated extrusion through microporous filters with 100 nm pores. Membrane permeability was evaluated by monitoring the leakage rates of various encapsulated fluorescent dyes. For example, the profiles in Fig. 1A show the leakage of entrapped fluorophore 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS) and its water-soluble quencher *p*-xylylenebis(pyridinium) bromide (DPX) from unilamellar vesicles at pH 7.4 and 25 °C.[‡] The difference is striking, the vesicles composed of **2** lose a

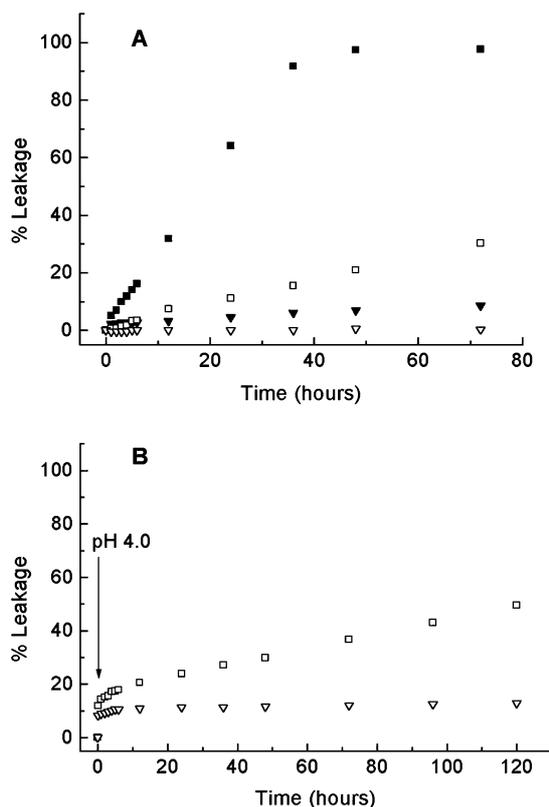
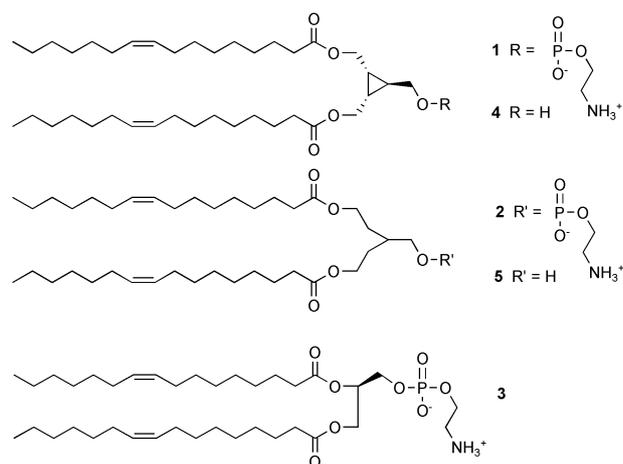


Fig. 1 Percent leakage of HPTS–DPX from vesicles (50 μM phospholipid) in 5 mM TES–100 mM NaCl. (A) Leakage at pH 7.4 from vesicles composed of **1** at 25 °C (empty triangles) and 37 °C (filled triangles) and from vesicles composed of **2** at 25 °C (empty squares) and 37 °C (filled squares). (B) Leakage induced upon acidification from neutral to pH 4. From vesicles at 25 °C and composed of **1** (empty triangles) and from vesicles composed of **2** (empty squares).

third of their fluorescent contents after three days, whereas there is essentially no leakage from vesicles composed of **1** over the same time period. Also shown in Fig. 1A are leakage experiments conducted at 37 °C where the rates of HPTS–DPX leakage are 0.09% hr⁻¹ for **1** and 2.46% hr⁻¹ for **2**. Thus, the vesicles composed of constrained analogue **1** are twenty-seven times less permeable than vesicles composed of **2**. Although vesicles containing phosphoethanolamines are generally known to become unstable upon acidification,⁹ this is not the case with vesicles composed of **1** or **2**. For example, lowering the pH from neutral to 4 at 25 °C has no apparent effect on vesicle leakage (compare Fig. 1A and 1B), or vesicle size as judged by dynamic light scattering. The leakage rate from vesicles composed of **2** does not change as the concentration of **2** is varied from 25 to 100 μM which indicates that leakage does not require vesicle collision.¹⁰

At room temperature, vesicles composed of **1** or **2** are in the fluid phase as judged by anisotropy measurements made with the fluorescent probes diphenylhexatriene (DPH) and trimethylammonium diphenylhexatriene (TMA-DPH) (Table 1). This is not surprising because **1** and **2** have identical non-polar tails containing *cis* double bonds. High-sensitivity differential scanning calorimetry measurements of the vesicles at neutral pH show no discernible phase transitions between 10–90 °C indicating that **1** and **2** have a strong propensity for the bilayer phase.

Table 1 Anisotropy measurements for fluorescent probes in different vesicle compositions at 25 °C.[§]

Probe	1	2	3 (fluid-phase) ^a	DPPE (gel-phase) ^b
DPH ^c	0.09	0.07	0.15	0.62
TMA-DPH ^c	0.16	0.18	0.22	0.57

^a Gel-fluid transition temperature for **3** is –33 °C.¹⁵ ^b Gel-fluid transition temperature for 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) is 63 °C.¹⁵ ^c DPH is diphenylhexatriene; TMA-DPH is trimethylammonium diphenylhexatriene.

The difference in leakage rates is notable for a number of reasons. Previous studies have shown that phospholipid bilayer membrane stability is increased when the long, non-polar tails are tethered at their ends to make a giant macrocycle.^{4,11} This tethering effect has been attributed to a reduced number of *trans*–*gauche* interconversions which stiffens the acyl chains, thus improving packing efficiency.¹¹ Such an effect cannot be invoked here because the tails are not tethered and the membranes are fluid phase. Compared to the gel phase, the fluid phase is characterized by a significantly expanded average phospholipid surface area, rapid *trans*–*gauche* isomerism in the non-polar tails, rapid rotation around the phospholipid long axis, and rapid but limited angular excursions of the phospholipid long axis.¹² Thus, it is surprising that such a subtle restriction in conformational flexibility at the phospholipid interfacial region has a profound effect on membrane permeability. At this point, a number of potential explanations are under consideration. For example, there may be a significant difference in bilayer thickness, or alternatively, it may be that the massive membrane reorganization required for passage of large, multiply-charged molecules (transient pore formation) is very sensitive to phospholipid packing around the interfacial region. Previously reported molecular dynamics calculations of bilayer aggregates appear to be consistent with this latter idea.¹³ In any case, our results show for the first time that subtle constraints on the structure and flexibility of the interfacial

region of a phospholipid can have a major effect on the mechanical strength of an assembled bilayer membrane.

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Notes and references

† Selected spectral data: **1**: *R*_f = 0.58 (CHCl₃–MeOH–H₂O 62:25:4); ¹H NMR (300 MHz) δ 5.39–5.26 (m, 4H), 4.35 (br dd, *J* = 12.3, 5.1 Hz, 2H), 3.95–3.82 (m, 4H), 3.79–3.64 (m, 4H), 2.27 (t, *J* = 7.5 Hz, 4H), 2.00 (br d, *J* = 5.7 Hz, 8H); ¹³C NMR δ 173.8, 130.0, 129.7, 68.0 (br), 67.4 (br), 63.1, 61.6 (br); FAB-HRMS (*m/z*) [*M* + *H* + *Na*]⁺ calcd for C₄₀H₇₄NO₈P 751.5128, found 751.5153. **2**: *R*_f = 0.58 (CHCl₃–MeOH–H₂O 62:25:4). ¹H NMR (500 MHz) δ 5.37–5.29 (m, 4H), 4.17–4.06 (m, 4H), 3.90 (br s, 2H), 3.78 (br t, *J* = 4.5 Hz, 2H), 3.71 (br s, 2H), 2.26 (t, *J* = 7.5 Hz, 4H), 2.00 (q, *J* = 6.5 Hz, 8H); ¹³C NMR δ 174.1, 130.0, 129.7, 67.3 (d, *J* = 6 Hz), 66.3, 62.3, 61.6 (br d, *J* = 5.5 Hz), FAB-HRMS (*m/z*) [*M* + 2*H*]⁺ calcd for C₄₀H₇₆NO₈P 731.5466, found 731.5437.

‡ The difference in vesicle leakage rates, shown in Fig. 1, was confirmed at neutral pH with experiments using entrapped carboxyfluorescein or the fluorophore–quencher mixture of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *p*-xylylenebis(pyridinium) bromide (DPX). The HPTS–DPX system is particularly useful because its fluorescence is pH independent at an excitation wavelength of 413 nm: see, D. L. Daleke, K. Hong and D. Papahadjopoulos, *Biochim. Biophys. Acta*, 1990, **1024**, 352.

§ Vesicles (250 μM phospholipid) containing 0.2 mol % of either DPH or TMA-DPH in 5 mM TES–100 mM NaCl. Interpretation of the anisotropy values is discussed in ref. 14, but simple inspection of the data in Table 1 shows that the values for vesicles composed of **1** or **2** are similar to the values for fluid-phase vesicles composed of **3** and are very different to the values for gel-phase vesicles composed of DPPE.

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