Cyclohexane Rings Reduce Membrane Permeability to Small Ions in Archaea-Inspired Tetraether Lipids

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Abstract: *Extremophile archaeal organisms overcome problems of membrane permeability by producing lipids with structural elements that putatively improve membrane integrity compared to lipids from other life forms. Herein, we describe a series of lipids that mimic some key structural features of archaeal lipids, such as: 1) single tethering of lipid tails to create fully transmembrane tetraether lipids and 2) the incorporation of small rings into these tethered segments. We found that membranes formed from pure tetraether lipids leaked small ions at a rate that was about two orders of magnitude slower than common bilayer-forming lipids. Incorporation of cyclopentane rings into the tetraether lipids did not affect membrane leakage, whereas a cyclohexane ring reduced leakage by an additional 40%. These results show that mimicking certain structural features of natural archaeal lipids results in improved membrane integrity, which may help overcome limitations of many current lipid-based technologies.*

*I*n nature, ion pumps, molecular transporters, and alterations of membrane composition are used to reduce membrane leakage and maintain gradients.[1–4] Archaeal organisms (halophiles, thermophiles, acidophiles, nitrifiers, and methanogens), one of the three domains of life, have evolved mechanically and chemically robust membrane compositions that allow survival in extreme environments.[5] For instance, Crenarchaeota, a kingdom of Archaea, have an optimal survival temperature above 80 °C.[6] Interestingly, the membranes of these hyperthermophiles are comprised of lipids containing cyclopentane rings, with a positive correlation found between the number of cyclopentane rings integrated

in their lipid membrane and environmental growth temperature.[1,7] This unique structural feature has been proposed to decrease membrane leakage by increasing lipid packing.[8] Additionally, Thaumarchaeota have cyclohexane rings incorporated into their lipids,[7] which have also been suggested to affect membrane packing.[9]

Modification of membranes by addition of cholesterol, PEG lipids, and lipids with high phase-transition temperatures is a common strategy to reduce membrane leakage in laboratory settings.[10] However, the incorporation of additives onto membranes can be problematic as a result of, for example, leaching,[11] potential long-term toxicity,[12–14] or difficulty with liposome preparation.[15]

Several attempts have been made to address problems with membrane leakage by using lipids extracted from archaeal species or through chemical synthesis of archaea-inspired lipids. For instance, polar lipid fraction E (PLFE) extracted from *Sulfobus acidocaldarius* exhibit low permeability, tight membrane packing, and high stability.[16,17] However, harvesting reproducible and large quantities of specific lipid compositions from cultured archaeal species can be challenging.[18] While a few groups have also reported the synthesis of singly tethered transmembrane spanning tetraether lipids,[19–23] the relationship between structure and function of these lipids remains unclear because of the limited data available on their membrane permeability properties. A systematic study of the effect of specific structural features (taking inspiration from archaeal lipids) on membrane leakage could make it possible to design lipids with improved integrity under a variety of environmental conditions.

Herein, we describe a series of synthetic lipids inspired by those found in archaeal organisms, namely glycerol mono-alkyl glycerol tetraether lipids with phosphocholine head groups (GMGTPC), which exhibit excellent membrane integrity without the necessity of additives (Scheme 1). Collectively, the structural features of these new synthetic lipids attempt to mimic lipids derived from Crenarchaeota or Thaumarchaeota.[6] By incorporating the essence of some key structural features (for example, the ether glycerol linkage, tethering of lipids, and incorporation of rings) found in natural archaeal lipids, we generated a set of synthetic lipids that formed stable liposomal membranes at room temperature with reduced leakage properties compared to commercially available EggPC lipids.

We designed the series of synthetic lipids shown in Scheme 1 to evaluate the effects on membrane permeability of two important structural components found in many archaeal lipids: 1) the effect of the tethering of alkyl tails to create bolaform amphiphiles capable of spanning the length

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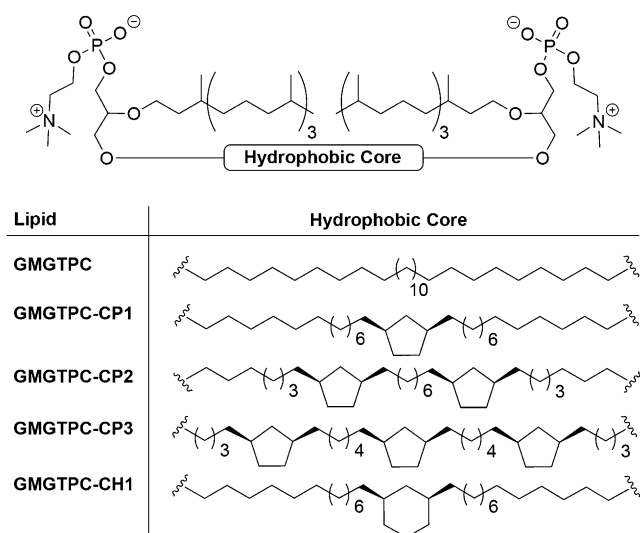
Scheme 1. Structures of synthesized tetraether lipids.

of the membranes, and 2) the effect of the incorporation of cyclopentane or cyclohexane rings within the tethered lipid chain. In all lipids synthesized, we included ether linkages instead of ester groups between the lipid tails and the head groups because ether functional groups are expected to be more chemically stable than the ester groups commonly found in eukaryotic and prokaryotic lipid membranes.

Tetraether archaeal lipids found in nature contain either a single transmembrane tether or are macrocyclic (that is, both transmembrane lipid tails are tethered).[24] Although one example of a macrocyclic tetraether lipid has been prepared by total synthesis,[25] the preparation required over 20 synthetic steps (without incorporation of rings).[26,27] Thus, obtaining access to a series of synthetic macrocyclic tetraether lipids containing rings was not practical. We therefore synthesized lipids containing a single tether, which made it possible to prepare a series of transmembrane spanning lipids containing zero to three rings in sufficient quantities (150–420 mg) to evaluate their leakage properties. Phosphocholine head groups were incorporated into all lipids because these zwitterionic groups are known to produce stable liposomes.[28]

We used a series of Wittig and SN2 reactions[29,30] to generate a set of five GMGTPC tethered lipids (Scheme 1) that differ from each other by the number and type of rings in the hydrophobic core. The preparation of each of the lipids required about 10 synthetic steps (see Figures S1–S6 in the Supporting Information). Phytanyl groups were incorporated as the untethered lipid chain in all GMGTPC lipids to avoid occurrence of the phase transition temperatures of the lipids near room temperature. Differential scanning calorimetry (DSC) measurements support that all newly synthesized lipids in liposome form do not undergo a phase transition over a temperature range of 5–70 °C (Figure S7).

We prepared liposomes from pure GMGTPC lipids by hydration of thin films of each lipid in buffer containing 5,6-carboxyfluorescein (CF), followed by extrusion. This preparation afforded liposomes with an average hydrodynamic

diameter of about 130 nm, as determined by dynamic light scattering (DLS; Figure S8). We expect the lipids in these liposomes to predominantly adopt a transmembrane configuration.[31,32] However, it is plausible that some fraction of the lipids can adopt a hairpin configuration to help stabilize the high curvature of the liposomes. The average size of these liposomes was stable over at least 6 hours, and we did not observe evidence of liposome aggregation or fusion when subjected to a liposome fusion assay[33] (Figures S8 and S9). The absence of a lipid phase transition or aggregation/fusion of liposomes near room temperature suggests that the GMGTPC lipids are suitable for evaluating the effects on membrane leakage of the incorporation of small rings into tetraether lipids.

To evaluate the relative permeability of membranes formed from these different lipids, we developed a modified pH equilibration assay that was previously reported by Kakinuma and co-workers.[20] In this assay, we encapsulated CF within the liposomes with an initial internal liposomal pH value of 7.2. The liposomes were then incubated in a buffered solution with an external pH value of 5.8, and the change in fluorescence intensity of the CF unit was monitored over time as the internal liposome pH equilibrated to pH 5.8. We chose to use pH values of 5.8 and 7.2 as external and internal liposomal pH values, respectively, because CF exhibits a linear correlation between its fluorescence intensity and the environmental pH value within this pH range.[34] We also chose to use CF as the fluorescent reporter of pH because we did not detect any appreciable leakage of CF from the liposomes formed from any of the lipids used in this study over the time required to observe pH equilibration at room temperature (Figure S10).

While we expect all of the membranes to be most permeable to protons over any other ionic species under these experimental conditions, it is possible the intraliposomal buffer and other ions, such as OH^-, Na^+, or Cl^-, could also contribute to the observed rate of pH equilibration.[35] We therefore consider the measured initial rates of pH equilibration from membranes composed of the different lipids to represent an estimate of their overall permeability to small ions rather than an estimate of their permeability to a specific single ionic species.

To understand whether tethering of lipids affected membrane leakage of small ions, we evaluated the detected initial rates of pH equilibration of liposomes composed of EggPC and GMGTPC (Figure 1 A; Figure S11). Similar to previously reported trends comparing the permeability of liposomes composed of lipids derived from archaea versus bacteria,[17] GMGTPC liposomes exhibited a decrease in the rate of leakage of small ions of about two orders of magnitude when compared to liposomes formed from EggPC. This result could arise, in part, through a combination of the absence of the ester linkage found in EggPC, the presence of the branched alkane network provided by the phytanyl group in GMGTPC, and the minimization of the small aqueous layer found in between the two lipid leaflets of a bilayer-forming lipid (which, presumably, would be sparse in a membrane comprised of pure tethered-GMGTPC lipids).[16,36,37]
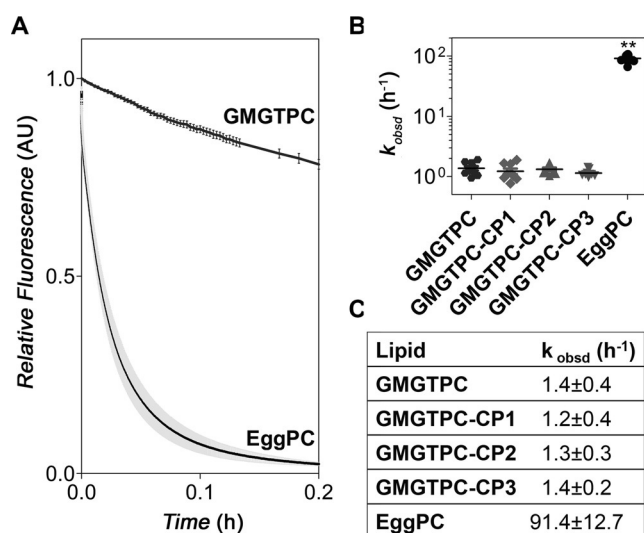
Figure 1. Observed rate of pH equilibration from liposomes formed from EggPC or synthetic lipids. A) The change in CF fluorescence intensity (monitoring at $\lambda(\text{Ex}/\text{Em}) = 485/517$ nm) from CF-encapsulated GMGTPC or EggPC liposomes versus time. B) Comparison of the observed initial rates of decreased CF fluorescence intensity from CF-encapsulated liposomes composed of different lipids. C) Average detected initial rates of pH equilibration in liposomes composed of different lipids. Standard errors of the mean are provided based on nine measurements each. Statistical analyses were performed using a paired t-test. ** indicate a *p*-value < 0.01.

Figure 2. Comparison of the detected initial rates of pH equilibration of liposomes composed of lipids with zero rings (GMGTPC), one cyclopentane ring (GMGTPC-CP1), or one cyclohexane ring (GMGTPC-CH1). A) Natural log of the relative fluorescence intensity of CF (first 15% of pH-dependent fluorescence decrease of CF) plotted against time and B) the detected initial rates of pH equilibration from liposomes composed of GMGTPC, GMGTPC-CP1, or GMGTPC-CH1. Statistical analyses were performed using a paired t-test. ** indicate a *p*-value < 0.01.

A common feature found in many natural lipids derived from Crenarchaeota is the presence of cyclopentane rings within the tethered transmembrane core of tetraether lipids.[38–40] To examine the effect of the integration of cyclopentane rings in the lipid on small-ion membrane leakage, we evaluated the rate of pH equilibration from liposomes formed from GMGTPC-CP1–3 (Figure 1 B and C), which contained 1, 2, or 3 *cis*-1,3-cyclopentane rings (Scheme 1). Interestingly, we found that the presence or number of rings had no detectable effect at room temperature on the rate of pH equilibration compared to GMGTPC (which has zero rings within its tethered hydrophobic core). These results are in contrast to reported computational studies suggesting that cyclopentane rings[41] in tethered lipids increased lipid packing.[7,42] Although the stereochemistry of the cyclopentane rings (*cis* versus *trans*) may account for the discrepancy between our experimental results and the reported computational studies, conformational analysis[43] and X-ray studies[44] of polymers containing multiple 1,3-cyclopentane rings support that the energy of interstrand packing of these polymers is independent of the *cis* or *trans* configuration of the rings. Nevertheless, the results shown in Figure 1 demonstrate that any structural effects to the lipid through introduction of *cis* cyclopentane rings is not sufficient to significantly affect membrane leakage of small ions under the conditions used in these studies.

To examine the effects on membrane leakage of a cyclohexane ring incorporated into tethered lipids, we synthesized GMGTPC-CH1 (Scheme 1) containing a *cis*-1,3-cyclohexane group. In this case, the molecule maintained the same *cis*-
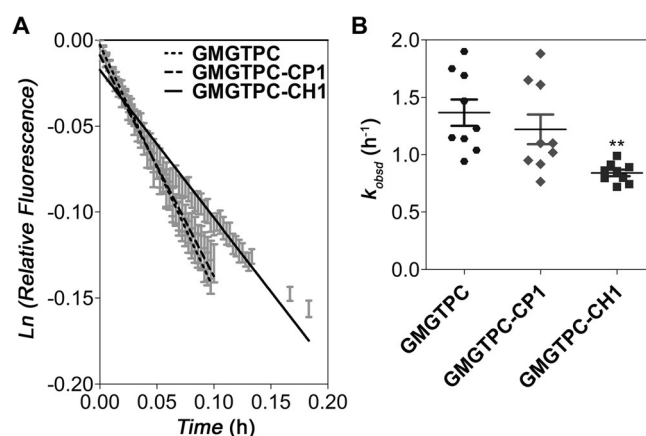
1,3 stereochemistry as in the GMGTPC-CP1–3 lipids, avoiding introduction of an additional variable when comparing their leakage properties. Interestingly, Figure 2 shows that liposomes composed of GMGTPC-CH1 lipids exhibited an additional circa 40% decrease in the rate of small-ion membrane leakage ($k_{\text{obsd}} = 0.8 \pm 0.1$ h^{-1}) compared to liposomes comprised of lipids with no rings (GMGTPC) or with one cyclopentane ring (GMGTPC-CP1). Presumably, the difference in flexibility of the cyclohexane ring compared to a cyclopentane ring affects lipid packing, which leads to decreased membrane permeability to small ions in GMGTPC-CH1 liposomes.[45] Additionally, intercalation of cyclohexane or cycloheptane between the hydrophobic tails of a bilayer lipid is believed to decrease proton permeability.[46] These results reveal one potential functional benefit of cyclohexane ring incorporation found in lipids derived from Thaumarchaeota, which grow optimally in environments between pH 5–8 and temperatures of 20–45 °C.[6]

We have thus presented a systematic study of the effects on membrane leakage of some key structural features inspired by natural archaeal lipids. As expected, the incorporation of phytanyl groups, the presence of tethering, and the incorporation of ether glycerol backbones in tetraether lipids substantially decreased the membrane permeability of small ions when compared to commercially available EggPC lipids. Surprisingly, we found that incorporation of *cis*-1,3 cyclopentane rings had no effect on leakage of small ions in GMGTPC lipids. In contrast, incorporation of a *cis*-1,3 cyclohexane ring significantly decreased small-ion membrane leakage compared to all other GMGTPC lipids studied. Such differences in small-ion permeability between lipids containing cyclopentane versus cyclohexane rings could reflect differences in ring flexibility as it relates to lipid packing. This work represents an important step towards establishing

some design principles, taking inspiration from nature, for generating lipids with low membrane permeability.

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