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Synthetic Lipids

Effect of Headgroups on Small-Ion Permeability across Archaea-Inspired Tetraether Lipid Membranes

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Abstract: This paper examines the effects of four different polar headgroups on small-ion membrane permeability from liposomes comprised of Archaea-inspired glycerol-monoalkyl glycerol tetraether (GMGT) lipids. We found that the membrane-leakage rate across GMGT lipid membranes varied by a factor of $\leq$1.6 as a function of headgroup structure. However, the leakage rates of small ions across membranes comprised of commercial bilayer-forming 1-palmitoyl-2-oleoyl-sn-glycerol (PO) lipids varied by as much as 32-fold within the same series of headgroups. These results demonstrate that membrane leakage from GMGT lipids is less influenced by headgroup structure, making it possible to tailor the structure of the polar headgroups on GMGT lipids while retaining predictable leakage properties of membranes comprised of these tethered lipids.

Eukaryotes and prokaryotes often respond to environmental stress by modifying the lipid composition of their membranes.[1–3] Archaea, a unique classification of prokaryotes, thrive in extreme environments, but can maintain their membrane integrity under environmental stress presumably due to the unique structural features integrated in their lipids.[4–6] These structural features include incorporation of: 1) phytanyl (isoprene) hydrocarbon chains, 2) tethered lipid chains, and 3) ether linkages between the glycerol backbone of the headgroup and the hydrophobic chains (Figure 1). Additionally, the mixtures of polar lipid headgroups in Archaea membranes are dependent on specific growth conditions.[4] For instance, under acidic conditions, the lipid composition of Archaea will include a high fraction of headgroups that can facilitate hydrogen bonding between the headgroups on adjacent lipids.[7,8]

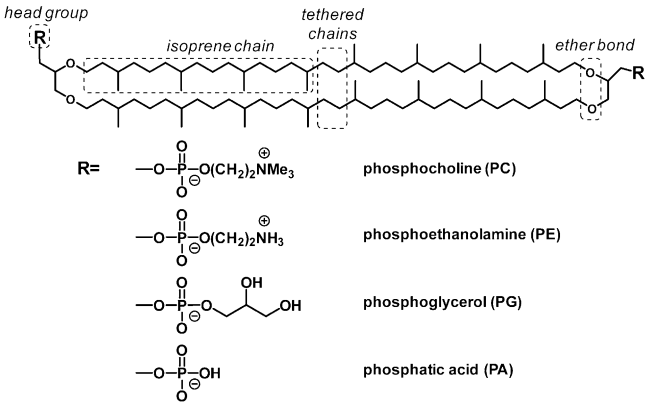

Figure 1. Examples of lipid headgroups found in Archaea.

Figure 1 shows the structures of four common polar lipid headgroups that are found naturally in Archaea.[5,9] The delicate balance in generating a precise mixture of lipids comprised of these different polar headgroups is thought to be essential in maintaining a viable membrane under harsh conditions.[10]

We recently reported a systematic study of the dependence of membrane leakage on the presence of small rings incorporated into the tethered, membrane-spanning segments of synthetic glycerolmonoalkyl glycerol tetraether (GMGT) lipids.[11] We found that incorporating a single transmembrane tether (with or without rings), phytanyl hydrophobic side chains, and ether linkages between the glycerol backbone made it possible to generate membranes with approximately two orders of magnitude slower rate of leakage of small ions (H^+, OH^-, Na^+, Cl^-, buffer ions) at room temperature compared to membranes generated from commercial EggPC lipids. These results demonstrate that altering the structure of the hydrophobic portion of lipids can dramatically affect membrane leakage. Additionally, previous computational[12] and experimental studies[13] suggest that lipid headgroups affect membrane permeation of water, but have little effect on permeability of small organic molecules across membranes.[14] However, to our knowledge no systematic study on the effects of headgroups on small-ion permeability has been reported.

Herein, we describe the synthesis of a series of Archaea-inspired GMGT lipid analogs containing four different headgroups found in nature (phosphocholine, PC; phosphoethanolamine, PE; phosphatic acid, PA; phosphoglycerol, PG; Figure 1)

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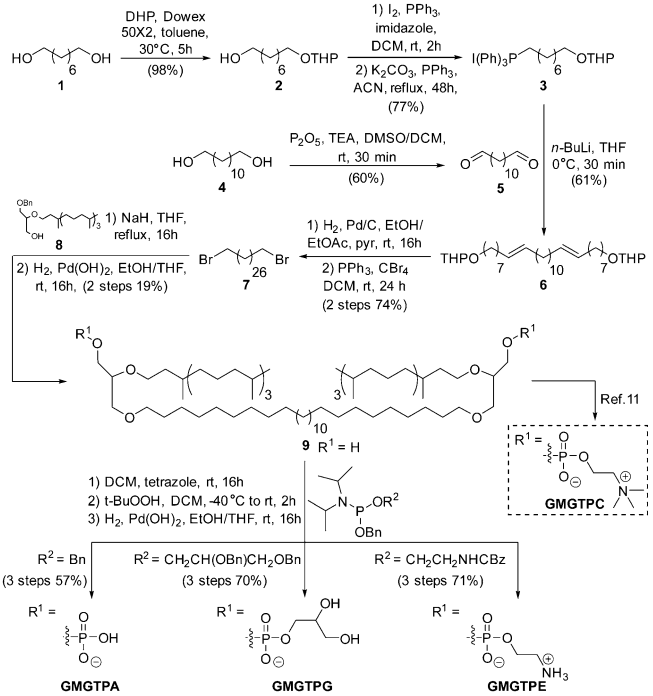

Scheme 1. Synthesis of GMGT lipid derivatives.

and investigate the effect of headgroups on small-ion membrane permeability across membranes comprised of these lipids. We prepared GMGT lipid analogs from the common diol **9** (Scheme 1), which comprised a 28-carbon aliphatic chain and two untethered phytanyl groups attached to a glycerol backbone through ether linkages. The phytanyl chains and ether groups were maintained throughout this series of lipids because these features were previously found to increase chemical stability and to reduce small-ion permeation.[11] Briefly, diol **9** was synthesized in seven steps from 1,12-dodecandiol, using a double Wittig reaction to form the key intermediate **6**. We then generated 1,28-dibromooctacosane **7** from **6** through a hydrogenation/bromination sequence, and reacted this dibromide with glycerol scaffold **8**, followed by hydrogenation to afford diol **9**. Finally, the syntheses of the different GMGT analogs were carried out using derivatives of benzyldiisopropyl phosphoramidite as phosphorylating agents.[15,16] The phosphite-triester products were then oxidized to the corresponding phosphate-triesters, and the protecting groups were removed by hydrogenation to afford lipids GMGTPA, GMGTPG, and GMGTPE in good yields as mixtures of stereoisomers. GMGTPC was generated by reaction of the diol **9** with 2-bromoethyl dichlorophosphate followed by nucleophilic displacement of the bromine with trimethylamine, as described previously[11] (see Figures S1–S3 in the Supporting Information for details of the syntheses of GMGT lipids).

We next assessed whether we could form liposomes comprised of pure GMGT lipids using a previously described procedure.[11] Though pure GMGTPC, GMGTPA, and GMGTPG readily formed stable liposomes of ≈120 nm diameter, (see Figure S4 in the Supporting Information), we were unable to form liposomes from pure GMGTPE lipids (similar results have been shown previously with standard diacylphospholipids with PE headgroups owing to relatively small headgroup size).[17] However, because we were able to form liposomes comprised of 1:1 mixtures between PC lipids and lipids carrying PA, PG, or PE headgroups, we used these mixtures to evaluate the effects of the headgroup on the relative membrane permeability (Figure 2). Differential scanning calorimetry (DSC) measurements show that suspensions of pure GMGTPC, GMGTPA, GMGTPG, or GMGTPE lipids in water maintained a liquid phase without an observed phase transition between 5 and 65 °C (see Figure S5 in the Supporting Information). Additionally, we did not observe any morphology changes by dynamic light scattering (DLS) within the timeframe of the leakage assays (see Figure S4 in the Supporting Information), suggesting that liposome fusion or aggregation did not significantly contribute to the observed rate of small-ion permeability.

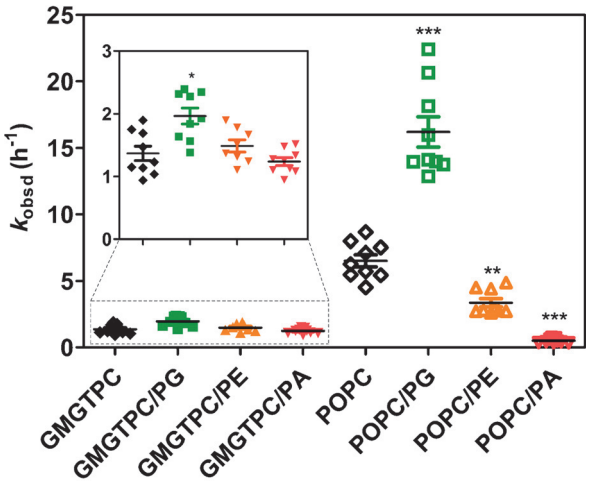

Figure 2. Observed initial rate of pH equilibration from liposomes formed from GMGT or PO series of lipids. Comparison of the observed initial rates of decreased carboxyfluorescein (CF) fluorescence intensity (monitored at $\lambda(E_x/E_m) = 485/517$ nm) from CF-encapsulated liposomes comprised of pure GMGTPC lipid or 1:1 mixtures of GMGTPC with GMGTPG, GMGTPE, or GMGTPA (the inset represents a zoomed-in graph of the observed initial rates of leakage from the GMGT lipids); and pure POPC lipid or 1:1 mixtures of POPC with POPG, POPE, or POPA. Statistical significance was determined using a paired Student *t*-test. *, **, *** indicate a *p*-value of <0.1, 0.01, 0.001, respectively, relative to the k_{obsd} of the analogous PC lipid.

We evaluated the relative permeability to small ions of membranes comprised of lipids carrying various headgroups using a pH-equilibration assay described previously.[11,18] Briefly, this method consists of an encapsulation of carboxyfluorescein (CF) inside liposomes with an intraliposomal pH of 7.2. The liposomes are then incubated in a buffered solution at pH 5.8, and the change in fluorescence intensity of CF is monitored over time as the pH between the inside and outside of the liposomes reaches equilibrium. We chose to use CF as a fluorescent reporter in this assay, in part, because we previously showed that CF does not leak from liposomes comprised of tetraether or diacyl lipids under these experimental conditions.[11] While we expect proton leakage to be the dominant contributor to the rate of pH equilibration,[19] other ions pres-

ent in the assay medium (e.g., OH^-, Na^+, Cl^-) could also contribute to the measured rate of leakage. The measured initial rates of pH equilibration, therefore, can be considered more generally as an estimate for membrane permeability to small ions rather than as an estimate of permeability to protons only. We also avoided addition of divalent cations in the buffer to prevent negatively charged lipid polar headgroups from inducing aggregation/fusion of liposomes.[20] To minimize error in our leakage-rate measurements from photobleaching and evaporation, we used initial rates (i.e., the first 15% change in CF fluorescence) to evaluate the rate of pH equilibration at room temperature (see Figure S6 in the Supporting Information). While this CF-based assay made it possible to estimate the relative effects of different headgroups on leakage of small ions from liposomes, other assays may offer additional advantages for estimating absolute permeability constants of specific ions across various lipid membranes.[19,21–23]

Figure 2 shows that there was no statistically significant difference between the observed rate of leakage of small ions from liposomes comprised of 1:1 mixtures of GMGTPC lipids with lipids containing PE or PA headgroups compared to liposomes comprised of pure GMGTPC lipids. Liposomes formed from a 1:1 mixture of GMGTPC:GMGTPG lipids, however, exhibited a 1.5-fold increased rate of leakage compared to pure GMGTPC liposomes (the numerical values of the observed first-order rates of membrane leakage are given in Table S1 in the Supporting Information). Overall, the rate of small-ion leakage from any two of the GMGT lipids differed by a factor of ≤ 1.6 as a function of headgroup.

To examine how these results compared to the effect of headgroups on standard bilayer-forming diacylphospholipids, we also examined leakage of small ions from 1-palmitoyL-2-oleoyL-sn-glycerol (PO) lipids. Because POPA and POPE have phase-transition temperatures near room temperature, we generated liposomes by mixing POPC lipids 1:1 with POPG, POPE, or POPA and compared their relative rate of leakage to liposomes comprised of pure POPC lipids (i.e., the analogous procedure we used to measure relative leakage from the GMGT lipids with the same four headgroups). Again, DSC measurements showed that liposomes comprised of 1:1 mixtures of POPC with POPA, POPE, or POPG maintained a liquid phase at room temperature and did not exhibit a phase transition between 5 and 65 °C (see Figure S5 in the Supporting Information), and POPC has a known phase transition at −2 °C.[24] DLS measurements also confirmed that lipid fusion or aggregation is not expected to significantly contribute to the observed rate of membrane leakage (see Figure S4 in the Supporting Information).

In the case of the PO series of lipids, we found significant effects of headgroups on the observed rates of small-ion membrane permeation (Figure 2). Similar to the series of GMGT lipids, the PG headgroup increased leakage compared to the PC headgroup. However, in the PO series, the PG headgroup had a much larger effect on membrane leakage (increased by a factor of 2.5) compared to liposomal membranes from lipids with PC headgroups only (see Table S1 in the Supporting Information). Furthermore, in contrast to the case with GMGTPE or GMGTPA (which did not affect membrane leakage compared to GMGTPC lipids), the rate of leakage from POPE- and POPA-containing membranes was a factor of 0.5 and 0.07 times slower, respectively, compared to membranes formed from pure POPC lipids. Surprisingly, liposomes comprised of a 1:1 mixture of POPC and POPA exhibited the lowest observed rate of small-ion leakage among all lipid mixtures tested under the experimental conditions used here. Figure 3 highlights that, in contrast to GMGT lipids, PO lipids show a strong dependence of headgroups on small-ion permeability.

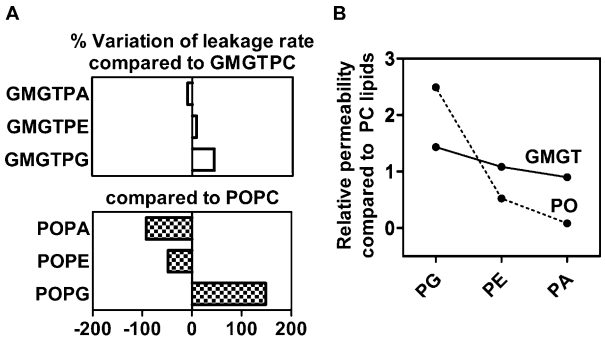

Figure 3. Relative effects of headgroups on small-ion membrane leakage. a) Graph of the relative variation of leakage rate from membranes comprised of 1:1 mixtures of PC with PA, PE, or PG lipids compared to membranes formed from pure PC lipids. Data represents the percent deviation of observed initial rates of membrane leakage compared to the observed initial rate of leakage from pure POPC or pure GMGTPC lipid membranes (zero percent). b) Graph showing the relative leakage rate of membranes comprised of 1:1 mixtures of PC with PA, PE, or PG lipids relative to the observed initial rate of leakage from membranes comprised of pure POPC or pure GMGTPC lipids (normalized to 1).

The reduced permeation of small ions observed for liposomes containing POPC mixed with POPA or POPE (compared to pure POPC liposomes) could arise from increased intermolecular hydrogen bonding between headgroups of neighboring lipids. Such intermolecular hydrogen bonding may lead to exclusion of water molecules near the membrane surface and increased membrane packing, as suggested through X-ray diffraction,[25] FT-IR,[26] and computation[27] studies. For the GMGT lipids, we previously showed that leakage of small ions from tethered lipids was significantly reduced compared to bilayer-forming lipids,[11] presumably as a result of favorable lipid packing of hydrocarbon chains in neighboring lipids within the membrane. Such inherently tight membrane packing in GMGT lipids, thus, may not be as influenced by membrane surface effects induced by the presence of PE or PA headgroups. On the other hand, for lipids with PG headgroups, the presence of multiple hydroxyl groups may lead to an increase in the number of water molecules in between lipid headgroups near the membrane surface, which could cause a decrease in lipid packing and an increase in membrane leakage.[13] We expect such an effect on leakage by PG headgroups would be more pronounced in PO lipids (which presumably are inherently more loosely packed) compared to GMGT lipids. The results from permeability experiments (Figure 2) support such a hypothesis.

We have, thus, presented a systematic study of the effects of lipid headgroups on the leakage properties of membranes comprised of GMGT or PO lipids. Liposomes containing PO lipids exhibited a strong dependence on headgroups for membrane leakage. Such effects on small-ion permeability from headgroups may limit the utility of PO (and possibly other bilayer-forming) lipids in applications and studies where maintaining a consistent membrane permeability is important across various headgroups. On the other hand, membrane leakage from liposomes comprised of synthetic GMGT lipids was not strongly influenced by headgroups. These findings suggest that GMGT lipids may offer greater flexibility for tailoring the functionality presented in the lipid headgroups, without significantly compromising membrane permeability. Such versatility in the design of lipid headgroups may open up opportunities to use GMGT lipids in a range of applications including the incorporation of receptor-targeting molecules on lipids for development of liposomal drug-delivery systems[28] or the incorporation of ligands as well as charged headgroups to attract specific analytes or binding partners to the membrane surface for biophysical studies.[29]

Acknowledgements

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