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Hybrid Lipids

Hybrid Lipids Inspired by Extremophiles and Eukaryotes Afford Serum-Stable Membranes with Low Leakage

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Abstract: This paper presents a new hybrid lipid that fuses the ideas of molecular tethering of lipid tails used by archaea and the integration of cholesterol groups used by eukaryotes, thereby leveraging two strategies employed by nature to increase lipid packing in membranes. Liposomes comprised of pure hybrid lipids exhibited a 5–30-fold decrease in membrane leakage of small ions and molecules compared to liposomes that used only one strategy (lipid tethering or cholesterol incorporation) to increase membrane integrity. Molecular dynamics simulations reveal that tethering of lipid tails and integration of cholesterol both reduce the disorder in lipid tails and time-dependent variance in area per lipid within a membrane, leading to tighter lipid packing. These hybrid lipid membranes have exceptional stability in serum, yet can support functional ion channels, can serve as a substrate for phospholipase enzymes, and can be used for liposomal delivery of molecules into living cells.

Reducing leakage of ions and small molecules across lipid membranes has been a major challenge for applications ranging from drug delivery to studies on membrane proteins.[1–4] For example, in drug delivery, encapsulated cargo in liposomes made from commercially available lipids typically suffer from rapid leakage, especially in the presence of serum.[3] The problem of content leakage has prompted many groups to steer away from liposomal delivery systems, and towards technologies based, for instance, on covalent attachment of drugs to polymeric backbones of nanoparticles.[5–10] However, unlike drug–polymer conjugates that are typically limited to chemically adhering only one type of drug to a polymeric backbone, the potential versatility of encapsulating different types of drugs without the need for chemical modification continues to make liposomal delivery systems an attractive argument for further development of membranes with low permeability.[1,11–14] In addition to drug delivery systems, a robust and stable membrane is required when designing nanopores for sensitive analytical assays using lipid membranes as platforms.[15]

In nature, several strategies are employed by organisms to address problems with membrane integrity. Eukaryotic organisms, for instance, typically fill defects in bilayer lipid membranes by incorporating free cholesterol (chol) molecules, which leads to increased membrane stability and reduced leakiness (Figure 1 A).[16] Archaea, on the other hand, generate lipids with ether linkages between the headgroups or covalently-tethered lipid tails (Figure 1 B), resulting in chemically stable membrane-spanning lipids that make it possible for the organisms to thrive in extreme environments.[17]

We previously reported a new class of archaea-inspired singly tethered lipids, namely, glycerol monoalkyl glycerol tetraether with phosphocholine (PC) headgroups (GMGTPC-CH,

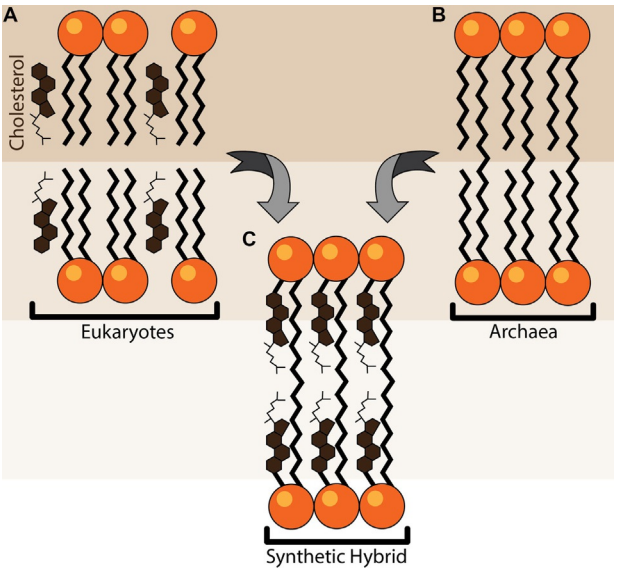

Figure 1. Comparison of strategies to improve membrane integrity used by eukaryotes (A) or archaea (B) with the novel strategy that combines both strategies in the synthetic hybrid lipid introduced here (C).

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Figure 2). These lipids readily formed stable liposomes and exhibited a reduction in small ion membrane leakage by ≈2 orders of magnitude when compared to the leakage properties of membranes formed from a commercially available diacyl

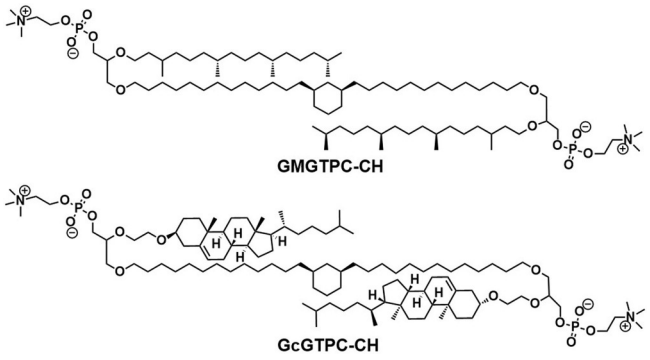
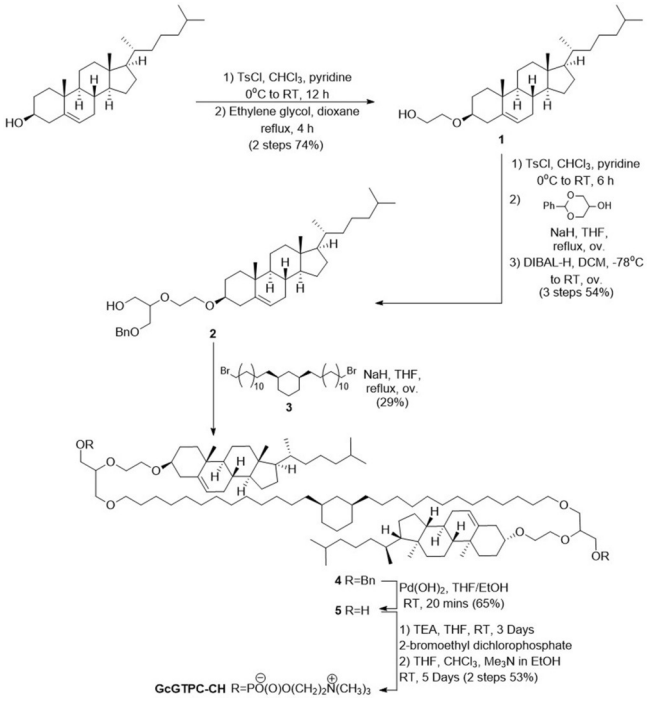


Figure 2. Structures of archaea-inspired synthetic lipids with (GcGTPC-CH) or without (GMGTPC-CH) covalent cholesterol integration.

lipid, Egg PC.[18] In contrast, the addition of free cholesterol to lipid formulations has been commonly used in research laboratories to reduce membrane leakage by improving membrane integrity (Figure 1 A).[19] Covalently attaching cholesterol to lipid tails has been shown to eliminate cholesterol leaching from liposomes.[20–22] Here, we explore the possibility to combine these two strategies used by nature for generating robust membranes by creating a hybrid lipid that incorporates covalent cholesterol integration into a tethered tetraether lipid (Figure 1 C). We compare the leakage properties of membranes comprised of this hybrid lipid to membranes formed from commercial diacyl lipids and an archaea-inspired lipid that did not comprise covalently attached cholesterol (both with and without incorporation of free cholesterol in the membranes). Molecular dynamics (MD) simulations provide a molecular interpretation for the observed differences in leakage properties between different lipid membrane compositions. We also examine the capability to incorporate functional ion channels into membranes from this hybrid lipid, explore if these lipids can be recognized as substrates of a membrane-active phospholipase-D (PLD) enzyme, assess the stability of liposomes comprised of these lipids in serum, and evaluate the uptake of such liposomes in cells. To the best of our knowledge, these studies represent the first reports of ion channel and PLD activities in pure tetraether lipid membranes, and, therefore, provide evidence that these unnatural membranes retain at least some of the functional properties of natural membranes.

Scheme 1 summarizes the synthesis of a cholesterol-integrated hybrid tetraether lipid, glycerol cholesterol-integrated glycerol tetraether lipid with phosphocholine headgroups (GcGTPC-CH, Figure 2). We designed the GcGTPC-CH lipid to comprise a cholesterol group attached to the lipid headgroup via an ethylene glycol spacer to improve flexibility and increase access for synthetic modification of the steroid. Tosylation of the 3β alcohol on cholesterol followed by substitution of the tosyl group with ethylene glycol afforded the modified cholesterol **1** with retention of stereochemistry on cholesterol.[23] The free terminal alcohol in **1** was then converted to the corresponding tosylate, which successively reacted with 2-phenyl-1,3-dioxan-5-ol followed by a selective ring-opening of the dioxolane moiety using diisobutylaluminium hydride (DIBAL-H) to form the benzyl protected glycerol backbone **2**. Dibromo alkane **3**[18] was then connected to the glycerol backbone in **2** to generate protected lipid **4** under basic conditions. The diol **5** was generated by deprotection of the benzyl ethers to generate the free alcohol groups. The formation of GcGTPC-CH was completed by reacting diol **5** with 2-bromoethyl dichlorophosphate, followed by displacement of the bromide by trimethylamine (see the Supporting Information and Figure S1 for details on the synthesis and characterization of **1**–**5**).



Scheme 1. Synthesis of hybrid lipid GcGTPC-CH.

We next probed the physical characteristics of GcGTPC-CH and examined whether replacing the phytanyl group (which prevents natural archaeal tetraether lipids from undergoing a phase transition between 0 to 80 °C)[24] in GMGTPC-CH with a cholesterol moiety would still make it possible to form stable liposomes within a useful temperature range. Differential scanning calorimetry (DSC) measurements revealed that GcGTPC-CH lipid remained in a liquid phase from 5 to 65 °C (see Figure S2 in the Supporting Information). This result was in agreement with previous reports, which showed that adding 40–50 mol% of free cholesterol to diacyl lipids[25] maintains a fluid phase and diminishes its phase transition. Dynamic light scattering (DLS) measurements demonstrated that we could generate liposomes with an average hydrodynamic diameter of ≈150 nm by extrusion through a 100 nm polycarbonate membrane (see the Supporting Information for details). Furthermore, liposomes remained structurally stable throughout the time frame of leakage experiments and we did not observe

any measurable changes in size by DLS (see Figure S3 in the Supporting Information).

Using a previously described pH equilibration method to estimate initial rates of leakage of small ions (e.g., H^+, OH^-, Na^+, Cl^-, and other buffer ions) from liposomes,[18,26] we first evaluated the observed initial rate of small ion membrane leakage to study whether the addition of free cholesterol (40 mol%) to 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), GMGTPC-CH, or GcGTPC-CH liposomal formulations affected membrane permeation of small ions (see Figure S4 in the Supporting Information). As expected, the addition of cholesterol to POPC resulted in a ten-fold decrease in small ion membrane leakage compared to cholesterol-free POPC membranes[27] (Figure 3A). However, the addition of cholesterol to GMGTPC-CH did not have a significant effect on the rate of small ion membrane leakage compared with the same lipid without added free cholesterol. To further probe this result, we examined whether free cholesterol was capable of integrating into the membranes comprised of GMGTPC-CH. The presence of free cholesterol in the GMGTPC-CH liposomes was confirmed by measurement of both total phosphorus content and free total cholesterol using a Bartlett assay[28] and an Amplex® Red cholesterol assay, respectively. The result showed a presence of 31±10 mol% of free cholesterol were indeed integrated in the GMGTPC-CH liposomes, which is within the expected range of cholesterol concentration for liposomal formulations.[29] In addition, cholesterol was also added to liposomes comprised of GcGTPC-CH lipids. However, we did not detect any free cholesterol associated with the GcGTPC-CH liposomes. This absence of free cholesterol incorporation in GcGTPC-CH lipid membranes is in agreement with previous reports that suggest that the maximum incorporation of total cholesterol in fluid lipid membranes is ≈50 mol% for liposomes prepared by extrusion.[29] In contrast with the observation that the addition of free cholesterol to GMGTPC-CH liposomes did not affect membrane leakage, liposomes comprised of pure GcGTPC-CH displayed a five-fold decrease in small ion membrane leakage compared with liposomes made with pure GMGTPC-CH (Figure 3A). This observed reduction in small ion membrane leakage from GcGTPC-CH is consistent with the hypothesis that covalently attaching cholesterol to the glycerol backbone in GcGTPC-CH allows cholesterol to appropriately orient within the membrane and results in tighter lipid packing than GMGTPC-CH (with or without added free cholesterol).

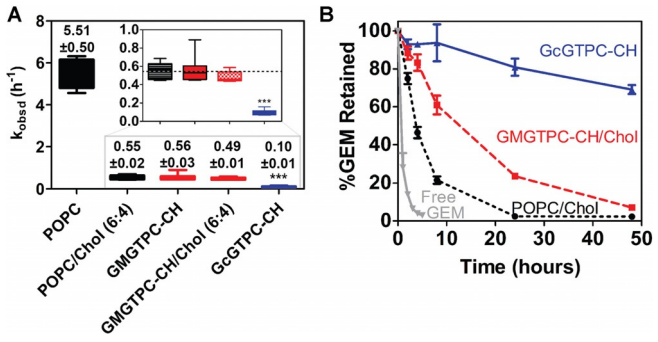


Figure 3. Membrane leakage results of small ions or gemcitabine (GEM, a neutrally charged drug) from liposomes formed from synthetic or POPC lipids with/without added free cholesterol. A) Comparison of the observed initial rates of decreased carboxyfluorescein (CF) fluorescence intensity (monitored at λ(Ex/Em) = 485/517 nm) from CF-encapsulated liposomes comprised of GMGTPC-CH, GcGTPC-CH, or POPC lipid with/without added 40 mol% cholesterol during liposome formation with averaged observed initial rates of membrane leakage noted over each box (unit = h^{-1}; N = 9). B) Graph of percent retention versus time of GEM encapsulated inside liposomes made from GcGTPC-CH, GMGTPC-CH with 40 mol% of free cholesterol, POPC with 40 mol% of free cholesterol, or free GEM (i.e., no liposomes) across a dialysis membrane upon incubation at 37 °C for 48 hours.

Secondly, we passively encapsulated gemcitabine (GEM, an anti-cancer drug with molecular weight of 263.2 g mol^{-1})[30] in liposomes to compare permeability of a neutrally charged molecule across membranes comprised of POPC/chol, GMGTPC-CH/chol or pure GcGTPC-CH. Here, we used a dialysis assay to compare the leakage of GEM over a 48 hour incubation at 37 °C (see Supporting Information for details). In contrast to the results from the small ion leakage assay (Figure 3A) that did not show a significant difference between POPC/chol and GMGTPC-CH/chol and a five-fold difference between POPC/chol or GMGTPC-CH/chol and pure GcGTPC-CH lipids, all 3 liposomal compositions exhibited significantly different leakage profiles for encapsulated GEM (Figure 3B). Remarkably, liposomes made from pure GcGTPC-CH retained 69.1±4.1% of GEM after 48 hours, whereas liposomes made from POPC/chol (2.3±0.1% retained GEM) or GMGTPC-CH/chol (7.1±1.2% retained GEM) exhibited almost complete loss of encapsulated GEM over the same period of time. These contrasting results between small ion versus small molecule leakage profiles for the different lipids could reflect differences in mechanism for leakage, in which small ions are believed to leak through transient membrane pores and neutral molecules could leak through both membrane pores and membrane partitioning.[31]

To provide a molecular interpretation for the observed trend in membrane permeabilities for POPC, GMGTPC-CH, and GcGTPC-CH, we performed molecular dynamics (MD) simulations of pure lipids in membranes (see Supporting Information for details). Figure 4A shows overlaid snapshots of the same individual lipids at 5 ns intervals revealing the conformational space explored by each lipid over 50 ns. The results highlight striking differences in the order of the lipid tails between these lipids. The reduced flexibility of lipid tails in the tetraether lipids translated to a decreased variance in the area occupied per lipid (Figure 4B), which could be represented quantitatively by the standard deviation (sd) around the mean area/lipid (Å^2). These calculations are in good agreement with the observed experimental trends for leakage of small ions and molecules across lipid membranes (Figure 3), supporting the concept that decreased variance in area per lipid leads to tighter lipid packing and lower permeability.[32] Furthermore, although our attempts at experimentally measuring permeability of water across different membrane compositions by an established light scattering[33] assay were inconclusive, MD simulations made it possible to estimate membrane water penetration for the different lipids.[34] We found 24 penetration events (i.e., transient events in which 3 or more water molecules resided anywhere within the hydrophobic region of a membrane over

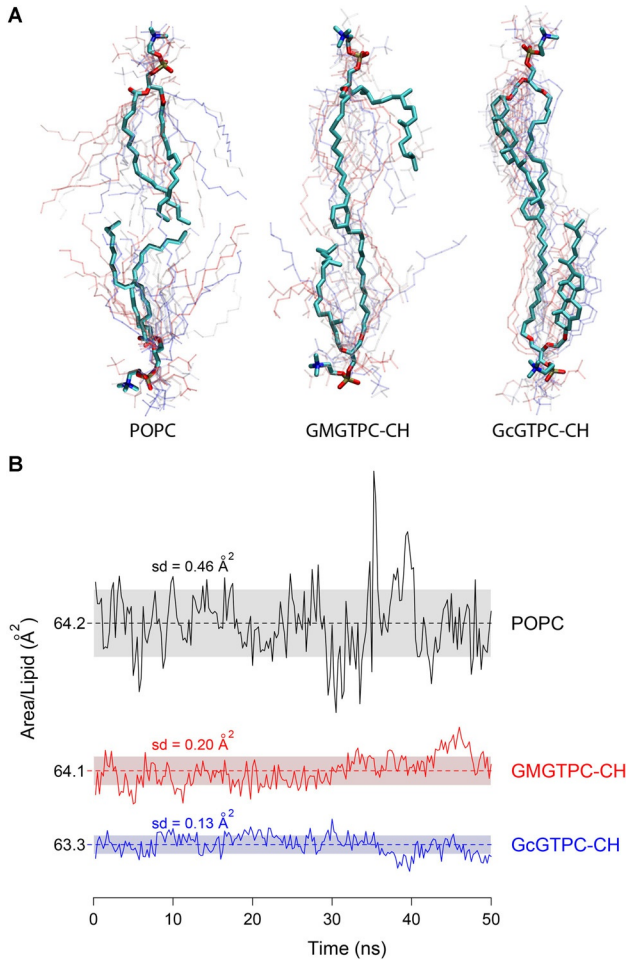


Figure 4. Results from molecular dynamics (MD) simulations of a membrane comprised of pure POPC, GMGTPC-CH, or GcGTPC-CH lipids. A) Overlay of 10 snapshots of individual lipids (taken in 5 ns intervals from MD simulations of pure lipid membranes) highlighting the increased ordering of the lipid tails from POPC to GMGTPC-CH to GcGTPC-CH. B) Graph of the area occupied by individual lipids (within membranes comprised of pure lipids) over a 50 ns membrane simulation highlighting the variance in area per lipid (represented quantitatively as standard deviation (sd)).

a 50 ns MD simulation) for pure POPC membranes, whereas we found 20 events for GMGMTPC-CH and only 1 such event for GcGTPC-CH under the same conditions (see Figure S5 in the Supporting Information). These differences in the number of observed water penetration events presumably reflect variations in both composition and conformational freedom between the different lipids tails, since all lipids contained the same phosphocholine headgroups.

To explore whether the membranes comprised of hybrid GcGTPC-CH lipids retained useful membrane properties for potential biological applications, we examined whether 1) GcGTPC-CH lipid membranes can act as a support for natural ion channel forming peptides such as gramicidin A (gA),[35,36] 2) GcGTPC-CH lipids can serve as a substrate for membrane active enzymes such as phospholipase-D (PLD),[37–39] 3) liposomes derived from GcGTPC are sufficiently stable in serum-containing buffers,[3,40,41] and 4) small molecules encapsulated in GcGTPC-CH liposomes can be delivered to living cells.[42–44]

Gramicidin A (gA) is an antibiotic peptide that forms well-defined ion channels in membranes.[15] Proper nanopore formation of gA in liposomal membranes allows monovalent cations to cross between the surrounding buffer and the intra-liposomal buffer. Using the pH equilibration assay that we used to measure passive membrane leakage, we added gA to a buffered solution at pH 5.8 containing GcGTPC-CH liposomes with encapsulated carboxyfluorescein (CF) and an intra-liposomal pH of 7.2. To determine whether gA was functional upon incorporation into the membrane, pH-dependent fluorescence emission of CF was monitored for a flux of monovalent cations (predominantly H^+ ion flux) into the liposome, causing an accelerated reduction of CF fluorescence when compared to liposomes without the addition of gA. Similar to previously reported results with commercial lipids,[35,36] the addition of gA to GcGTPC-CH liposomes reduced 95% of the fluorescence of CF after 30 minutes (Figure 5 A), suggesting that the ion channels were functional within the membrane. To ensure the addition of gA did not cause the liposomes to rupture, the presence of stable liposomal structures throughout the experiment was confirmed using DLS measurements (see Figure S6 in the Supporting Information).

Next, we assessed whether phospholipase-D (PLD), a biological membrane-active enzyme that cleaves phosphodiester bonds in lipids, could recognize synthetic GcGTPC-CH lipids as a substrate. Although phospholipase-C (PLC) requires an ester

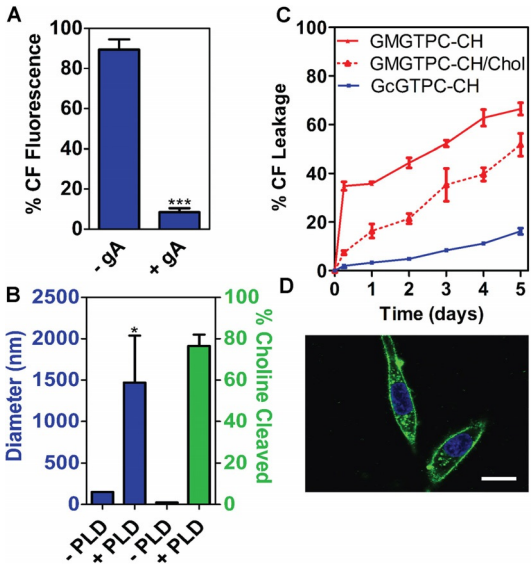


Figure 5. Demonstration of different membrane properties of GcGTPC-CH liposomes. A) Percent fluorescence of CF after incubation with/without 1 μM of gramicidin A at 37 °C for 30 min (monitored at λ(Ex/Em) = 485/517 nm). B) Liposome diameter measured using dynamic light scattering (DLS) after incubation with/without phospholipase-D (PLD) at 37 °C for 30 min (blue, left axis; $N = 10$); Percent choline cleaved from GcGTPC-CH liposomes with/without the presence of PLD after 30 min incubation at RT (green, right axis; $N = 2$). C) Percent leakage of CF after incubation with 30% serum in PBS at 37 °C over 5 days (monitored at λ(Ex/Em) = 485/517 nm). D) Fluorescence microscopy image of KB cells after incubation with calcein (green)-encapsulated GcGTPC-CH liposomes with added 0.5 mol% of DSPE-PEG-folate lipid for 6 hours. Scale bar = 20 μm. Hoechst nuclear stain (blue) was added to stain the nuclei. Statistical significance was determined using a paired Student *t*-test. *, *** indicates a *p*-value of < 0.1, 0.001, respectively.

functionality on the C-2 carbon of the glycerol backbone[45] for lipid recognition, the structural requirements necessary for PLD to recognize a lipid substrate are not known. Following reported protocols for probing the activity of PLD in liposomes comprising commercial diacyl lipids,[37–39] we added PLD to a liposomal suspension comprised of pure GcGTPC-CH lipids in the presence of Ca^{2+} ions at 37 °C. We observed a large morphological change in the liposomes by DLS, beginning with liposomes with average diameter of approximately 150 nm and ending with about 1500 nm diameter objects after a 30 min incubation of GcGTPC-CH liposomes with PLD (Figure 5B). A control experiment in the same Ca^{2+}-containing buffer, but without PLD, showed no change in liposome diameter (see Figure S7A in the Supporting Information). The change in liposomal diameter after incubation with PLD was also accompanied by a large variability in size of objects measured by DLS (see Figure S7B). This result is consistent with previous reports on the effects of PLD on liposomes comprised of commercial PC diacyl lipids,[37–39] which is attributed to PLD-catalyzed cleavage of the choline group from the PC headgroup and subsequent aggregation of liposomes upon interactions of phosphatidic acid (PA) headgroups with Ca^{2+} present in solution. Using a commercial choline detection assay, we also confirmed that PLD can indeed cleave the choline from the lipid headgroups in pure GcGTPC-CH liposomes, resulting in 76.5 ± 7.8% release of choline from the lipid headgroups after 30 minutes, whereas no detectable free choline was cleaved from the lipids in the absence of PLD (Figure 5B).

To examine the potential for GcGTPC-CH liposomes to exhibit improved stability in protein-rich environments for potential drug delivery applications, we used a standard self-quenching leakage assay of CF from GMGTPC-CH (with or without added cholesterol) and GcGTPC-CH liposomes in serum-containing solutions.[46] Liposomes were introduced into a buffered solution with fetal bovine serum (30% in PBS) at 37 °C and the increased fluorescence of CF upon leakage from liposomes was monitored over time. We observed an initial burst of CF fluorescence when CF-encapsulated GMGTPC-CH liposomes were incubated in serum-containing solutions (Figure 5C). CF-encapsulated GMGTPC-CH liposomes incubated with 40 mol% free cholesterol, however, exhibited a more gradual CF leakage profile than GMGTPC-CH liposomes that did not contain free cholesterol. After 5 days, the extent of CF leaked from GMGTPC-CH liposomes was similar, to the extent leaked with or without added free cholesterol. In contrast to these results from GMGTPC-CH liposomes, pure GcGTPC-CH liposomes exhibited a significantly reduced leakage profile of CF compared with GMGTPC-CH liposomes (with or without added cholesterol), with an estimated 80% retention of encapsulated CF after 5 days of incubation in serum (Figure 5C).

Lastly, after observing that GcGTPC-CH liposomes were relatively stable in serum-containing solutions, we examined whether GcGTPC-CH liposomes could be used to deliver small molecule cargo into living mammalian cells. After confirming that GcGTPC-CH liposomes (without encapsulated cargo) were not toxic at total lipid concentrations up to 100 μM (see Figure S8 in the Supporting Information), we examined whether pure GcGTPC-CH liposomes could deliver liposome-encapsulated calcein to KB cells (a HeLa-derived, epithelial-like cell line). We used calcein as a model for a small molecule drug in these studies because it is well known that calcein is not taken up by cells as a free molecule in cell medium, and the fluorescent properties of this molecule can be used to study its uptake in cells. In these studies, we also incorporated 0.5 mol% of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-polyethylene glycol (DSPE-PEG)-folate into the liposomal membranes to facilitate folate-mediated endocytosis into KB cells (see Supporting Information for details).[47] The fluorescence micrograph in Figure 5D shows that calcein-encapsulated GcGTPC-CH liposomes are indeed internalized into cells, demonstrating proof-of-concept that this novel synthetic lipid may have potential utility in liposomal drug formulations that exhibit good stability in serum-containing solutions (see Figure S9 in the Supporting Information for the results of cell uptake control experiments).

We have, thus, presented the design and synthesis of a hybrid GcGTPC-CH tetraether lipid that incorporates design elements inspired from two different strategies used by nature to improve membrane robustness through modifications of membrane composition and lipid structure. We demonstrated that membranes formed from pure GcGTPC-CH lipids exhibit a 50-fold reduction in membrane permeability to small ions when compared with membranes comprised of a typical diacyl lipid, POPC, and a five-fold reduction in membrane permeability compared to a previously reported archaea-inspired tetraether lipid, GMGTPC-CH, that lacks a covalently attached cholesterol group.[18] We also demonstrated that pure GcGTPC-CH liposomes exhibited a ≈30-fold reduction in leakage of a neutrally charged drug, gemcitabine, compared to POPC liposomes with 40 mol% added cholesterol, and a ≈10-fold reduction in leakage of the drug compared to GMGTPC-CH/chol liposomes. MD simulations provided some mechanistic insights that both tethering of lipid tails and incorporation of covalently attached cholesterol significantly increases the order of the lipid tails, which decreases the variance in area per lipid and tightens lipid packing. These MD simulations also predict that the GcGTPC-CH lipids would exhibit the lowest penetration of water molecules of all 3 lipids examined, which is in agreement with the experimental results of reduced permeability of small ion and molecules across GcGTPC-CH lipid membranes. Although GcGTPC-CH lipid membranes exhibit remarkably low permeability and exceptional stability in solution (with or without serum), we also showed that these synthetic membranes retain properties that are typically found in natural membranes, such as the capability to incorporate functional biomolecules (here, ion channels formed from gramicidin A) and act as substrates for membrane-active enzymes (here, phospholipase-D). We also presented initial results on the cellular uptake of GcGTPC-CH liposomes containing encapsulated small molecule cargo (here, calcein as a model for a small molecule drug). Although it remains to be seen whether the apparently advantageous properties of these lipids for drug delivery applications will translate to an in vivo setting, the results presented here support the concept that integrating covalently attached

cholesterol groups to a tethered tetraether lipid, which combines lipid design strategies exploited by eukaryotes and archaea, leads to membranes with significantly improved stability and reduced permeability compared membranes formed from common bilayer-forming lipid formulations.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: archaea · biomembranes · cholesterol · hybrid lipids · liposomes

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