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Water Permeability and Elastic Properties of an Archaea Inspired Lipid Synthesized by Click Chemistry

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Supporting Information

Archaea have evolved mechanically and chemically robust membranes composed of unique lipids to survive in extreme environments (i.e., high temperature, low pH, and high osmotic strength).[1] At high temperatures, the polar membrane lipids are mainly bolaamphiphile tetraether scaffolds with branched phytanyl side chains attached via ether bonds to the glycerol carbons at the sn-2,3 positions.[2] Archaeal lipids have garnered biological and technological interest as vaccine adjuvants and drug delivery systems because of their non-immunogenic properties and ability to form membranes with reduced permeability.[3−5] To obtain lipids with robust and unique membrane properties and overcome the limitations associated with extracting lipids,[6] synthetic chemistry has been commonly used.[7] Several research groups have designed synthetic routes to access a hemicyclic tetraether scaffold that mimics structural features found in archaeal tetraether lipids.[8−10] Synthetic archaea-inspired bolalipids have been experimentally investigated for a long time, mainly for their leakage properties. Thus, compared to liposomes made from commercially available diacyl lipids, liposomes comprised of pure tetraether lipids generally showed high stability in membrane disrupting conditions[8,11] and enhanced retention of both ions[12] (up to 100-fold) and organic small molecules[13,14] (up to 9-fold). However, little information is known regarding the relationship between lipid packing and permeability of membranes made of archaea-inspired tetraether lipids.

Herein, we report the synthesis of a new archaea-inspired tetraether lipid and study the mechanical elastic properties of membranes made from this bolalipid. To access structural diversity in the hydrophobic moiety of the lipids and to tune membrane properties, we used Huisgen 1,3-dipolar cycloaddition strategy (click chemistry) to design and synthesize a tetraether lipid incorporating phytanyl side chains and polar 1,4 triazole rings known to change lipid packing (Figure 1).[15] Notably, the [3+2] azide-terminal alkyne cycloaddition approach has been used for lipid functionalization[16] and tetra-acyl phospholipid synthesis.[17] However, in this work we demonstrate the first example of the synthesis of phytanyl side chain incorporated tetraether lipids with 1,4-triazole rings. We next confirm the ability of lipids incorporating 1,4-triazole rings to form stable small and giant vesicles and examined their physical and mechanical properties using a micropipette aspiration technique. To probe the effect of the 1,4 triazole rings on membrane properties, we also explore the mechanical properties of a previously reported tetraether phospholipid with phytanyl side chains, **GMGTPC**,[12] which lacks 1,4-triazole ring (Figure 1). This work presents the first report of elasticity measurements for synthetic archaea-inspired lipids and provides evidence that tuning the hydrophobic core of tetraether bolalipids result in dramatic change in membrane elasticity, which likely arises from changes in lipid packing.

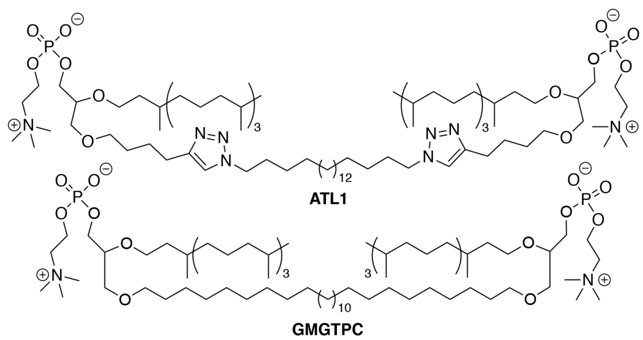

Figure 1. Chemical structures of tetraether phospholipid incorporating phytanyl side chains and triazole rings (Archaea-type Lipid 1- **ATL1**) and glycerol monoalkyl glycerol tetraether lipid with phosphocholine head groups (**GMGTPC**).

While Egushi et al. previously reported 32 step total syntheses of archaeal 32- and 72-membered macrocyclic tetraether lipids,[18,19] current synthetic strategies to synthesize hemicyclic tetraether bolaamphiphiles with phytanyl side chains involve either *O*-alkylation[20] or dimeric metathesis[21,22] of phytanylated glycerol units. Here, we directed our efforts to design a synthetic scheme that would allow for the facile modulation and tuning of the hydrophobic lipid core to the glycerol scaffold while reducing the number of synthetic steps to access new tetraether lipids.

With our design, we envisioned that using a bis-azide hydrophobic linker, we could tether two alkyne-bearing glycerol

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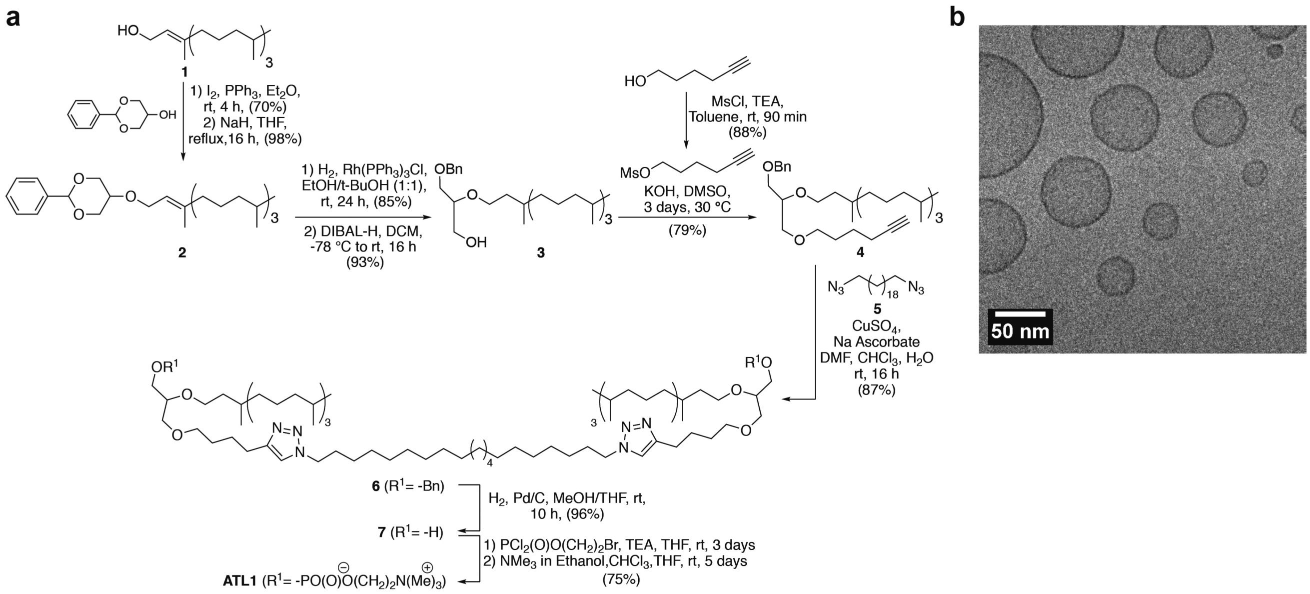

Figure 2. (a) Synthesis of a tetraether phospholipid incorporating phytanyl side chains (Archaea-type Lipid 1 - **ATL1**) and (b) Cryo-TEM image of extruded SUV prepared with **ATL1**.

units using click chemistry to easily form the tetraether scaffold (Figure 2a). The length of the hydrophobic linker (20 carbons) between the two triazole rings has been chosen to maintain an adequate hydrophilic−lipophilic balance that is crucial for the formation of stable vesicles. The synthesis begins with the preparation of alkyne phytanylated glycerol **4**. First, phytanyl iodide **2** was prepared by the iodination of phytol in the presence of triphenylphosphine and imidazole, and then reacted with 2-phenyl-5-hydroxy-1,3-dioxane to generate the ether derivative **2** in 98% yield.[22] After hydrogenation of the phytanyl olefin using Wilkinson's catalyst and *tert*-butanol as a solvent, a selective ring-opening of the dioxane moiety using diisobutylaluminum hydride (DIBAL-H) led to the formation of the benzyl protected glycerol backbone **3** (79% yield, 2 steps). The primary alcohol of **3** was finally reacted with hex-5-yn-1-yl methanesulfonate using KOH in DMSO to give glycerol **4** with 79% yield. Organic bisazide **5** was prepared from the corresponding dibromide. Bis-azide derivative **5** was then reacted with four equivalents of alkyne glycerol **4** using copper(II) sulfate ($CuSO_4$) and sodium ascorbate in a mixture of dimethylformamide/water/chloroform to ensure complete solubility of reagents and product. The resulting benzylated tetraether scaffold **6**, obtained by click chemistry with good yield (87%), was hydrogenated in the presence of palladium on carbon to produce diol **7**. The final phospholipid, termed the Archaea-type Lipid 1 (**ATL1**) was generated in 75% yield by the reaction of diol **7** with 2-bromoethyl dichlorophosphate, which was followed by a nucleophilic displacement of the bromine with trimethylamine, as described previously.[12] Therefore, this synthetic strategy enables the facile synthesis of a tetraether phospholipid lipid incorporating phytanyl side chains in high yields and only in 8 steps starting from phytol **1**.

The new lipid was first characterized by differential scanning calorimetry (DSC), which confirmed that **ATL1** lipids do not undergo a phase transition above room temperature (Figure S1). The absence of phase transition temperature is in good agreement with reported literature that shows lipids incorporating phytanyl side chains remain in liquid phase at room temperature.[23] We next explored if stable liposomes can form with pure tetraether lipids incorporating 1,4-triazole rings. Cryo-electron microscopy analysis revealed that ATL1 lipid could form ∼3.7 ± 0.6 nm thick membrane and ∼50 nm diameter small unilamellar vesicles (SUVs) using a standard thin-film hydration followed by extrusion method (Figure 2b and Figure S2).

We next examined whether small molecules could be encapsulated and retained by SUVs made from **ATL1** lipids and different mole percentages of cholesterol (10−50%). Calcein was encapsulated in liposomes at a self-quenching concentration of 80 mM and relief of self-quenching due to dilution on leakage was measured by monitoring calcein fluorescence at 515 nm, with excitation at 495 nm.[24] Results from leakage experiments revealed that the presence of cholesterol in liposomal membrane dramatically increases calcein retention whereas liposomes made from pure **ATL1** lipids were not able to retain the small molecule (Figure S3). Of all liposomal formulations tested, the greatest retention over 12 h was found when cholesterol content was 30 mol % whereas high and low cholesterol content displayed rapid release of the dye (e.g., 80% of leakage within 2 h with 50 mol % of cholesterol). In contrast, tetraether phospholipids incorporating phytanyl side chains, such as **GMGTPC** lipids[12] that lack 1,4-triazole rings (Figure 1), have shown high retention for small molecules without the need for cholesterol while having similar membrane thickness to **ATL1** (Figure S2).[14] The reduced capability of **ATL1** liposomes to retain encapsulated small molecules suggests that 1,4-triazole rings may create kinks in the hydrophobic core, which results in looser lipid packing when compared with liposomes made of **GMGTPC** lipids but still allows liposome formation. To test this hypothesis further, we fabricated giant unilamellar vesicles (GUVs) of both **ATL1** and **GMGTPC** lipids (see Supporting Information for more details). The giant size of these vesicles (usually 20−40 μm diameter, Figure S4) enabled us to measure the area compressibility modulus, the lysis tension, and the water permeability in a direct way, i.e., application of force on the membrane.

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We used the micropipette aspiration technique to assess the mechanical properties of these GUVs. In this technique, the suction pressure applied to a fluid membrane results in a uniform and isotropic membrane tension. The changes in the aspiration length inside the micropipette can then be related to mechanical and physical quantities such as stretching modulus and water permeation coefficient (see Supporting Information for more details). GUVs composed of **GMGTPC** lipids were used as a point of comparison to probe the effect of 1,4-triazole rings on membrane properties. GUVs of both **ATL1** and **GMGTPC** tetraether phospholipids were formed by electroformation (Figure S4).[25,26] Water permeability of lipid membranes of both **ATL1** and **GMGTPC** GUVs were measured via micropipette aspiration setup by increasing the solute (that is glucose) concentration in the surrounding hypertonic solution and then monitoring the rate at which water permeated through the membrane (Figure 3a and Figure S5).[27] The permeability coefficient from at least 15 vesicles for each type of tetraether lipid were obtained (Figure 3b). The measured water permeability was 54 ± 3 μm/s for **ATL1** and was substantially greater than the value of 21 ± 0.2 μm/s for **GMGTPC**. **GMGTPC** tetraether lipids showed lower water permeability than typical phosphocholine based diether lipids (≤18 carbons) with the reported permeability of membranes within the range of 30 to 150 μm/s.[27] Hence, the presence of triazoles makes **ATL1** more than 2-fold permeable to water compared with **GMGTPC**; we return to this point below.

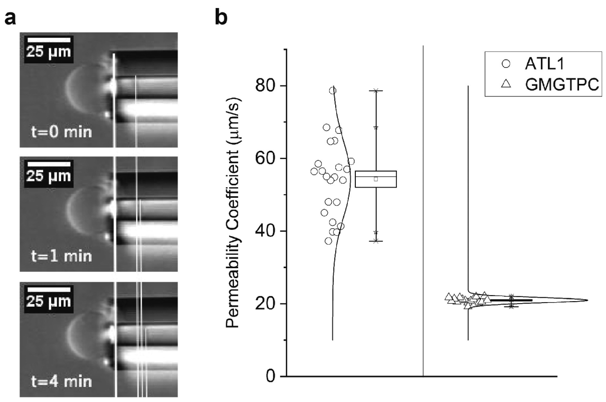
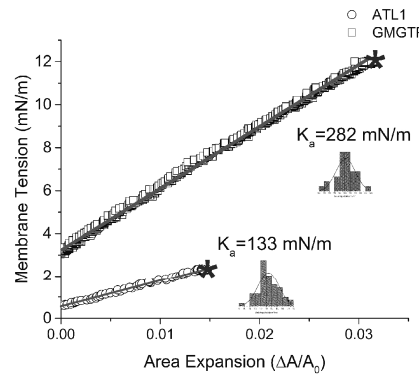


Figure 3. (a) Sequence of images of a single giant unilamellar vesicle made of **ATL1** during a water permeation experiment. The increase in aspiration length is proportional to the decrease in volume of the vesicle (white lines show the position of the aspirated tongue inside the micropipette). (b) Coefficient of apparent water permeability for both **ATL1** (n = 23) and **GMGTPC** (n = 17) bolalipids measured by osmotic filtration at 23 °C against 15% hypertonic glucose solution.

Higher water permeability could also be a direct effect of looser lipid packing. Therefore, we compared the elastic properties of both lipids **ATL1** and **GMGTPC** to study the effect of 1,4 triazole rings on lipid packing. Figure 4a shows the membrane tension versus fractional area expansion, obtained from the aspiration of **ATL1** and **GMGTPC** GUVs as described in the Supporting Information. The increase in membrane tension is directly proportional to the areal expansion with the area compressibility modulus (K_a) being the proportionality constant. Cumulative results for stretching modulus measurements revealed a K_a of 134 ± 3 mN/m for GUVs made from **ATL1**. For comparison, GUVs made from

a



b

Membrane properties	ATL1	GMGTPC
coefficient of apparent water permeability (µm/s)	54 ± 3	21 ± 1
stretching modulus (mN/m)	134.1 ± 2.6	291.3 ± 3.3
lysis tension (mN/m)	2.4 ± 0.1	11.0 ± 0.3
cohesive energy density (mJ/m^2)	0.025	0.18
area strain at the rupture point (τ^*/K_a)	~ 0.018	~ 0.038

Figure 4. (a) Membrane tension as a function of fractional area dilation of a single bilayer **GMGTPC** and **ATL1** vesicle. A linear fit is used to extract the stretching modulus, the slope of the superimposed line. Asterisk (*) denotes the rupture point of the vesicle. (b) Elastic properties and water permeability of the **ATL1** and **GMGTPC** lipids.

GMGTPC lipids K_a was 291 ± 3 mN/m, which is more than twice stiffer than **ATL1** lipids (Figure 4 and Figure S6). For both **ATL1** and **GMGTPC**, the distribution of K_a values followed a unimodal distribution (Figure S6). This result indicates that all of the vesicles were unilamellar, because the value of K_a is expected to be proportional to the number of lamellae. This assertion is consistent with cryo-TEM images obtained for **ATL1** lipids (Figure 2b). For both **ATL1** and **GMGTPC** lipids, K_a lies within the range reported for various lipid systems (K_a = 135−380 mN/m) in parallel comparison.[28] Furthermore, compared to the crystalline or gel states of the reported lipid systems,[29] the moduli of both **ATL1** and **GMGTPC** lipids can be considered soft, representing a liquid-like chain disorder for both lipids.

We then measured the maximum tension that could be sustained by the GUVs without rupture (the lysis tension, τ^*). For **ATL1** GUVs, the measured value was τ^* = 2.4 ± 0.1 mN/m (±SEM). For **GMGTPC**, the value was 5-fold larger, τ^* = 11.0 ± 0.3 mN/m (Figure 4b and Figure S7). Considering the values of K_a and τ^*, the area strain at the rupture point (τ^*/K_a) drops from ~0.038 for **GMGTPC** to ~0.018 for **ATL1** (Figure 4b). The cohesive energy density (toughness of the membrane) were obtained by considering the area under the stress−strain plots. For **ATL1** and **GMGTPC**, the measured values were 0.025 and 0.18 mJ/m^2, respectively (Figure 4b and Figure S7).

The micropipette measurements show that the 1,4 triazole moieties in **ATL1** membranes increased the water permeability by a factor of 2.6 ± 0.2, and decreased the stretching modulus K_a by a factor of 2.2 ± 0.1 and the lysis tension by a factor of 4.6 ± 0.2 relative to **GMGTPC**. We now turn to a discussion of these results based on the packing of the lipids and the polarity of the 1,4 triazole moieties. In general, the permeability

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coefficient is directly proportional to the partition coefficient of water between the membrane phase and aqueous solution, and also to the diffusion constant of water within the membrane phase.[30] Triazole group is known to interact with biological molecules through hydrogen bonding and dipole interactions,[31] thus we propose that due to these capabilities, the 1,4 triazole moiety enhances the water partition coefficient relative to **GMGTPC**. We also propose that 1,4 triazole rings perturb lipid packing, thus decreasing the mechanical strength. Interestingly, when we doped the **ATL1** membrane with 30 mol % **GMGTPC**, a 36% increase in stretching modulus was observed. The cohesive energy of the tetraether lipid incorporating the 1,4 triazole groups was 0.018 mJ/m^2, which is a factor of 7.0 ± 0.2 lower than **GMGTPC** and low compared to natural phospholipids (0.05 to 0.5 mJ/m^2).[32] On a per-molecule basis, these energy densities are very close to thermal energy. Thus, thermal fluctuations should cause significant variations in density and increase in local lateral compressibility, which all would enhance the diffusion of water through the membrane and lower the work necessary to form molecular-packing defects.[33] Therefore, this potential of defect formation can manifest itself with a higher membrane permeability to water and to small solutes.

Further, we base our reasoning for the compromised stretching modulus observed for **ATL1** on the to decrease in the interfacial tension. We rule out the effect of membrane thickness on stretching modulus, because both lipids show similar thicknesses (Figure S2). We may follow Flory's model, in which the area compressibility modulus of a bilayer is derived to be $K_a = 6\Pi$ (where Π being the surface pressure of the monolayer). In a flat, tension-free membrane, the surface pressure of a monolayer is a constant of interfacial energy, γ for the exposure of the hydrocarbon to water, thus $\Pi = \gamma$.

In conclusion, we used click-chemistry to develop a hemicyclic tetraether scaffold incorporating phytanyl side chains and 1,4 triazole rings. A new tetraether phospholipid with 1,4 triazole rings in the hydrophobic core was readily prepared and successfully used for small and giant vesicle preparation suggesting that the new lipid scaffold offer great flexibility for tailoring the functionality presented in the lipid hydrophobic core, without compromising the ability to form stable unilamellar vesicles. Whereas membranes made of the new lipid displayed low retention for organic molecules in small liposomes, water permeability in giant liposomes was found to be similar to bilayer systems. We elucidated that the incorporation of the relatively hydrophilic 1,4 triazole rings in the hydrophobic core of an amphiphilic molecule could alter the macroscopic properties of a membrane by increasing permeability by a factor of 2.6 ± 0.2, and decreasing the stretching modulus K_a by a factor of 2.2 ± 0.1 and the lysis tension by a factor of 4.6 ± 0.2. We postulate that the hydrophilic nature of the triazole moiety and having large dipole moments probably results in higher partitioning of water molecules, thus compromising the membrane integrity. This work introduces new design principles for producing lipid membranes with distinct properties in a synthetically tractable manner.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemmater.8b00992.

DSC measurements; membrane thickness measurement; calcein release assay; DIC images of GUVs; water permeability data; elastic area compressibility modulus; cohesive energy density; lysis tension; experimental details; lipid synthesis; NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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