Structure-Function Relationships in Pure Archaeal Bipolar Tetraether Lipids

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ABSTRACT: Archaea, the third fundamental domain of life are distinguished from bacteria and eukaryotes due to the presence of unique lipids in their cell membranes. Archaeal bipolar lipids are among the most unusual lipids occurring in nature because of their presumed ability to span the entire membrane. They are challenging to extract in pure form from natural sources or synthesize chemically, and as a result, prior studies on pure lipids have been limited. Here we have utilized synthesis to enable in-depth biophysical investigations on a series of archaeal glycerol dialkyl glycerol tetraether (GDGT) lipids having symmetric or unsymmetric combinations of polar head groups. We showed that these lipids self-assemble to form membrane-bound vesicles in aqueous media, encapsulate polar molecules, and reconstitute a functional integral membrane protein. Membrane thicknesses and electron density profiles were investigated by performing small-angle X-ray scattering (SAXS) studies and cryogenic electron microscopy (cryo-EM) imaging on unilamellar vesicles of GDGT lipids. SAXS studies on bulk aqueous dispersions of GDGT lipids over a large temperature range (10-90 °C) allowed us to identify lamellar and non-lamellar phases and their interconversions under various buffer conditions. We also studied how the propensity to form various mesophases is reflected in the functional behavior of the GDGT membranes. Specifically, we asked whether vesicles overwhelmingly composed of GDGTs can undergo fusion as it is difficult to conceptualize such behavior with the assumption that such membranes have a monolayer structure. Interestingly, we observed that GDGT vesicles undergo fusion with influenza virus with lipid mixing kinetics comparable to that with vesicles composed of typical monopolar phospholipids. Our results suggest that GDGT membranes may consist of regions with a bilayer structure which facilitates fusion and thus offer insight into how archaea may perform important physiological functions that require dynamical membrane behavior.

INTRODUCTION

Archaea are an enigmatic group of prokaryotic organisms. Originally thought to be extremophiles, archaea have been found in every realm of the biosphere including the human microbiome.3 Archaea constitute ~1.3% of the total biomass on the Earth,2 yet they have remained quite underexplored. While archaea resemble bacteria in certain superficial ways, fundamental differences in their biochemistry led to their classification as a separate domain of life. For instance, archaeal lipids differ significantly from the lipids found in bacteria (and eukaryotes) in terms of structural features, such as, absolute stereochemistry of the glycerol moiety, the nature of the hydrocarbon chains and their linkages to the glycerol moiety.3 These distinctions between the lipids of Archaea and Bacteria (and Eukarya) form the basis of a dichotomy known as the “Lipid Divide” (Figure 1A).4 In archaeal lipids, the hydrocarbon chains have multiple methyl branches and are of isoprenoid origin - typically lacking any unsaturation. Their hydrocarbon chains are connected to the sn-glycerol-1-phosphate backbone via ether linkages. In bacterial lipids, the hydrocarbon chains are fatty acid-based, contain various degrees of unsaturation, and the hydrocarbon chains are connected to the sn-glycerol-1-phosphate backbone through ester linkages. Lack of unsaturation and the presence of ether linkages make the archaeal lipids extremely stable to aerial oxidation and hydrolysis respectively. Due to this exceptional chemical stability, archaean lipids serve as excellent biomarkers for biogeochemical and climate studies.5

Remarkably, a major class of archaeal lipids has a bipolar structure – which is analogous to two monopolar lipids co-valently joined at the ends of their hydrocarbon tails. The bipolar nature of such archaeal lipids gives rise to the question of how these molecules are organized within a membrane? Three types of limiting molecular arrangements can be imagined (Figure 1B): (1) stretched – the lipid molecules are fully stretched (i.e., O-shaped) and span the entire width of the membrane to form a monolayer; (2) looped – the lipid molecules are bent (i.e., U-shaped) at the hydrocarbon region so that the two headgroups face the same side of the membrane, so the membrane more closely resembles a bilayer; and (3) mixed – where both O- and U-shaped lipids coexist. There is considerable evidence that suggests that at least a majority of the lipids occur in a stretched conformation.6-8 Although there is a report mentioning exclusive formation of U-
shaped bilayers on a solid substrate via transfer of Langmuir films, evidence of such structures in biological membranes or model vesicles is lacking. Therefore, it is generally assumed that the archaeal bipolar lipids adopt a membrane-spanning conformation, although the possibility of a small percentage of U-shaped lipids coexisting with O-shaped lipids has been speculated.

Figure 1. A. Schematic diagram illustrating the concept of the “Lipid Divide” between bacterial/eukaryotic and archaeal lipids. The carbons on glycerol moieties are marked (1–3) according to stereochemical numbering (sn) convention. (R) and (S) denote the absolute stereochemistries of the glycerol moieties. The blue spheres denote phosphate-containing polar headgroups. For simplicity, the stereochemistries of the branched chains are not shown. B. Three possibilities of shapes adopted by archaeal bipolar lipids in a membrane.

Bipolar lipids render archaeal membranes mechanical stable and impermeable even at high temperatures and at highly acidic pH. Although bipolar lipids are found in some bacteria, those are fatty acid-derived, and usually do not constitute the major lipid species in a membrane. Glycol dialkyl glycerol tetraethers (GDGT) are one of the primary bipolar lipids found in archaea. Thermoacidophilic archaea possess GDGTs alongside other bipolar lipids while methanogenic archaea possess GDGTs alongside monopolar lipids. Fundamental understanding of structure-function relationships in GDGTs through biophysical studies will further our understanding of the basis of the adaptability of archaea to extreme environments and could aid in the design of synthetic lipids with desired properties. Archaeal bipolar lipids have long been considered as excellent building blocks of liposomes for drug delivery or artificial membranes with exceptional long-term structural and thermal stabilities. However, the scarcity of available pure GDGT lipids poses a roadblock to comprehensive biophysical studies which typically require many milligrams of lipids. Although a few model monopolar archaeal lipids in the form of ester- or ether-linked diphytan(o)yl phospholipids are commercially available, pure GDGT lipids are difficult to access. Also, the presence of multiple stereocenters make these lipids challenging to synthesize chemically. Only a handful of examples exist where polar GDGT lipids have been synthesized via total synthetic or semisynthetic approaches. To date, no GDGT lipids can be purchased from commercial vendors in pure form. Therefore, most biophysical studies until now have been performed on crude lipid extracts or model bipolar lipids. In this work we took advantage of synthetic chemistry to generate a series of four parallel GDGT-0 lipids in multi-milligram quantities (Figure 2). Among these, two lipids are symmetric and two are unsymmetric in terms of head group composition. The symmetric lipids P2 and PG2 consist of phosphate and phosphoglycerol headgroups respectively on both ends. In the unsymmetric lipids P1Glc and PG1Glc a glucose group is present on one end and phosphate and phosphoglycerol groups respectively at the other end. We studied the self-assembly behavior of these GDGT lipids in various aqueous buffers and identified conditions in which membrane-bound giant vesicles are formed. We characterized the structural properties of GDGT membranes using small-angle X-ray scattering (SAXS) and cryogenic electron microscopy. We used SAXS also to characterize the
transformations between various mesophases under a range of conditions of ionic strengths, pH, and divalent cations over a wide temperature range. Building on the physical characterization data, we decided to revisit the question of shapes of GDGT lipids in membranes. Here we offer functional evidence that the GDGT membranes contain a mixture of two kinds of molecular conformations which allows them to undergo dynamical processes such as fusion.

**EXPERIMENTAL SECTION**

**Preparation of buffers.** The following buffers were used and the osmolalities were measured using an Advanced Instruments Model 3320 osmometer:

- **Tris buffer**: 50 mM Tris, pH 7.5. Osmolality: 88 mOs-mol/kg.
- **HB buffer**: 20 mM HEPES-Na, 150 mM NaCl, pH 7.2. Osmolality: 316 mOs-mol/kg.
- **Vesicle buffer**: 10 mM Na-phosphate, 90 mM sodium citrate, and 150 mM NaCl (pH 7.4). Osmolality: 545 mOs-mol/kg.
- **Fusion buffer**: 10 mM Na-phosphate, 90 mM sodium citrate, and 150 mM NaCl (pH 5.1). Osmolality: 510 mOs-mol/kg.

**Optical microscopy.** Fluorescence images were acquired with a Nikon Ti-U microscope using a 100X oil immersion apochromat TIRF objective (NA = 1.49) (Nikon Instruments, Melville, NY). A Spectra-X LED Light Engine (Lumencor, Beaverton, OR) was used for illumination, and an Andor iXon 897 EMCCD camera (Andor Technologies, Belfast, UK) with 16-bit image settings. Images were captured with Metamorph software version 7.10.4 (Molecular Devices, Sunnyvale, CA).

**Negative-staining transmission electron microscopy (TEM).** Formvar-coated Cu grids (300 mesh, Catalog #FCF300-Cu, Electron Microscopy Sciences) were glow-discharged using a Denton BenchTop™ Turbo setup and used fresh. 5 µL of a vesicle sample was added to formvar-coated Cu grid surface and allowed to sit for 3 min. The grids were washed by touching the surface of 2 drops of Milli-Q placed on a parafilm sheet. 3 drops of 1% uranyl acetate in a glass vial by evaporating solvents from 80 µL of 5 g/mL solution of PG2 in 1:1 CHCl3:MeOH. The vial was kept in the desiccator for 12 h. The film was hydrated by gently tapping with 400 µL of a solution consisting of 10 mM Tris (pH 8.0), 30 mM octyl glucoside, 0.26 mM LDAO, and 6 µM RC. The protein to GDGT lipid ratio was about 1:100. The lipid/detergent/protein mixture was added to 18 mg of pre-cleaned wet BioBeads SM-2 resin (soaked in Milli-Q water) and allowed to tumble for 30 min at RT. Afterwards, the solution was separated from the beads and taken in a 10 kDa MWCO dialysis cassette. Dialysis was carried out against 10 mM Tris buffer (pH 8.0) for 48 h with 2 changes of buffer in between and a clear light purple dispersion is collected. Removal of detergents was verified by mass spectrometry. Transient (fs/ps) absorption spectroscopy measurements for RCs shown in Figure S3 were obtained in the laboratory of Professor Dewey Holten and Christine Kirmaier by Dr. Kaitlin Faries at Washington University using previously described methods. We are indebted to them for providing this data.

**Surface pressure-area isotherms.** Surface pressure-area isotherms of monomolecular layers were recorded at room temperature (22 °C) using a KSV NIMA KN 2002 (Biolin Scientific) Langmuir trough equipped with a teflon trough (243 cm2 or 555 cm2) and symmetric delrin barriers. A Wilhelmy plate made of a 30 mm×14 mm piece of Whatman #1 filter paper was used to monitor the surface pressure. At first, the trough surface was rinsed with methanol. Next, Milli-Q water was used to thoroughly clean the trough. Typically, a solution of the lipids of known concentration in chloroform (or 9:1 chloroform: methanol) was slowly added dropwise onto the surface of the water using a glass microsyringe. The solvent was allowed to evaporate for at least 20 min. The barriers were then compressed at a rate of 10 mm/min and pressure-area isotherms were recorded.

**Small-angle X-ray scattering (SAXS).** SAXS data were collected at the Beamanlne 4-2 of Stanford Synchrotron Radiation Lightsource. The distance between the sample and the detector (Pilatus3 X 1M) was 1.2 m and the energy of the X-rays was 11 keV (λ = 1.127 Å). Momentum transfer (q) was measured over a range of 0.01-0.75 Å⁻¹, where q is modulus of the scattering vector defined as follows:

\[ q = \frac{4\pi\sin\theta}{\lambda} \]

where θ = scattering angle, λ = wavelength of the X-rays.

Two kinds of experiments were performed:

1. **Scattering from unilamellar vesicles.** Small unilamellar vesicles of P2, PG2, and PG1Glc were prepared by extruding a multilamellar dispersion in Tris buffer through a 50 nm polycarbonate membrane filter. In the case of P1Glc, the lipid dispersion contained a significant amount of insoluble aggregates which could not be fully dispersed by bath sonication and those stuck to the membrane filters during extrusion. Therefore, the dispersion was subject to probe ultrasonication which helped to break down the larger aggregates. ~30 µL volumes of the vesicle dispersions were taken in 0.2 mL microtubes and loaded onto an automated sample loader at the beamline. Samples were exposed for a variable time (1s or 2s) and variable number of scans (24-50) were taken depending on the concentration of the lipids. Each sample was measured after storing at room temperature for up to a day and identical scattering profiles were obtained. Details of the SAXS data fitting is provided in the Supplementary Information ("Fitting of SAXS data from unilamellar vesicles" section).

2. **Scattering from bulk dispersions.** In these experiments, a thin film of a GDGT lipid was deposited on the walls of a
glass vial. The film was hydrated with an aqueous buffer (typically 50 mM Tris, pH 7.5) by a combination of vortexing and bath sonication. Additional components such as salt (NaCl), or MgCl₂ were included in the hydration buffer. The resulting lipid dispersion was loaded into capillaries (Quartz capillaries from Hampton Research having the following specifications: length ~ 80 mm, outer diameter ~ 1.5 mm, wall thickness ~ 0.01 mm) and placed in a custom-designed temperature-controlled capillary holder. Temperature was raised typically in steps of 10 °C over the range 10-90 °C. About 20 min was allowed at each temperature before collection of data. Typically, 20 consecutive 1 s scans were taken and averaged. Lipid phases were identified from the ratios of q-values of the Bragg peaks.

Cryogenic transmission electron microscopy (cryo-TEM). Unilamellar vesicles were prepared in a manner identical to that for SAXS experiments. Cryo-TEM was carried out at the Stanford Cryo-Electron Microscopy Center (cEMc). 3-5 µL of an SUV dispersion was applied to a Quantifoil holey carbon grid (Quantifoil Micro Tools GmbH) that was previously glow discharged, blotted for 1-2 seconds, and plunged-frozen in liquid ethane using a Vitrobot Mark IV System (Thermo Scientific). The frozen grids were imaged with an FEI Tecnai F20 electron microscope (FEI Company) equipped with a Gatan K2 Summit direct detection device (Gatan Inc) with a dose rate of 6-10 e⁻/px/s. A low pass filter of 0.1 and mean shrink of 4 were applied to the raw images for visualization only. The raw images were processed with RELION 3.0. Overlapping regions of the bilayers were picked with the manual picking tool, extracted, CTF-corrected, and 2D averaged. The 2D classes with good alignment were reclassified into a single 2D class. The electron scattering profile of the bilayer was calculated by taking a line profile of the 2D classes in FIJI (ImageJ).

Fusion of GDGT vesicles with influenza virus. Previously described experimental design and methodology of data analysis for viral membrane fusion was employed here.32,33

Influenza virus preparation. IAV (strain X-31, A/Aichi/68, H3N2) grown in the allantoic cavity of SPF eggs was purchased from Charles River Laboratories (Wilmington, MA). The main stock (HA titer: 32768/0.05 mL, EID₅₀: 10⁶.⁵/mL, protein content: 2 mg/mL) was stored as 20 µL aliquots at -80 °C till use. IAV is a Biosafety Level 2 agent and was handled following an approved biosafety protocol at Stanford University. Texas Red-DHPE was incorporated into the IAV envelope by incubating virus sample with 10 µM of Texas Red-DHPE in HB buffer at 4 °C on ice overnight such that the total EtOH (necessary for dissolving Texas Red-DHPE) concentration in the labeling mixture did not exceed 2% v/v. The labeled virus suspension was directly diluted in vesicle buffer for use in lipid mixing experiments. For content mixing experiments, the commercially available virus suspension was directly diluted in vesicle buffer.

Vesicle preparation and fusion chamber: Large unilamellar vesicles (compositions summarized in Figure 7A) were prepared by extrusion of a multilamellar dispersion through 100 nm polycarbonate filters. For content mixing experiments, self-quenching concentration (30 mM) of sulforhodamine B was included in the hydration buffer and the vesicles were separated from the free dye by size exclusion chromatography. All viral experiments were carried out using a simple microfluidic flow cell prepared from glass and polydimethylsiloxane (PDMS) by plasma-bonding. The glass surfaces of the flow cell’s channels were passivated with co-polymers PLL-PEG/PPLL-biotin-PEG. The vesicles were tethered to the passivated surface via NeutrAvidin, and excess vesicles were removed by flowing vesicle buffer through the channel.

Viral fusion experiments. Virus particles (labeled or unlabeled) were added to the flow cell and allowed to bind to the tethered vesicles for 15 min. Unbound virus particles were removed by flowing in vesicle buffer. pH was dropped by adding fusion buffer (pH 5.1) and a continuous video stream was acquired over 1200 frames at 3.47 fps. In the event of a lipid mixing or content mixing, signal from self-quenched Texas Red-DHPE or sulforhodamine B respectively turned brighter. The wait times between lowering of pH and appearance of a bright spot were calculated and compiled into cumulative distribution functions.

![Figure 2](https://i.imgur.com/3J5Q5.png) Structures of the GDGT lipids described in this study. The carbons on glycerol moieties are marked (1–3) according to stereochemical numbering (sn) convention. It is notable that in isocaldarchaeol, the isopranoid chains are linked in a parallel fashion (2 → 2′ and 3 → 3′). The glucosyl derivatives were synthesized as mixtures of anomers.
RESULTS AND DISCUSSION

Synthesis of GDGT lipids. In earlier work led by the Burns group, an efficient synthetic route to the core structure of parallel GDGT-0 (iso-caldarchaeol) was described. In the same work, the synthesis of P2 and P1Glc were reported. In this work, along with P2 and P1Glc, the synthesis of two additional GDGT lipids—PG2 and P1Glc—was achieved (Figure 2, Supplementary Schemes S1–7). It should be noted that the glucosyl groups in P1Glc and P1Glc are present as a mixture of anomers.

Self-assembly of GDGTs in aqueous media. We found that the GDGTs spontaneously self-assembled into giant vesicles (1–20 µm diameter) in aqueous media upon hydration of a thin lipid film and the membranes were visualized by staining with the lipophilic dye-labeled Texas Red-DHPE (Figure 3A, Figure S1). If a water-soluble fluorescent dye such as pyranine (HPTS) was added to the hydration solution, the vesicles encapsulated the dye in the inner aqueous volume (Figure 3A, Figure S1). The GDGT vesicle membranes were observed under the microscope to display fluid-like behavior such as shape fluctuations (Figure 3B). However, it is noteworthy that unlike typical fluid phase phospholipids (e.g. POPC), the GDGT lipid films required increased mechanical forces (i.e. scratching, vortexing, sonication) to disperse into vesicles, suggesting that the lipids are tightly packed in the thin films. Thin films of the glucose-containing lipids (P1Glc and P1Glc) were harder to hydrate likely because of extensive intermolecular hydrogen-bonding among the sugar headgroups. We observed that the efficiency of hydration of the lipid films depended on the nature and ionic strengths of the buffers. Tris buffer (50 mM, pH 7.5) was found to be the most suitable for hydration for all the lipids studied. Only PG2 films could readily hydrate in several other buffers like HEPES, PBS, and even in pure water to form vesicles. Also, only PG2 could hydrate to form giant vesicles in the presence of proteins such as superfolder green fluorescent protein (sfGFP) and encapsulate the protein (Figure 3C). Other GDGT lipids formed aggregates in the presence of protein. Next, we tested whether the multilamellar lipid dispersions could be extruded into smaller vesicles via extrusion through membrane filters. Using negative-staining transmission electron microscopy we confirmed the presence of vesicles having diameters less than 200 nm (Figure S2).

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Self-assembly of GDGTs in aqueous media. A. Giant vesicles of P1Glc formed by hydration of a thin film in Tris buffer (50 mM, pH 7.5) imaged by laser-scanning confocal microscopy. Membranes were stained with 0.1 mol% Texas Red-DHPE (left) or the water-soluble dye pyranine (HPTS) was encapsulated inside (right). Scale bar: 10 µm. A single giant unilamellar vesicle (GUV) indicated in a box is enlarged and the corresponding fluorescence intensity profiles along a line across the center in two channels are shown. B. GDGT vesicles undergo shape fluctuations as observed with a P1Glc GUV. Membranes were visualized with Texas Red-DHPE. Scale bar: 10 µm. C. Encapsulation of sfGFP in giant vesicles of PG2 imaged by laser scanning confocal microscopy. Scale bar: 15 µm. D. Negative-staining TEM images of PG2 vesicles reconstituting photosynthetic RC prepared by detergent removal method. Scale bar: 100 nm. E. Ground state absorption spectrum of photosynthetic reaction center (from Rhodobacter spheroides) reconstituted in PG2 vesicles. For comparison, the spectrum of the same protein in detergent (LDAO) micelles is overlaid.

Given the high microviscosity of tetraether membranes and large difference in intermolecular packing as compared to typical phospholipids, it is not obvious whether integral membrane proteins of non-archaeal origin can be reconstituted into GDGT vesicles. There are only a few examples of reconstitution of non-archaeal integral membrane proteins into crude archaeal GDGT lipid membranes. Elferink et al. reconstituted bovine cytochrome c-oxidase in...
lipid extracts from *Sulfolobus acidocaldarius* primarily composed of bipolar lipids. Curiously, archaea are not known to be involved in photosynthesis, which is a heavily membrane-dependent process. Therefore, we chose to reconstitute a bacterial photosynthetic reaction center (RC) protein into GDGT membranes. First, we dissolved the protein in a mixture of octyl glucoside and PG2. Next, the detergents were removed by treatment with BioBeads followed by extensive dialysis and detergent removal was confirmed by mass spectrometry. We then performed negative-staining TEM on the protein-lipid dispersion and detected spherical or nearly spherical structures of diameters 40-370 nm (mean: 119 ± 63 nm, n = 108) corresponding to the proteoliposomes (Figure 3D). The RC reconstituted in GDGT membranes were next used for the spectroscopic studies. To ascertain that the RC is still intact and functional after reconstitution into archaeal lipid, we first recorded the UV-Vis spectra, which confirmed that the protein is still properly assembled, and that the chromophores are still in place (Figure 3E). Finally, we performed ultrafast transient absorption spectroscopy to see whether the RC is still able to perform primary electron transfer in this new environment. The results showed that RC can still undergo charge transfer, and the kinetics and mechanism are largely unchanged (Figure S3A, B).

**Self-assembly of GDGTs at the air-water interface.** Amphiphilic molecules self-assemble at the air-water interface to form insoluble monomolecular layers with the polar headgroup facing water and the hydrophobic region positioned in air. Since GDGTs have two polar head groups we were curious to test how they self-assemble at the air-water interface and therefore understand how flexible these molecules are conformationally. Using a Langmuir-Blodgett trough, we found that GDGT lipids form stable monomolecular layers at the air-water interface as evident from measurements of surface pressure-area (π-A) isotherms obtained by the Wilhelmy plate method (Figure 4A, Figure S4). The isotherms exhibited stable liquid expanded behavior lacking any first order transitions. In the case of P2, P1Glc, PG2, and PG1Glc, the surface pressure reached a plateau at significantly high values of 36 mN/m, 30 mN/m, 42 mN/m, and 41 mN/m respectively, which is indicative of monolayer stability.

The results were compared with those obtained with monopolar phospholipids having straight chains (POPC, DPPC) and archaeal-inspired branched-chain phospholipids (DPhPA, DPhPC, DoPhPC, DPhPG) (Figure 4A, Figure S4). We found that the GDGTs display significantly higher mean molecular areas (MMA) as compared to monopolar lipids (Figure 4B). At the lift-off point, the GDGTs display MMA considerably higher than 300 Å² while at a surface pressure of 25 mN/m, all GDGTs display MMA of approximately 250 Å². The corresponding values for monopolar lipids are at least 3-4 times smaller. The high MMA values of the GDGTs can be explained by suggesting that these lipids have the conformational flexibility to loop into a U-shaped conformation at the air-water interface – i.e., with both headgroups facing water and the hydrocarbon chains forming an arch (Figure 4C).

**Measurement of structural properties of GDGT membranes.** There are well-established X-ray based methods for elucidating the structure of lipid bilayers. Here we measured the thicknesses of the GDGT membranes from small-angle X-ray scattering (SAXS) on unilamellar vesicles in solution. In this method, only diffuse scattering is obtained from uncorrelated membranes and therefore no Bragg peaks are observed. The observed scattering intensity profiles result from the difference in scattering length densities between the lipids and the buffer. In a typical experiment, we obtained scattering intensity profiles from GDGT unilamellar vesicles characterized by three broad lobes (Figure 5A). The profiles were fitted to a well-established model based on a simulated annealing algorithm that has been previously used to compute asymmetric bilayer electron density profiles (EDP), all fitting parameters are provided in Table S1. A typical EDP is characterized by two peaks corresponding to the electron-rich lipid headgroup regions separated by a trough corresponding to the electron-deficient hydrocarbon region (Figure 5B). Therefore, the head-to-head distances in a lipid membrane can be readily measured from the peak-to-peak distances of the

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**Figure 4.** A. Surface pressure-area (π-A) isotherms of various monopolar and bipolar (GDGT) lipids measured using a Langmuir-Blodgett trough and Milli-Q water as subphase. B. Table showing lift-off areas (Å2) and mean molecular areas (MMA) measured at 25 mN/m. C. Schematic diagram showing the likely arrangements of monopolar and bipolar lipids at the air-water interface.
EDPs which ranged between 38-40 Å for the GDGT lipids (Figure 5B).

Cryogenic transmission electron microscopy was used to measure the thicknesses of the hydrocarbon regions of the membranes and confirm the unilamellar nature of the vesicles. Small unilamellar vesicles of GDGTs were vitrified on holey carbon grids using standard techniques and imaged by cryo-EM. The overwhelming majority of the vesicles were unilamellar displaying uni-vesicular or multi-vesicular (or “vesicle-in-vesicle”) architectures (Figure 5C, Figure S5A, B). In addition, some sponge-like morphologies comprising of an interconnected network of membranes were observed in case of P2 and PG1Glc which were excluded from bilayer profile analysis (Figure S5C). We measured the thickness of each membrane by segmenting vesicles into small regions and aligning and averaging the segments to derive 2D class averages (Figure 5C). The linear profile of each membrane was measured across the center of the aligned and averaged images to obtain corresponding electron density profiles (Figure 5D). In agreement with the SAXS results, we found that the membrane thicknesses for all GDGT lipids were similar, with PG2 slightly thinner than the rest. Congruent with previous work, measured membrane thicknesses were approximately 8 Å thinner than those measured by SAXS (Figure 5D).41 We note that the membrane profiles have a different shape than those obtained from SAXS because they represent the projection of the spherical density rather than the EDP normal to the plane of the bilayer. Together, the SAXS and cryo-EM data demonstrate that the thicknesses of GDGT membranes is principally determined by their hydrocarbon chains rather than their headgroups.

![Figure 5](https://doi.org/10.26434/chemrxiv-2023-05gjp)

**Characterization of mesophases formed by GDGTs.** Lipid molecules self-assemble into supramolecular structures called mesophases (e.g. lamellar, hexagonal, cubic) depending on their molecular geometry and external factors. Functional roles of lipids are dictated by their propensity to form specific mesophases. For example, the tendency of phosphoethanolamine lipids to form inverted non-lamellar phases has been associated with their role in facilitating fusion of biological membranes via induction of negative curvature.42 SAXS on bulk lipid dispersions produces...
We sought to establish a link between the mesophase behavior of the lipids across a wide temperature range and their probable functional roles in archaeal cell membranes. Differential scanning calorimetry (DSC) studies on bulk GDGT lipid mixtures from extracts typically revealed that the lipids exist in fluid phases at all temperatures and do not exhibit any endothermic phase transitions. Moreover, each DSC measurement requires a significant amount of material that is not always practical to recover. Therefore, we characterized the mesophases formed by aqueous GDGT dispersions using synchrotron SAXS over 10-90 °C under the influence of (i) varying salt concentration; (ii) presence of Mg$^{2+}$; and (iii) acidic pH. The results are summarized in Table 1.

**Table 1**: Summary of lipidic mesophases formed by the GDGT lipids under various buffer conditions over 10-90 °C. “Buffer” refers to 50 mM Tris.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>P2</th>
<th>P1Glc</th>
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<th>PG1Glc</th>
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<tr>
<td>Buffer only</td>
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<td>Disordered lamellar</td>
<td>Disordered lamellar</td>
<td>Disordered lamellar</td>
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<tr>
<td>(pH 7.5)</td>
<td></td>
<td>(10-30 °C) Hexagonal (40-90 °C)</td>
<td></td>
<td></td>
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<tr>
<td>Buffer + 0.1 M NaCl (pH 7.5)</td>
<td>Disordered hexagonal (10-20 °C) Hexagonal (30-70 °C) Hexagonal + cubic Pn3m (80-90 °C)</td>
<td>Disordered lamellar (10-40 °C) Hexagonal (50-90 °C)</td>
<td>Disordered lamellar</td>
<td>Disordered lamellar</td>
</tr>
<tr>
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<td>Lamellar (10-40 °C) Cubic la3d (50 °C) Hexagonal (60-90 °C)</td>
<td>(Not dispersible)</td>
<td>Disordered lamellar</td>
<td>Disordered lamellar (10-20 °C) Hexagonal (30-90 °C)</td>
</tr>
<tr>
<td>Buffer + 1.0 M NaCl (pH 7.5)</td>
<td>Lamellar (10-30 °C) Lamellar + hexagonal (40-60 °C) Hexagonal (70-90 °C)</td>
<td>(Not dispersible)</td>
<td>Lamellar (10-20 °C) Disordered lamellar (30-90 °C)</td>
<td>Hexagonal</td>
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<tr>
<td>Buffer + 10 mM Mg$^{2+}$ (pH 7.5)</td>
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<td>Lamellar phase 1 Lamellar phase 2</td>
<td>Disordered lamellar</td>
<td>Hexagonal</td>
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<tr>
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</table>

Figure 6. Characterization of lipidic mesophases using SAXS. **A.** Concentric diffraction rings (2D detector image) from a lipid dispersion are characteristic of underlying mesophases. **B.** Dispersions of the lipid P2 in 50 mM Tris, pH 7.5 do not exhibit any characteristic features over 10-90 °C. **C.** SAXS intensity profile from P2 dispersion in 50 mM Tris, pH 7.5 containing 0.1 M NaCl at 90 °C showing a mixture of inverted non-lamellar phases (hexagonal and cubic).
In a low ionic strength buffer such as 50 mM Tris (pH 7.5), P2, PG2, and PG1Glc exhibited no Bragg peaks (Figure 6B, Figures S6, S8, S9) over the entire temperature range while P1Glc exhibited a weak hexagonal phase at 30 °C which gradually increased in intensity with temperature (Figure S7). These results suggest that the lipids mostly formed uncorrelated bilayers in Tris buffer due to repulsive forces between the charged headgroups. Repulsive forces may also arise from mutual steric hindrance between undulating membranes stacked in a liquid crystalline multilayer. Addition of salts is expected to screen the electrostatic repulsion and generate more ordered structures. Therefore, we investigated the effect of 0.1 M, 0.5 M, and 1 M NaCl added to 50 mM Tris buffer (pH 7.5). Among all lipids, P2 exhibited the most complex behavior at varying salt concentrations (Figure S6). In presence of 0.1 M NaCl, somewhat broad and low intensity H2 peaks were observed at 10-20 °C which became sharper and more intense over 30-70 °C. Over 80-90 °C, an Pn3m cubic phase coexisted with the H2 phase (Figure 6C). In 0.5 M NaCl, strong lamellar peaks were seen at 10 °C which fully transitioned to H2 peaks at 60 °C and above via an Ia3d cubic phase at 50 °C. In 1.0 M NaCl, strong lamellar peaks existed over 10-30 °C which coexisted with an H2 phase at 40-60 °C and an H2 phase only was observed at higher temperatures. P1Glc was difficult to disperse even in the presence of 0.1 M NaCl and a hexagonal phase was detectable at 50 °C and above (Figure S7). P1Glc film could not be hydrated at higher salt concentrations. PG2 exhibited disordered phases at all salt concentrations except a small degree of lamellar ordering over 10-30 °C in the presence of 1 M NaCl (Figure S8). These observations are in good agreement with microscopy studies where we see facile formation of PG2 vesicles in buffer having a similar range of ionic strengths. PG1Glc displayed disordered phases in 0.1 M NaCl, but a hexagonal phase started to appear above 30 °C in the presence of 0.5 M NaCl (Figure S9). In the presence of 1 M NaCl, a small hexagonal phase peak was detectable even at 10 °C which increased in intensity with temperature (Figure S9).

Next, we studied the effect of Mg2+ ions on the phase behavior of GDGTs (Figure S10A-D). Both the phosphate-containing lipids P2 and P1Glc exhibited strong lamellar ordering at all temperatures in presence of MgCl. P2 exhibited sharp Bragg peaks at all temperatures with lamellar d-spacing of 4.95 nm at 10 °C which monotonically decreased to 3.93 nm at 90 °C. P1Glc exhibited Bragg peaks corresponding to a mixture of two lamellar phases having lamellar d-spacing of 4.67 nm and 4.49 nm at 10 °C which monotonically decreased to 3.99 nm and 3.95 nm respectively at 90 °C. On the other hand, the phosphoglycerol-containing lipids PG2 and PG1Glc exhibited hexagonal phases in the presence of MgCl. In the case of PG2, a weak and broad hexagonal phase peak appeared at 50 °C and it became more prominent at higher temperatures. For PG1Glc, a poorly resolved broad 1st H2 peak appeared at 10 °C which increased in prominence with temperature, and the 2nd and 3rd H2 peaks could be unambiguously assigned only above 50 °C. We reason that upon binding of Mg2+ to the PG headgroup phosphates, they become dehydrated and bridge together. Consequently, the volume of the headgroup region becomes smaller in comparison to the hydrocarbon region and the molecules organize into a hexagonal phase. Interestingly, we also observed Mg2+-induced H2 phase formation in the monopolar diphytanyl archaeal lipid DPhPG (which has a negative spontaneous curvature) at all temperatures (Figure S11), which supports the idea that bridging of PG headgroups by Mg2+ ions is the primary driver of H2 phase formation.

Since GDGT lipids occur in thermoacidophilic archaea, we investigated the effect of acidic pH on the mesophase behavior of these lipids. The phosphate containing lipids P2 and P1Glc could not be dispersed in 50 mM Tris (pH 2.8) likely because of partial protonation of the phosphate groups. Interestingly, both the phosphoglycerol containing lipids PG2 and PG1Glc were found to form hexagonal phases over the temperature range (30-90 °C) surveyed (Figure S10E-F). For PG1Glc, the H2 peaks were sharp and three H2 peaks (ratios of 1:√3:√4) were detected at all temperatures. Similar to Mg2+-induced hexagonal phase formation, we reason that due to protonation of the PG groups (pK<sub>a</sub> ~ 3) they too become dehydrated and therefore decreases in volume. Notably, we did not observe hexagonal phase formation in PG2 and PG1Glc at pH 5.1, where the PG headgroups are expected to be fully deprotonated.

**Fusion behavior of membranes composed of GDGTs.** Like any organism, archaeal membranes must undergo dynamic physiological processes like fission and fusion during cell division, membrane vesicle budding, or viral entry/egress. However, there is a lack of clarity regarding how archaeal membranes primarily composed of bipolar lipids can undergo fusion (or fission). In a canonical model of bilayer membrane fusion, the outer leaflets of apposing membranes first merge to form a stalk-like hemifusion intermediate. This step is triggered by fusogenic agents like divalent cations, acidic pH, polyethylene glycol, or fusion peptides which help overcome the energetic barriers associated with various intermediate steps along the fusion pathway. In the next step, the inner leaflets merge via transient non-lamellar intermediates to open up a fusion pore and the two membranes completely fuse along with mixing of inner aqueous contents. In the case where GDGT lipids form a monolayer membrane where all lipids are membrane-spanning, there will be high energy barriers and geometrical constraints to forming various fusion intermediates as compared to bilayer membranes. Therefore, we hypothesize that at least some regions of the GDGT membrane should have a bilayer structure which is only possible if some lipids adopt a looping or U-shaped conformation (Figure 1B).

Whether bipolar lipid membranes can undergo fusion or not and how such behavior is related to the conformations (O vs U) of lipids has been a subject of intense investigation. Lerich and coworkers demonstrated that vesicles composed purely of an acyclic bipolar lipid can undergo Ca2+-induced fusion without loss of contents. They further probed lipid ordering using 1H-NMR and concluded that the bipolar lipids adopt both O- and U-shapes in membranes although precise percentages were not determined. 1H-NMR experiments on model acyclic bipolar lipid membranes suggest that 90% of the lipids exist in spanning or O-shape and 10% in U-shape. Many studies on archaeal lipid mixtures attempted to establish a rational link between the lipid structures and membrane fusion (or the lack thereof) induced by Ca2+ or PEG. For example, Relini et al. noted...
that vesicles formed from archaeal lipid extracts which displayed transformations between lamellar and non-lamellar phases underwent fusion implying that lipids that can form non-lamellar phases are necessary to induce membrane curvature during fusion. In previous sections, we showed that under the influence of temperature, salt concentration, pH, or divalent cations, GDGTs show diverse mesophases, especially the formation of inverted non-lamellar phases such as hexagonal and cubic via dehydration of headgroups. Although those lamellar to non-lamellar phase transitions were observed in bulk lipid dispersions, the presence of such lipids in vesicle membranes likely increase the propensity to form local transient non-lamellar structures essential for fusion to take place. Therefore, we asked whether the tendency of GDGT lipids to form non-lamellar phases is related to membrane fusion. To test the fusion behavior of GDGT vesicles, we chose influenza virus as a fusogenic partner as the latter possesses an efficient fusion machinery embedded in a bilayer lipid membrane that triggers a fusion pathway representative of many biological fusion processes.

Figure 7. Low pH-induced fusion of GDGT vesicles with influenza virus labeled with a self-quenching concentration of Texas Red-DHPE. A. Schematic diagram showing vesicles tethered to glass surface via Biotin-DPPE binding to influenza virus particles via a sialoglycolipid receptor GD1a. Upon lowering of pH, the Texas Red-DHPE undergoes dilution, and the fluorescence turns on. B. Fluorescence intensity trace (Texas Red channel, 561 nm emission) of a single fusion event between influenza virus and “PG2 mix” vesicles are shown along with corresponding images at various time points. C. Microscopy images showing many fusion events (indicated in yellow boxes) between influenza virus particles and “PG2 mix” vesicles are observed within a field of view. Scale bar: 5 µm. D. Cumulative distribution functions corresponding to wait times to individual lipid mixing events for viral fusion with 100 nm vesicles composed of monopolar lipids (Phospholipid mix) and bipolar lipids (PG2 mix and PG1Glc mix).

We followed a widely utilized fluorescence microscopy assay to observe fusion of viruses with lipid vesicles on a single particle level. In this method, the vesicles are allowed to fuse with bound influenza virus labeled with self-quenching concentrations of Texas Red-DHPE by acidification of the medium (Figure 7A). 100 nm vesicles composed overwhelmingly of GDGT lipids (PG2 mix - PG2:GD1a:Biotin-DPPE:Atto 647N-DMPE 98.45:1:0.5:0.05; PG1Glc mix - PG1Glc:GD1a:Biotin-DPPE:Atto 647N-DMPE 98.45:1:0.5:0.05 by molar ratio) were prepared in a pH 7.4 buffer. The vesicles were tethered to a functionalized glass slide through interaction of neutravidin with Biotin-DPPE (Figure 7A). Next, influenza virus particles labeled with a self-quenching concentration of Texas Red-DHPE were added. The α2,3-sialic acid containing sialoglycolipid receptor GD1a served as the receptor for influenza hemagglutinin binding. The pH was lowered by flowing in an acidic buffer (pH 5.1). Upon hemifusion, the outer leaflets of viral and vesicle membranes merge and the Texas Red-DHPE gets diluted. Consequently, its fluorescence turns on as evident from a sudden spike in fluorescence intensity (Figure 7B). Interestingly, we observed on a single particle level that many dim fluorescent spots (corresponding to a virion labeled with self-quenched TR-DHPE) turned brighter (Figure 7C). We plotted the time to the onset of lipid mixing into cumulative distribution function for many vesicle populations and

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found that the fusion of GDGT vesicles follow a kinetics comparable to that with vesicles composed of monopolar phospholipids and cholesterol (“Phospholipid mix”) (Figure 7D). Next, we asked whether the fusion events led to full fusion or whether they were arrested at the hemifusion stage. To address this question, we prepared “PG2 mix” vesicles encapsulating self-quenching concentration of the water-soluble dye sulforhodamine B (SRB) (Figure S12A). In the event of a full fusion, the interior of the virus and vesicle compartments become one and the fluorescence of SRB is enhanced upon dilution. We allowed influenza virus particles (unlabeled) to bind to tethered GDGT vesicles and then lowered the pH. We observed many events where a dim fluorescent spot turned bright indicating transfer of SRB from vesicle to virus through the fusion pore with nearly identical kinetics as compared to vesicles composed of monopolar phospholipids (“Phospholipid mix”) (Figure S12B, C).

To explain this fusion behavior, we reason that the membranes are composed of two types of domains – monolayers of O-shaped lipids and bilayers of U-shaped lipids. Formation of U-shaped bilayer domains is likely dictated by the conformational flexibility of the hydrocarbon region in GDGT-0 lipids which allows the lipids to fold in the middle. Previous reports suggest that viral fusion peptides can induce inverted non-lamellar phases in phosphoethanolamine lipids which are known to form hexagonal phases at high temperatures. Similarly, the tendency of GDGT-0 lipids to form inverted non-lamellar phases further allows dehydration of the lipid headgroups upon interaction with influenza viral fusion peptide to stabilize various intermediates key to fusion. Examples from archaeal biology indeed support the idea that GDGT-0 lipids have a greater tendency to adopt U-shapes and play a pivotal role in membrane dynamics. For instance, it has been observed that archaeal viruses such as SSV1 consist of a disproportionately larger fraction (68.1%) of GDGT-0 lipids as compared to its host Sulfolobus solfataricus (0.8%). The presence of high fraction of GDGT-0 lipids in the viral membranes likely facilitates their fusion with the host membranes both during entry and budding.

CONCLUSIONS

In summary we described the synthesis of a series of chemically pure GDGT-0 lipids and their self-assembly into vesicles in aqueous media. We addressed the question of what conformations those lipids adopt in a membrane and how it impacts the functional behavior of the membranes. While it is generally considered that archaeal bipolar lipids adopt a membrane-spanning conformation (O-shape), we have provided structural and functional evidence that GDGT-0 lipids possess the conformational flexibility to fold into U-shapes in a membrane. First, we showed that the GDGT-0 lipids adopt U-shapes in monomolecular films at the air-water interface as evident from the significantly higher mean molecular area occupied by these lipids in comparison to monopolar lipids. Next, we showed that vesicles primarily composed of GDGT lipids can undergo pH-induced fusion with influenza virus particles at kinetic rates similar to that measured with conventional monopolar lipid vesicles. Such behavior can be explained by considering that the GDGT membranes at least partially consist of bilayer regions composed of U-shaped lipids that act as sites of fusion. Conformational flexibility is further evident from the rich mesophase behavior for each GDGT lipid which were found to transform between lamellar and non-lamellar phases at various combinations of buffer and temperature conditions. One matter that will need careful consideration in the future is the impact of the cycloalkane rings in the hydrocarbon region of the higher order GDGT-n (n ≥ 1) lipids. GDGTs containing n cyclopentane rings are expected to be conformationally rigid and less likely to loop into a U-shape. Ongoing work is focused on synthesizing such lipids to probe their structural and dynamical behavior.

ASSOCIATED CONTENT

Supporting Information. Supplementary schemes S1-S7, Synthetic procedures, Supplementary figures S1-S12, and NMR spectral data

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Notes

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REFERENCES


