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The role of squalene in the organization of monolayers derived from lipid extracts of *Halobacterium salinarum*

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Abstract

We have studied interfacial compressibility and lateral organization in monolayer configurations of total (squalene containing) and polar (squalene-devoid) lipid extracts of *Halobacterium salinarum* NRC-1, an extremely halophilic archaeon. Pressure-area isotherms derived from Langmuir experiments reveal that packing characteristics and elastic compressibility are strongly influenced by the presence of squalene in the total lipid extract. In conjunction with control experiments using mixtures of DPhPC and squalene, our results establish that the presence of squalene significantly extends elastic area compressibility of total lipid extracts, suggesting it has a role in facilitating tighter packing of archaeal lipid mixtures. Moreover, we find that squalene also influences spatial organization in archaeal membranes. Epifluorescence and atomic force microscopy characterization of Langmuir monolayers transferred onto solid hydrophilic substrates reveal unusual domain morphology. Individual domains of microscopic dimensions (as well as their extended networks) exhibiting a peculiar bowl-like topography are evident in atomic force microscopy images. The tall rims outlining individual domains indicates that squalene accumulates at the domain periphery in a manner similar to the accumulation of cholesterol at domain boundaries in their mixtures with phospholipids. Taken together, results presented here support the notion that squalene plays a role in modulating molecular packing and lateral organization (i.e., domain formation) in the membranes of archaea analogous to that of cholesterol in eukaryotic membranes.

Keywords

Squalene; archaea; domains; monolayers; halophiles

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Author Contributions

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Supporting Information

Additional material includes figures S1-S4 and their accompanying legends. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

1. INTRODUCTION

A diverse range of microbial organisms exist that thrive under conditions that are challenging to most life on Earth.^{1,2} These organisms, primarily bacteria and archaea, survive under environmental conditions spanning extreme ranges of pH (acidophile, alkaliphile), temperature (thermophile, psychrophile), pressure (piezophile), salinity (halophile), dehydration (xerophile), and sugar concentrations (osmophile). Collectively referred to as extremophiles, they have evolved highly specialized biomolecular machineries that allow them to cope with their unique environments.

Membranes of extremophiles- the first line of defense against environmental assaults - have evolved to withstand particular environmental hardships. For instance, archaeal membranes are composed of lipids that have ether linkages and isoprenoid tails (e.g., phytanyl and bis-phytanediyl chains), contrasting sharply with the ester linkage and aliphatic acyl tails, common in non-archaea lipids. Moreover, common phospholipids with choline, serine, ethanolamine, and inositol head-groups are conspicuously absent in archaeal membranes. Because of their ability to protect the membrane under extreme environmental conditions, archaeal lipids (as well as their mixtures) are attracting considerable attention as model systems to determine molecular (e.g., tail structure, saturation, head-group electrostatics, etc.) and material (e.g., elasticity, permeability, fluidity, etc.) properties that help membranes maintain their integrity under harsh environmental conditions.³ They are also being investigated for a variety of medical and biotechnological applications.^{4,5} For instance, liposomal formulations derived from the lipids of extremophiles, also called archaeosomes, are proving to be a valuable resource for designing robust drug and gene delivery systems in demanding applications.⁵

The archaeon *Halobacterium salinarum* is an extremophile that has adapted to live in some of the most saline environments on Earth, such as salt flats and salterns. Their membranes tolerate high salt concentrations (2.5M to 5.2M) on the extra-cellular side and some, such as *H. salinarum*, accumulate potassium in their cytoplasm (~2M).⁶⁻⁸ Moreover, archaea membranes have evolved to withstand rapid and abrupt fluctuations in environmental salinity, such as caused by events rainfall or evaporation of water in dry environments. Moreover, the membrane of this organism (and other extreme halophiles) must protect essential intra-cellular components, such as DNA, from UV and UV-generated oxidative radicals, as they are exposed to relatively high doses of UV light such as in salt pans.⁹⁻¹¹ In particular, it has been shown that *H. salinarum* increases production of carotenoid pigments, which are present in the membrane, when the organisms encounter superoxide (O₂⁻).¹² Together, these conditions necessitate a membrane structure that is both physically and chemically robust, in addition to being tolerant of high and variable salt concentrations.

The polar lipid portion of the membrane of *H. salinarum* is primarily composed of phospholipids and glycolipids based on archaeol, a glycerol diether lipid containing phytanyl chains derived from isoprenoids. Primary amongst them are phosphatidylglycerol (PG) and phosphatidylglycerophosphate methyl ester (PGP-Me), which together account for 85% of the polar lipid content.³ The polar fraction also includes bacterioruberin, a 50-

carbon, bi-polar, membrane-spanning pigment, thought to be a photoprotectant. The non-polar lipids of *H. salinarum* are also based on isoprenoids, presumably synthesized by the same enzymes, which catalyze synthesis of the phytanyl chains of archaeol. Squalene, a non-polar, 30-carbon isoprenoid, is the primary component of the non-polar fraction, known primarily for its association with bacteriorhodopsin.

Although questions of which of the abundant components of archaeal membranes and by what mechanisms confer them the extra-ordinary combination of properties such as adaptability, salt tolerance, and photoprotection are of obvious importance, studies purportedly aimed at understanding relations between composition, organization, and properties are sparse. In a recent study, Tenchov and coworkers isolated and reconstituted polar lipid extracts of *H. salinarum* (and other halophiles) lipids into large unilamellar vesicles (LUVs).³ Comparing the solute retention and aggregation properties of LUVs from fractions with and without PGP-Me under strong salinity gradients, they attributed the salinity tolerance of archaeal membranes to originate from the properties of PGP-Me. Although PGP-Me possesses a double negative charge, the ability of archaeal membranes to resist aggregation under high salt concentrations is not likely to stem from head-group electrostatics because of strong screening. The authors suggest that the structural integrity of archaea membranes stem from a combination of steric repulsion between molecules of PGP-Me due to their large head-groups. However, this proposition remains unverified.

In this regard, squalene, another ubiquitous component of archaea membranes and a sterol intermediate, can be expected to play a role. In eukaryotes, membrane composition adjusts to cope with environmental stresses. Trees accumulate isoprene-conjugated derivatives in their lipid membrane in response to elevated heat.¹³ Similarly, mammals tune the levels of cholesterol in their cellular membranes to modulate their elastic properties, which influence their fluidity and permeability.^{14,15} Moreover, in mammalian cells cholesterol does not distribute uniformly across the cell-surface lipid species; it preferentially partitions with saturated components thus producing dynamic domain texture, including caveoli and rafts, at cellular surfaces.^{16,17} Model studies, such as carried out using molecularly tailored lipid-cholesterol mixtures organized as Langmuir monolayers at the air-water interface, suggest that cholesterol also has a peculiar area condensing effect on monolayer packing. In fact, these two effects of cholesterol on membrane structure and organization, namely its effect on average molecular packing and non-uniform distribution, are manifestations of the ability of cholesterol to insert itself in the intermolecular space between neighboring lipids. This steric interaction then locally orders the acyl chains in lipid mixtures, allowing tighter packing, and thus greater stability and reduced fluidity for the bilayer as a whole.¹⁸⁻²⁰ While this adaptability is well-established and understood for eukaryotes, the mechanisms by which membranes of archaea cope with environmental stresses are less well understood. Because of its ubiquitous presence among archaea, and since it may adopt a sterol-like structure, squalene can be expected to play a role analogous to cholesterol in archaeal membranes.²¹

In the work reported here, we compare two physical-chemical characteristics, namely the interfacial compressibility and lateral organization (both influenced pronouncedly by cholesterol in mammalian cells), of squalene containing (total) and squalene-devoid (polar)

lipid extract of *Halobacterium salinarum* NRC-1. Pressure-area isotherms derived from Langmuir experiments map onto other similar amphiphiles and suggest packing characteristics and elastic compressibility properties to be strongly modulated by the presence of squalene in the total lipid extract: the incorporation of squalene dramatically extends elastic area compressibility and allows for tighter packing of archaeal lipid mixtures. Further evidence of squalene induced area condensation is observed in interactions with DPhPC. Squalene also appears to influence lateral organization of archaeal membrane components. Epifluorescence and atomic force microscopy characterization of Langmuir monolayers transferred onto solid hydrophilic substrates reveal pronounced phase separation characterized by an unusual domain morphology. Individual domains of microscopic dimensions (as well as their extended networks) exhibit a peculiar bowl-like morphology. The presence of tall rims surrounding individual domains is consistent with the accumulation of squalene at the domain periphery. Taken together, these lines of evidence supports the notion that squalene might have a role analogous to cholesterol in phospholipid bilayers by modulating lipid packing and lateral organization (i.e., domain morphology) in the archaeal lipid mixtures.

2. EXPERIMENTAL SECTION

2.1. Culturing *Halobacterium salinarum*

All *Halobacterium salinarum* NRC-1 cells were cultured at 37 °C on a New Brunswick Scientific G-53 Shaker (New Brunswick Scientific, NJ, USA) at 150 rpm in complex media (CM) consisting of 250 g/l NaCl, 20 g/l MgSO₄ * 7H₂O, 2 g/l KCl, 3 g/l Na-Citrate and 10 g/l Oxoid™ Neutralized Peptone (Fisher Scientific: New Hampshire, USA).²² To prepare NRC-1 cells for lipid extraction, cells were revived from freezer stock to an optical density measured at 600nm (OD₆₀₀) of 0.7 in 4 ml of CM and then diluted to a starting OD₆₀₀ of 0.001 in 2 l of CM in a 4 l Pyrex Erlenmeyer flask (No: 4980, Stopper #10). Cells were grown for 3 days to stationary phase, when the OD₆₀₀ was greater than 1.0. The culture was aliquoted and stored at -40 °C until use.

2.2. Lipid extraction

As needed, the culture was thawed, centrifuged at 4000 rpm, and the media discarded. Lipids were extracted using the Bligh & Dyer method.²³ The chloroform phase was evaporated and the extract weighed. The mixture was then re-dissolved in chloroform for a final concentration of ~1 mg/ml, and the mixture was placed in a freezer for storage until use.

2.3. Preparation of Langmuir monolayers at the air-water interface

Langmuir monolayer experiments were carried out using a computer-controlled, Teflon Langmuir trough (Nima, Coventry, UK) equipped with teflon-coated moving barriers. A stationary Wilhelmy balance was used to measure the surface pressure with a resolution of (0.5 mN/m) employing a 10 × 20 mm paper slip suspended at the air-water interface. The sub-phase was deionized water. ~Lipid solution at 1-2 mg/ml for lipids harvested from *H. salinarum* was applied dropwise to the water surface using a glass syringe (Hamilton Company, Reno, Nevada), and the organic solvent was allowed to evaporate for 15 minutes.

Likewise, solutions consisting of DPhPC (Avanti Polar Lipids, Alabaster, AL) and squalene (Sigma-Aldrich, St. Louis, MO) were applied at 10 mg/ml. The barriers were then compressed with a linear speed of 6 cm²/min, and isotherm measurements in the form of surface pressure (mN/m) versus area per trough area were recorded at 1 second intervals until the monolayer reached its collapse pressure. All measurements were conducted at 21 °C.

2.4. Acetone Fractioning

To separate polar and non-polar lipid components, acetone fractionation was performed.²⁴ The total lipid extract in chloroform was dried down to a cake using nitrogen gas. Approximately 40 µl of chloroform was added to dissolve the cake. 1.5 ml of cold acetone was added and the mixture was vortexed and then centrifuged at 12,000 rpm for 5 minutes. The supernatant was then removed and saved. The precipitate was washed again in the same manner, and the second supernatant was discarded. The precipitate was then dried down further with nitrogen gas, and re-dissolved at a concentration of 1 mg/ml. The acetone in the supernatant was evaporated using nitrogen gas. The supernatant portion was then redissolved in chloroform to a concentration of 2 mg/ml. After acetone fractioning was performed on the total lipid extract, the polar and non-polar portions were both mixed with TR-DHPE (approximately 1 mol%).

2.5. Deposition of Langmuir-Blodgett monolayers & imaging

Spreading solutions were prepared by adding fluorescent dyes, namely, Texas Red-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium Salt (Texas Red-DHPE) or naphthopyrene or perylene to 100 µl of chloroform solution of a desired lipid mixture. The approximate concentration of each dye was approximately 1 mol percent of the lipid mixture. 25 µl aliquots of the spreading solution were then deposited drop-wise at the surface of a Langmuir trough, and the chloroform allowed to evaporate for 10 minutes. The barriers were compressed to 35 mN/m at a rate of 20 cm²/min and monolayers were deposited onto a freshly piranha-etched, number one cover slip at 21 °C. Additional Langmuir-Blodgett films were prepared by vertically transferring Langmuir monolayers compressed to 26 mN/m and 36 mN/m at 6 cm²/min onto glass coverslips (Supporting Material 3) at a rate of 5 mm/min. Cover slips were cleaned previously with a piranha etch solution consisting of 4:1 sulfuric acid and hydrogen peroxide at 90 °C, and were used within 1-2 days. Any residual water on the sample was allowed to evaporate and the sample imaged using either an inverted fluorescence microscope (Nikon, Melville, NY) or a combined confocal (Olympus, Center Valley, PA) & atomic force microscope (Asylum Research, Goleta, CA). Samples were scanned with a silicon nitride tip (Veeco Metrology, Santa Barbara, CA), using a 0.01 N/m cantilever.

2.6. Thin-layer chromatography for Lipid analysis

TLC plates (silica gel 60A, 20 cm × 10 cm, layer thickness 0.2 mm) were obtained from Merck (Darmstadt, Germany). Plates were either washed twice with chloroform-methanol (1:1; v/v) and activated at 180°C for 25 minutes before use, or used as is.²⁵ In both cases the end results were similar. Lipids were eluted with chloroform-methanol-90% acetic acid;

65:4:35 (v/v/v),²⁵ and detected by spraying with 5% sulfuric acid in water, followed by charring at 180°C for 4-5 min.²⁶ Visualization was done in a UV cabinet equipped with a UV lamp model ENF-260C (Spectroline, NY, USA) under UV light at 365nm.

3. RESULTS AND DISCUSSION

3.1. Characterization of polar lipid extracts derived by acetone fractionation

To confirm that squalene is indeed separated from the polar fraction during acetone precipitation of the total extract, we performed thin layer chromatography (TLC) on the total extract, as well as the polar and non-polar fractions. Comparing individual lipid extracts to squalene standard (S) (Figure 1) clearly establishes that squalene is present in both total lipid extraction (T) and non-polar lipids (N) fractions, but it is conspicuously absent from the polar fraction (P). This result also confirms that squalene is successfully separated from the polar lipids during the acetone washing process.

At present, we are not able to assign identities to the other separated lipids in each lane due to a lack of availability of suitable standards. It is notable, however, that while the lane for the total extract displays 4 visible spots, one of which corresponds to squalene, the polar lane only reveals three readily discernible spots, with two possible weak spots directly below. While the removal of squalene is the most obvious difference between the polar and total extract, it is possible that other apolar components may also be removed from the polar mixture.

3.2. Isotherms and compressibility characteristics of Langmuir monolayers with lipids derived from *Halobacterium salinarum*

Surface area-pressure (π -A) isotherms of total lipid extracts of *H. salinarum* (bacterioruberin positive and negative strains) are shown in Fig. 2a. Surface pressures (mN/m) are measured experimentally using the Wilhelmy plate method (See experimental). Because average molecular weight of the lipid mixtures are unknown, we report the areal axis for the lipid extracts of *H. salinarum* in terms of area per weight, cm²/μg. For comparison, the isotherm for DPhPC is also presented here in the same manner. Isotherms for the total lipid extracts consist of four well-resolved regimes, qualitatively consistent with those seen typically for insoluble amphiphiles at the air-water interface.²⁷ The initial slope from 0 mN/m to ~5 mN/m corresponds to the two-dimensional gas (G) phase (regime I). This is followed by the first limb of increasing surface pressure at ~5 mN/m for both cases signaling the emergence of the liquid-expanded (LE) phase (regime II). Upon further compression, an apparent and reproducible shoulder can be observed. This could be a partial collapse of one of the components, and also marks the transition to the liquid-condensed (LC) phase (regime III) at ~35 mN/M. Subsequently, a high-pressure plateau, consistent with the monolayer collapse, (regime IV) at ~40 mN/m is observed.

To better appreciate the elastic properties of monolayers of archaeal lipid extracts, we analyze the isotherm data in terms of monolayer compressibility, K_s , given by Equation 1 and 2 given below.²⁸

$$k_s = - \frac{1}{A} \frac{dA}{d\pi} \quad (1)$$

$$k_s(\pi_i) = - \frac{1}{A_i} \frac{A_{i+1} - A_{i-1}}{\pi_{i+1} - \pi_{i-1}} \quad (2)$$

Surface pressure-dependent compressibility profiles, so obtained (Fig 1, panels b and c) provide a measure of two dimensional bulk modulus ($\beta=1/K_s$). Because β and K_s represent second derivatives of the surface free energy with respect to area, these bumps might be associated with the discontinuities during both first- and second-order transitions. Although independent factors might explain their appearance in our data, these bumps are nevertheless consistent with (but do not conclusively establish) the appearance of the LC phase. Weak, but unmistakable bumps, are also evident in compressibility profiles at 30-35 mN/m, consistent with the appearance of the LC phase.

Next, we consider the isotherm and compressibility data for the polar lipid fraction alone. These fractions, isolated by acetone extraction are devoid of squalene (See TLC results below). Isotherm data for the polar lipid fraction from the bacterioruberin positive strain, shown in Fig. 1, reveal a significant departure from the behavior of the total lipid extract. Specifically, we find that the collapse in the isotherm as signaled by kinks reflecting abrupt drops in film pressure upon compression occurs at a measurably lower surface pressure (36 mN/m) than that observed for total lipid mixtures (> 40 mN/m). Partial collapses are observed at both 30 mN/m and 35 mN/m for the polar fraction. Thus, the polar lipids appear to remain in an expanded phase for all π before collapse and do not exhibit the formation of liquid-condensed phase. Moreover, at surface pressures corresponding to those typically associated with biological membranes (e.g., 30-35 mN/m),²⁹ both bacterioruberin positive (~ 2 cm²/μg) and negative strains (~ 2 cm²/μg) reveal molecular areas that are noticeably smaller than those typically associated with phospholipid molecules, such as DPhPC (~ 5 cm²/μg). Together, these observations suggest a significant contrast in the amphiphilic behavior of polar and total lipid extracts, already implicating non-polar components (e.g., squalene) in modulating packing and elastic compressibility behavior of the archaea lipid extracts in dense, LE or LC, phases.

To determine if squalene introduces “area condensation effect”, such as induced by cholesterol in mammalian membranes,^{18,20} through its steric association with phytanoyl or phytanyl-tailed lipids, we designed experiments using a simplified model system. This is because isolating any area condensation effect in the complex lipid extracts is not trivial; the effects may be further exacerbated by variability in lipid composition, which can be caused by small differences in conditions during culturing, extraction methods, and even in intermediate steps, such as cold storage.³⁰ To emulate the interaction between squalene and the isoprene chains of the lipids in the *H. salinarum*, we chose diphytenoylphosphatidylcholine (DPhPC), a purified isoprene-tailed lipid. This lipid has phytanoyl tails, which are similar in structure and length to the phytanyl tails of the dominant components of *H. salinarum*, PG and PGP-Me. Mixing DPhPC with squalene, we

can prepare a well-defined set of systematically tuned molecular compositions for the lipid mixtures. Model studies of the kind above have been reported previously for mixtures of squalene with acyl-tail linked phospholipids, which reveal both area-condensation and an area-expansion effects, depending on squalene content and film pressure.^{31,32} Studies aimed at mimicking association between squalene and lipids possessing isoprene chains, however, have not been previously reported to our knowledge.

Isotherm data of DPhPC (Fig. 3) reveals that at 30-35 mN/m, molecular areas are ~70-75 Å²/molecule, suggesting that the isoprene chains achieve packing densities comparable to the acyl chains of phospholipids (~50 Å²/molecule for DPPC).³³ In contrast, squalene remains in the gaseous state for molecular areas above 20 Å²/molecule. Further compression reveals the appearance of liquid-expanded phase. This information can be used in conjunction with the simple lever rule for binary monolayer mixtures to get some insight into any condensation effect. Here, the weighted sum of molecular areas ($A = A_1X_1 + (1 - X_1)A_2$ where A_i and X_i represent the area and the mole fraction of component i) is compared with the experimentally determined areas and departure from the ideality noted. A positive deviation provides a measure of intermolecular repulsion (expansion effect) and the negative indicates attraction or condensation effect.

Using this method,³¹ we plot the molecular area of mixtures of squalene and DPhPC as a function of the concentration of squalene used in Figure 3.³¹ Because squalene, in its pure phase does not condense to the surface pressure of interest (30 mN/m), we used an unphysical value of 1 Å²/molecule as a lower bound. Each line in Figure 3b represents the molecular area measured at a given pressure. The dashed lines between pure DPhPC and pure squalene represent the predicted molecular areas at each pressure for no interaction between DPhPC and squalene. Negative departures—actual measured values falling below these lines—then represent an area condensation effect at that pressure, while positive deviations—points above the line—reflect an areal expansion effect. As the squalene content is raised above 10 mol%, an area condensation effect is clearly evident for all plotted pressures. Note that a previous study by Castelli and coworkers carried out a similar comparison for mixtures of squalene with DMPC, an acyl-tailed phospholipid, and found area expansion effect as opposed to the condensation effects we observe for mixtures with DPhPC. This contrast further highlights that squalene interacts differently (more favorably) with phytanoyl chains.

It is now well-established that mammalian cells adjust their cholesterol content to achieve such area condensation effect when met with harsh environmental conditions.^{14,34} Given that squalene is composed of isoprene subunits and is situated within the hydrophobic core, it is not surprising that it may have a similar steric contribution in the organization of archaea lipids. Such area condensation at concentrations above 10 mol% might explain its partitioning into the purple membrane of *H. salinarum* (concentrations as high as 20% of total lipids), where tight packing of lipids and bacteriorhodopsin trimers are reported.³⁵ Note also that our results also reveal that both bacterioruberin positive and negative strains exhibit the condensation effect suggesting that bacterioruberin is not required to achieve condensation, but may play a secondary role, augmenting the area condensation effect.

Taken together, the isotherm and compressibility measurements above establish that the total lipid mixtures, both bacterioruberin positive and negative, are significantly more compressible than singular eukaryotic lipids. These mixtures attain a remarkably low film area at elevated surface pressures. In polar lipid fractions, this effect is far less pronounced suggesting the role of squalene in area condensation effect in total lipid extracts. Further, in our model system, squalene is shown to have an area-condensation effect on DPhPC for all measured pressures above 10 mol%. This implies that squalene may have a similar interaction with the comparable isoprene chain based amphiphiles, such as those that constitute the lipidome of *H. salinarum*.

3.2. Epifluorescence, confocal, and atomic force microscopy

To determine if the condensation effect - seemingly produced by the local association of squalene with major lipid components - produces co-existing phases, as suggested by the observed first-order phase transitions in the compressibility plots, we performed optical and atomic force microscopy experiments. To enable visualization by fluorescence microscopy, the monolayers were doped with phase-sensitive fluorescent probes and subsequently transferred to glass cover slips. We used Texas Red-DHPE, which partitions preferentially into fluid or disordered lipid phases, and a complementary naphthopyrene (or perylene) probe, a spectrally-distinct dye that incorporates into both ordered and disordered phases.³⁶ In particular, naphthopyrene has enhanced preference for the denser, ordered phase.³⁶ Epifluorescence experiments were carried out using both a wide-field, inverted fluorescence microscope, as well as a combined confocal fluorescence-atomic force microscopy system. Results from the confocal imaging are shown in Figure 6 and typical results of wide-field epifluorescence in Figure 4 and 5.

To examine if the addition of dyes influenced the isotherms of the extract, we carried out further Langmuir trough experiments. Isotherms obtained for Texas Red-DHPE and Texas Red-DHPE & perylene doped lipid solutions reveals a slight areal expansion effect (Supporting Material 1); the presence of perylene increases the surface pressure at collapse by 2-3 mN/m, while addition of Texas Red-DHPE alone to the lipid extract decreases the surface pressure at collapse by ~2-3 mN/m. We observe that the isotherms shift to lower densities by about 0.2-0.4 cm²/μg.

For monolayers derived from Langmuir films of total lipid extracts on a trough and transferred to glass cover slips, the images acquired for the Texas Red channel reveal a striking pattern of fluorescence heterogeneity (Fig. 4). Randomly distributed microscopic domains devoid of fluorescence texture the image. Because Texas Red-DHPE partitions preferentially into fluid phase, the domains seen in Figure 4, panel **a**, can be attributed to the existence of an ordered phase as discrete domains in the surrounding fluid phase.³⁶ To confirm that the dye-depleted domains are not physical defects (or holes), we carried out companion experiments using the complementary naphthopyrene or perylene dye. Epifluorescence images shown in Figure 5, panel **a** and Figure 6 reveal a complementary fluorescence pattern with the fluorescent dye decorating the domains.

A closer examination of the domain structure reveals that the ordered phase domains adopt two distinctly different sizes and morphologies, both of which co-exist in single samples.

Smaller domains measure approximately 15-25 μm in diameter. These domains, sometimes horseshoe-shaped, are more rounded in morphology and often possess a “nucleus,” in which naphthopyrene concentrates. Those with irregular shapes appear to be oligomeric (e.g., dimers, trimers, and tetramers) clusters of rounded monomers. The rounded edges of these domains suggest the domain interface is governed by line-tension, which would tend to minimize the domain perimeter.³⁷ Additionally, the images reveal sparse distribution of a second type of larger domains, $\sim 50 \mu\text{m}$ -wide, which are dendritic in appearance. Similar dendritic structure of domains has been previously reported for many widely different lipid planar lipid configurations.³⁸ To quantify the relation between domain size and the degree of branching, we examined the dependence of the fractal dimension, D , estimated using simple box counting method, on the size of the domains, shown in Figure 4. These results, summarized in Fig. 4c clearly show that as domains grow larger, the spread in D values narrow and average domain dimension converges toward the value of 1.67, associated with diffusion-limited aggregation in two dimensions.

Additionally, many of the domains in the monolayer appear grouped together forming extended networks or superstructures as seen in Figure 5. These networks are comprised of domains that are proximal to one another, but have not merged. The domain superstructures often span several millimeters and can contain hundreds of domains.

Similar domain networks were also observed in epifluorescence imaging of untransferred Langmuir monolayers at the air-water interface that were not compressed, but were cooled from 50-10° C (Supporting Material 2). The inter-domain attraction can be attributed to van der Waals attraction at the domain boundaries, presumably due to the accumulation of non-polar, membrane-spanning lipids at the domain edges.

Previous studies of domain morphologies in Langmuir-Blodgett monolayers establish that several experimental factors influence shapes and sizes of the condensed phase domains. These include temperature, surface density of the precursor Langmuir monolayers at the air-water interface, kinetics of compression and rates of transfer, sub-phase composition and pH, and even the influence of the trace concentrations of the fluorescent dye. Generally, slow isothermal compression produces round domains while a high compression speed produces dendritic domain shapes.³⁹ Additionally, dendritic domain shapes may also result at equilibrium due to competing influences of short-range line tension at the domain boundary and a long-range electrostatic repulsion. At present, we cannot isolate the influence of all of the above factors. Because the domain structures we observe are independent of compression rates we speculate that they are equilibrium structures rather than non-equilibrium kinetic structures produced by diffusion-limited aggregation. We postulate that as the smaller, and better equilibrated, rounded domains ($\sim 400 \mu\text{m}^2$) increase in size, they cross over a threshold domain dimension, beyond which the electrostatic long-range, electrostatic repulsions between polar head-groups also rises.⁴⁰ At the same time, the short-range attractive line-tension remains constant. As a consequence, beyond the threshold domain sizes, the balance between the two shifts in favor of long-range repulsive interactions favoring extended dendritic structure.⁴⁰ Additional work, beyond the scope of the current study, is needed to fully validate this proposition.

A closer examination of smaller domains was afforded by a combined confocal fluorescence and atomic force microscope. These measurements revealed finer topographical features within the smaller domains. These are illustrated in images shown in Figure 6. AFM data reveal that the domains are invariably characterized by a tall outer rim, as well as densely-packed peaks further in the domain, giving rise to a rough interior. The domain interior is almost 1 nm thicker than the surrounding monolayer, while the difference between the domain edge and the surrounding monolayer is 2 nm. At the intersection of these domains, the tall, outer rims juxtapose, but do not merge. Also of note, in the area where perylene dye has concentrated, near the center of the domain, for which there are no noticeable, corresponding features in the domain topography.

Additional Langmuir-Blodgett transfers were carried out below and above the shoulder at 35 mN/m. AFM results reveal an increase in domain height for samples transferred above the shoulder (Supporting Material 3). Below the shoulder, domains are nearly equal in height to the surrounding bilayer, but the domain edge is visible. Above the shoulder, domains increase in height by 2-3 nanometers.

The polar lipid mixture was also spread on the trough, and deposited onto coverslips (see experimental). In the deposited monolayers of the polar extract samples (data not shown), fluorescence images appear mostly uniform suggesting that domains did not form at optically resolvable size scales. This matches well with the observed properties of the isotherm as the polar mixture does not have a transition to the LC phase, as the compressibility plots in Figure 2 indicate that the shoulders are actually partial collapses, rather than transitions into the LC phase.

The large domain periphery thickness, which is enhanced by as much as 2 nanometers (Figure 6, panel **b**), is a surprising and intriguing observation. Our leading hypothesis has been that this is due to the aggregation of a particular membrane component to the domain periphery. The prevalent isoprene chains of the polar lipids in this extract (C_{20}) are approximately 2.1 nm in length. Both squalene (C_{30}) and bacterioruberin (C_{50}) are significantly longer at 2.9 nm and 3.9 nm, respectively. The extended length of these molecules would be detectable by AFM over the shorter chains of the polar lipids. Given their large maximum chain length, these lipids were considered as the primary contributors to this measured height at the domain periphery. To determine whether the bi-polar and membrane-spanning bacterioruberin is responsible for the thicker domain periphery, the lipid extract of the bacterioruberin-negative strain were doped with Texas Red-DHPE and perylene. Monolayers obtained by Langmuir transfer using these lipid mixtures reveal domain morphology and organization (including enhanced domain edge thickness) comparable to those obtained for samples prepared from lipid extracts of bacterioruberin-positive strains (Supporting Material 4). Moreover, it has been previously suggested that bi-polar lipids such as bacterioruberin bend in the middle into a U-shape at the air water interface, effectively reducing the thickness of the bi-polar monolayer.⁴¹ Thus, the effective length for bacterioruberin would only be about 1.9 nm, making it shorter than the isoprene chains of the polar lipids.

While we have demonstrated that bacterioruberin is not responsible for the domain edge thickness, we have no direct evidence to attribute this to squalene either. However, squalene is longer than the isoprene chains of the polar lipids, and given that it possesses no polar group, it would be situated entirely within the chain-region of the monolayer. The exact orientation and form of squalene within living archaeal membranes is unknown, but possibilities have been proposed. In addition to orienting perpendicular to the midplane of the bilayer, squalene may also dwell in an extended form at the interface between the two leaflets.⁴² This arrangement of squalene has been suggested to help prevent transient ion transfer across the membrane.⁴³ It has also been speculated that squalene may adopt a coiled or “sterol-like” structure within membranes.²¹

The AFM data presented here, displaying a 2 nanometer tall domain periphery, is consistent with an orientation of squalene that is perpendicular to the air-water interface. Although molecular preference in monolayer configurations may be significantly altered in bilayer motifs, it seems plausible that squalene in opposed monolayers may orient perpendicular to the mid-plane of the bilayer (rather than parallel to it), thus providing the archaeal membranes a mobile topographic divide and a means for lateral compartmentalization. This preference for the domain-bulk interface of squalene in archaeal lipid monolayers bears a resemblance to the organization of cholesterol in mono- and bilayers of ternary lipid mixtures. Previous studies suggest that cholesterol partitions to the interface of the fluid phase and the denser, ordered domains, in order to reduce the line-tension, which is caused by the hydrophobic mismatch at the domain boundaries.⁴⁴ Squalene might have an analogous structural role in the archaeal membranes.

4. CONCLUSIONS

The lipid extract of *H. salinarum* is an intriguing mixture due to the presence of lipids that have unusual structure and are highly charged (PG and PGP-Me), as well lipids that are non-polar and span both leaflets (squalene and bacterioruberin). Both the isotherms of these separate groups, as well as fluorescence analysis of Langmuir-Blodgett deposited monolayers, reveal that the domain structure in the total mixture is strongly influenced by the non-polar components. Squalene, a dominant non-polar component, appears to have a function similar to that of cholesterol in mixtures with eukaryotic lipids, in that it helps to orient the lipid tails away from the interface.¹⁹ This in turn allows the lipid tails to pack closely together, lowering the average area per molecule. Additionally, the enhanced order and accompanying packing characteristics among the lipid tails can then lead to the observed domain formation in the presence of squalene. This notion is supported both by the significantly increased average height at the domain edges, due to squalene orienting the lipid chains away from the interface, as well as the condensation of the average area per molecule in the presence of squalene. Additionally, the domains observed through fluorescent observation of doped monolayers deposited onto glass cover slips are seen to form large superstructures. These domain networks, composed of domains linked together at their edges, or in some cases, partially merged domains, can span distances into the millimeter range. This interesting domain motif might also have important implications for the organization of lipids of *H. salinarum* *in vivo*. The explicit partitioning of squalene to the

domain periphery may also occur in the membrane of the organism, particularly within the purple membrane, which is known to have a slightly higher squalene concentration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Thin-layer chromatography (TLC) of the different lipid fractions extracted from *H. salinarum*: Total lipids (T), Non-polar lipid fraction (N), Polar lipid fraction (P). Purified squalene (S) is shown in the far right lane. When present, squalene shows as dark- brown spot under UV light, and is indicated with white circles.

Figure 2.

Pressure-area isotherms and compressibility plots for the extracts of *H. salinarum*, as well as DPhPC. In (a), isotherms of the total lipid extract from *H. salinarum*, the total lipid extract from a bacterioruberin-negative strain of *H. salinarum*, the polar lipid fraction of the total extract of *H. salinarum*, and DPhPC. In (b), the plotted lipid compressibilities, k , of the isotherms from panel (a). In (c), a closeup of (b) showing the diminishing compressibilities with increasing pressure.

Figure 3.

Pressure-area isotherms for monolayers of mixtures of squalene and DPhPC, as well as corresponding molecular area as a function of squalene. In (a), isotherms for mixtures of DPhPC and squalene, where squalene content was modulated systematically. In (b), the molecular area as a function of squalene content for the mixture, plotted for three pressures. The dashed lines connect the molecular areas for pure DPhPC and pure squalene for each pressure.

Figure 4.

The relationship between domain size and fractal dimension. **(a)**, An epifluorescence image of a monolayer of total lipid extract formed on the Langmuir trough that was compressed at $20 \text{ cm}^2/\text{min}$ to 35 mN/m and transferred to a glass cover slip at the pulling rate of 5 mm/min . The dye is Texas Red-DHPE, $20\times$ scale bar $50 \mu\text{m}$. **(b)**, a histogram of the domain sizes in panel **(a)**. **(c)**, a plot of the domain area versus the calculated fractal dimension.

Figure 5.

An image obtained from epifluorescence microscopy showing extended domain networks and monomers. (a), fluorescence images of domains in a monolayer of the total lipid extract that was compressed at 20 cm²/min to 35 mN/m on a Langmuir trough and subsequently transferred to a glass cover slip at a rate of 5 mm/min, 40x, scale bar 50 μ m. Dyes are Texas Red-DHPE and naphthopyrene.

Figure 6.

Data from combined atomic force microscopy and confocal fluorescence microscopy measurements. **(a)**, fluorescence images (60x) revealing domains in a monolayer of total lipid extract that was compressed at 20 cm²/min to 35 mN/m on a Langmuir trough and was then transferred to a glass cover slip at the pulling rate of 5 mm/min. scale bar 15 μm. Dyes are Texas Red-DHPE and perylene. **(b)**, an AFM micrograph of a selection of domains seen in **(a)**. **(c)**, a linescan of the area demarcated in red in **(b)**.