Biochimie 91 (2009) 711–717

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Mini-review

Archaeal tetraether bipolar lipids: Structures, functions and applications

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ARTICLE INFO

Article history:
Received 28 November 2008
Accepted 15 January 2009
Available online 27 January 2009

Keywords:
Archaea
Lipids
Tetraether
Biotechnical applications

ABSTRACT

Archaea have developed specific tools permitting life under harsh conditions and archaeal lipids are one of these tools. This microreview describes the particular features of tetraether-type archaeal lipids and their potential applications in biotechnology. Natural and synthetic tetraether lipid structures as well as their applications in drug/gene delivery, vaccines and proteoliposomes or as lipid films are reviewed.

1. Introduction

In 1977, the discovery of the Archaea domain as a new taxonomy for living systems revealed the existence of a novel class of microorganisms encountered in exceptional ecological niches such as high (thermophiles and hyperthermophiles) or low (psychrophiles) temperatures, acidic media (acidophiles and thermoacidophiles), anaerobic atmosphere (methanogens) and high salinity (halophiles) [1]. A variety of phylogenic arguments has led several authors to suggest that Archaea may have played a key role in the early history of life [2]. The question most commonly asked of the Archaea is related to the nature of the molecular adaptations responsible for their ability to survive and grow in extreme environments. Indeed, these organisms have developed a range of tools to adapt their biological systems to each type of environmental living conditions. The modulation of the DNA/RNA structures by increasing the G–C base pair ratio as well as the supercoiling and the association with cationic proteins exemplify the strategies exhibited by Archaea to maintain the structure and integrity of their nucleic acids at high temperatures [3]. Additionally, the replacement of some amino acids was found to have a significant effect on protein stabilization. For instance, acidophilic proteins contain fewer acidic amino acids on their surface. This results in a low charge density, which is thought to prevent electrostatic repulsion among the protein at low pH [4]. Another distinguishing feature of Archaea from eukaryotic cells and bacteria is the unique chemical structure of their core membrane lipids. The archaeal lipids are composed of saturated chains containing methyl branches, attached to glycerol by ether linkages with a stereochemistry in 2-position of the glycerol opposite to that of conventional mesophilic lipids. Of particular interest are the unusual bipolar tetraether lipids present as a mixture of regioisomers in thermoacidophilic and methanogenic species [5]. Tetraether-type lipids mainly consist of macrocycles composed of two membrane-spanning alkyl chains that connect the two glycerol backbones (Fig. 1). These atypical bipolar lipids play a key role to the adaptation of Archaea to extreme habitats by optimizing membrane organization and properties in direct response to the growth conditions of the organisms. The maintenance of membrane fluidity, transport functions, intracellular solute concentrations, chemiosmotic gradients and membrane protein stability are a few examples of the functions of these lipids. The uniqueness of archaeal lipid structures and functions within membranes has prompted a great deal of interest for natural lipids or synthetic analogues as innovative materials for the development of biotechnical applications. In this microreview, we will focus on the structures and functions of natural tetraether lipids as well as the design of synthetic analogues and will discuss their applications in various potential domains.

2. Structures and functions of natural archaeal tetraether-type lipids

The archaeal tetraether lipid structures found in methanogens, thermophiles (thermoacidophiles) and psychrophiles are

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doi:10.1016/j.biochi.2009.01.006

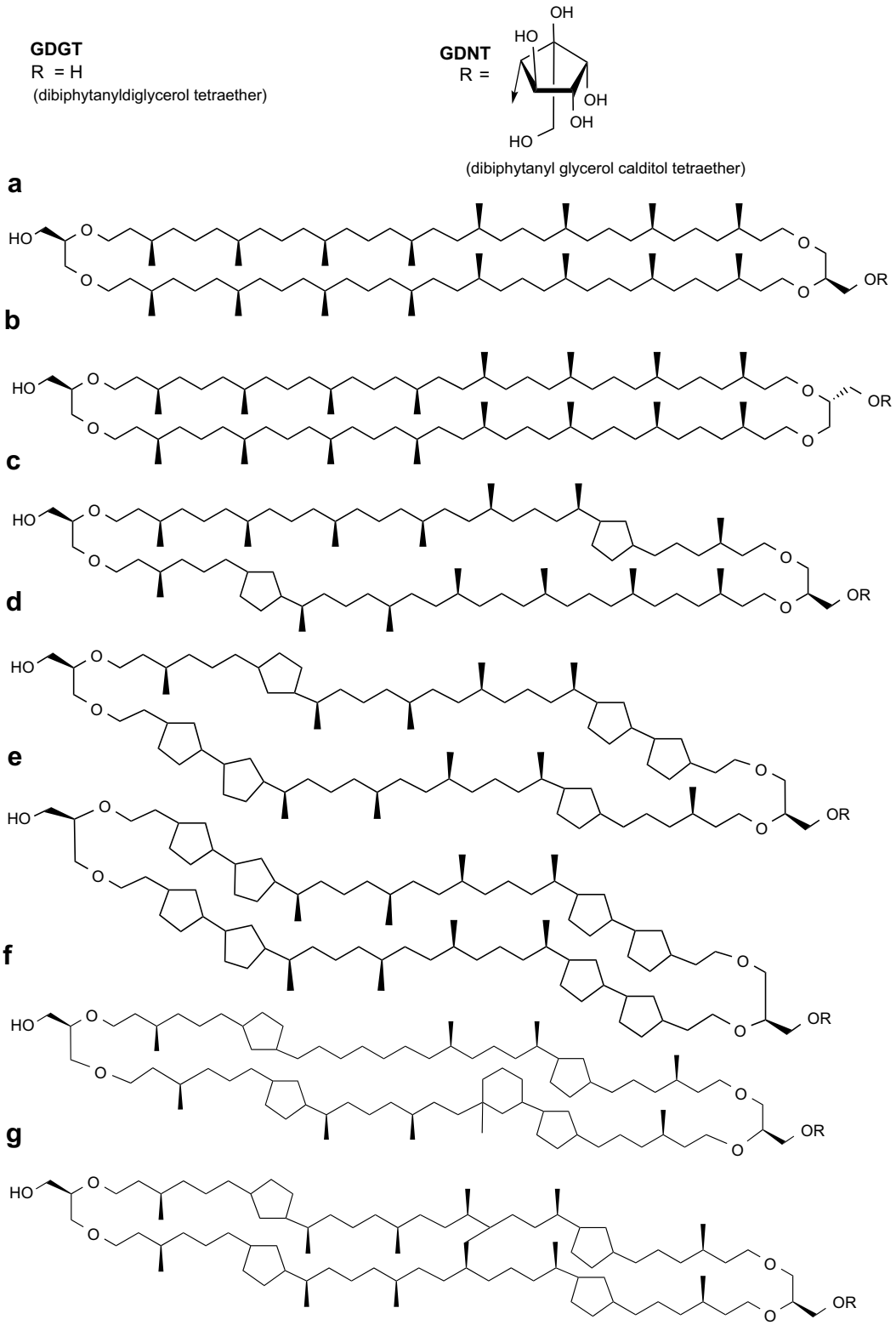

Fig. 1. (*modified from* Ref. [8]). Archaeal tetraether lipid architectures. (a–b) dibiphytanyl diglycerol (or glycerol calditol) tetraethers, (c–e) internal cyclization (cyclopentane rings) in dibiphytanyl diglycerol (or glycerol calditol) tetraethers, (f) internal cyclization (four cyclopentane rings and one cyclohexane ring) in dibiphytanyl diglycerol (or glycerol calditol) tetraethers, (g) H-shaped lipid structure containing four cyclopentane rings in dibiphytanyl diglycerol (or glycerol calditol) tetraethers.

characterized by two types of lipid backbones. The first one is called glycerol-dialkyl-glycerol-tetraethers (GDGT, Fig. 1; R = H) and is formed by two biphytanyl ether chains linked at both ends to a glycerol unit [6]. In the second class of molecules, glycerol-dialkyl-nonitol-tetraethers (GDNT, Fig. 1; R = calditol), a polyhydroxylated cyclopentanic calditol group comes at one end of the lipid. Variants of the dibiphytanyl diglycerol tetraether lipid skeletons were also identified in some Archaea. The

increasing proportion of cyclopentane rings (up to four pentacyclic rings in each of the biphytanyl chains) in thermoacidophilic lipids with increasing environmental temperatures [7], the presence of cyclohexane ring in lipids isolated from Archaea living in relative low temperature environments or the existence of a covalent cross-link at the centre of the isoprenoid chains in H-shaped tetraethers found in some thermoacidophilic species from deep sea hydrothermal vents are representative examples of the remarkable variations of the archaeal tetraether lipid structures. These bipolar compounds generally bear two different polar head groups at opposite ends of the hydrophobic backbone leading to unsymmetrical lipid structures. Archaea show a large variety of polar lipid classes, including phospholipids, glycolipids, phosphoglycolipids, sulpholipids and aminolipids. Because of the variety, specific information on the head group characteristics can help to identify an organism. Aminolipids are widely distributed and prevalent in the methanogens, but are totally absent in the thermophiles. Seemingly, unusual carbohydrates, that is, β-D-galactofuranosyl units [8] have been found in methanogens, but these acidic pH-sensitive five-membered rings are totally absent in thermoacidophilic Archaea.

The existence of unusual tetraether lipid structures in Archaea raises the question as to how these lipids are biosynthesized and how they function in the membrane of these organisms. The biosynthesis of these macrocyclic lipids is believed to proceed via (1) head-to-head coupling of two geranyl-geranyl residues with reduction to form biphytanyls and (2) cyclization within coupled geranyl-geranyl residues with reduction to form five (or six)-membered cyclic biphytanyls [9]. More recently, the isopropylene terminal of geranyl-geranyl group of monomeric precursor was found to be important for the C–C bond formation at the hydrophobic end. A mechanism involving a radical trigger at the allylic methyl group was then proposed for this C–C bond formation [10]. Numerous *in vivo* and *in vitro* studies conducted on polar lipid in Archaea have solved the general synthetic pathway [11]. However many biosynthesis mechanisms leading to archaeal lipids remain to be investigated and most enzymes involved in the biosynthesis pathways have never been characterized.

The large variety of tetraether lipid structures reflects the need for Archaea to adjust their core lipid structures in order to be able to ensure membrane functions despite harsh destabilizing environmental conditions. Several characteristics may be pointed out in that purpose. The ether linkages are more stable than esters over a wide range of pH, and the branching methyl groups help both to reduce crystallization (membrane lipids in the liquid crystalline state at ambient temperature) and membrane permeability (steric hindrance of the methyl side groups). The saturated alkyl chains ensure increased stability towards oxidative degradation. The unusual stereochemistry of the glycerol backbone (opposite to mesophiles) would impart resistance to attack by phospholipases released by other organisms and would thus have a survival value for the organisms. More specific functions of tetraether lipids have also to be considered: (1) the tetraether structure is thought to span the membrane from the inner to the outer side to form a monolayer membrane organization instead of the standard bilayer model [12]. Thus, a covalent linkage operates in the middle of the lipid layer: each bipolar tetraether molecule is completely stretched and spans its entire thickness. This monolayer organization would rigidify the membrane especially at the high growth temperatures of thermophilic, methanogens and of thermoacidophiles [13]; (2) the addition of cyclic structures (in particular five-membered rings) in the transmembrane portion of the lipids appears to be a thermoadaptative response, resulting in enhanced membrane packing and reduced membrane fluidity [6]; (3) the presence of a covalent bond between the alkyl chains in H-shaped tetraethers might protect the cells against membrane lysis at high temperature by reinforcing the strength of the monolayer membrane; and (4) high proportions of glycosyl polar head groups both in methanogenic and thermoacidophilic lipids may further stabilize the membrane structure by interglycosyl headgroup hydrogen bonding. The presence of large sugar heads towards the convex surface of the membrane may assume an asymmetric orientation, making therefore the monolayer organization easier [13].

3. Design of synthetic archaeal tetraether lipid analogues

The intriguing properties of the tetraether lipids of Archaea have stimulated the scientific community in projects that aim to study these atypical lipids. The extractions of natural lipids still conduce to mixtures of compounds that broaden the conclusions of the studies. To fully evaluate and understand the function of this type of lipids, several synthetic analogues have been developed. Indeed many bipolar lipids are reported in the literature [14], however, only few have retained the main structural features of natural archaeal tetraether lipids. The range of analogues can be divided into two main types: macrocyclic [15–21] and acyclic [22–35] structures. The first type, described in Fig. 2, is derived from two glycerol moieties that are linked together by two linear [15–18] (**1–4**), diacetylenic (**5–7**) [19,20] or branched [21] (**8–9**) chains. The polar head groups are usually derived from phosphate or phosphatidylcholine (PC), and until now only symmetrical structures have been designed and synthesised.

Thanks to easier synthetic approaches, acyclic tetraether lipids are more exemplified (Fig. 3). They are also based on two glycerol moieties linked by a single aliphatic chain through ether linkages. This chain can be a simple oligomethylene chain (**10–13**) [22–26] or it can incorporate branched residues (**14**) [27], biaryl (**15**) [28] groups or even deuterated methylenes (commonly used in ^{2}H NMR conformational studies) [29,30]. The quasimacrocyclic structures are then quasiclosed by two arms usually derived from phytanyl or oligomethylene chains. It is noteworthy that our group has developed the preparation of tetraether lipids that incorporate moreover a cyclopentane ring included in the hydrophobic chain (**16–21**) [31–35]. These analogues have therefore a very close structure to natural tetraether lipids (thermoacidophiles for instance).

For both macrocyclic and acyclic structures, the main polar head group that has been introduced into archaeal lipid analogues is a phosphatidylcholine moiety that is sufficiently polar to balance the hydrophobic core. Then phosphate, cationic glycine betaine residue or neutral sugar-type groups have been also used.

4. Biotechnical applications of natural and synthetic tetraether lipids

Owing to such originalities, natural and synthetic archaeal lipids are promising tools for medical, biological and biotechnological applications. Interest in these areas is stimulated primarily by the tetraether lipid self-assembling properties leading to supramolecular structures such as lipid films or liposomes.

4.1. Applications of tetraether lipid films

Monolayer lipid films, also termed black membranes have been prepared from archaeal tetraethers for better understanding the membrane behaviour and for potential applications in nanotechnology. Despite the unusual archaeal bipolar structure, the Langmuir–Blodgett technique for film formation at air/water interface can be applied to these amphiphiles. However, the construction of an unambiguous model is difficult to assess mainly due to variability in lipid extraction methods and/or monolayer preparation,

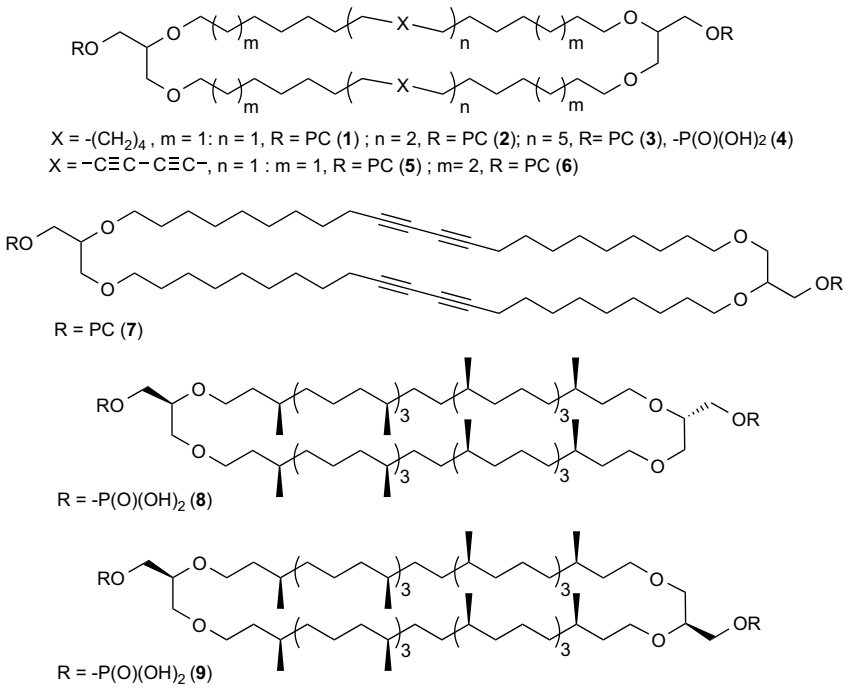

Fig. 2. Synthetic macrocyclic archaeal lipid analogues.

that could explain the strong differences observed in the results reported in the literature [36]. These studies have given rise to either models in which the molecules adopt an extended conformation (with or without tilt compared to the water interface) or models based on U-shaped molecules with the two polar heads in contact with the water phase [37]. The development of technological methods have made possible the production of highly organized films on the basis of archaeal lipids with remarkable long term stability [38], intrinsically good insulating properties [39] and low permeability [40].

The film deposition onto a solid support opens an easy way to change the properties of surfaces and to develop new biomimetic materials. For example, Rothe et al. [41] have coated nanoporous aluminium oxide membranes with ultrathin films of natural tetraether derivatives with the aim to change the filtration characteristics for biological applications. Thanks to the archaeal lipid properties, the coated membranes have lower permeability and could be easily sterilized.

Another application field of bipolar molecules is the preparation of ultrathin layers for biosensor design [42]. Indeed, the α-headgroup can be used to bind the lipid chemically to a solid surface, the hydrophobic core may serve as a barrier, and the second head group will be in contact with the environment and available for molecular recognition of solutes [14]. In this way, the stability problem encountered with bilayer lipid membranes is avoided. Furthermore, archaeal lipids offer novel structural opportunities in terms of protein or peptide/lipid interactions [43] and provide a natural environment for the immobilization of biomolecules. Different biological molecules can be thus embedded or adsorbed into the monolayers to produce biologically active systems. Valinomycin, a well described natural antibiotic peptide, is one example of functional molecule deposited on archaeal films. Because the LB

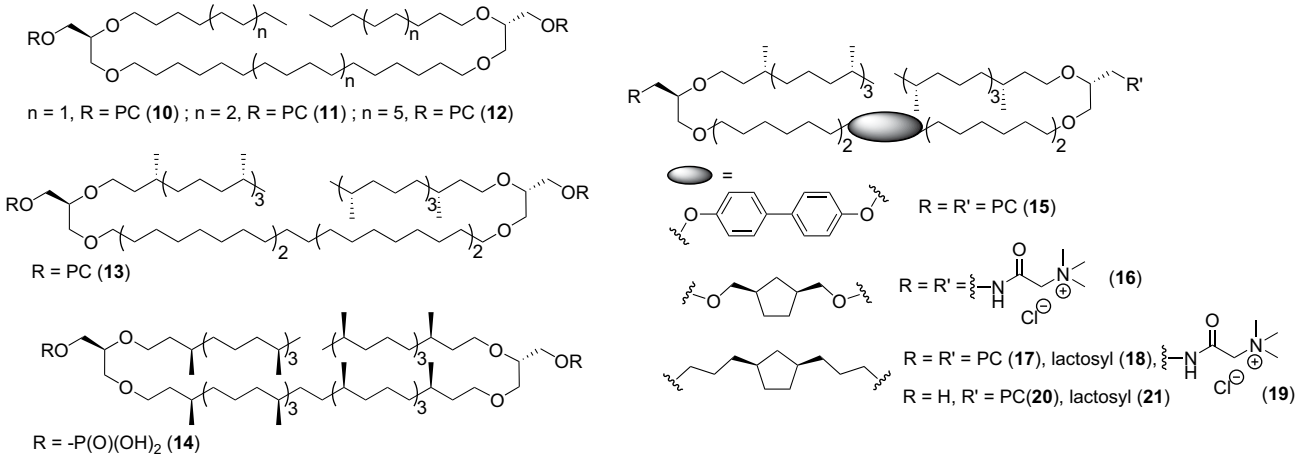

Fig. 3. Synthetic acyclic archaeal lipid analogues.

films of valinomycin are unstable with time, Berzina et al. [44,45] have used protective bilayers of barium salt of semisynthetic archaeal tetraether. Their results prove that selective interaction of potassium ions with valinomycin molecules takes place in the film and show a potential application as potassium ion sensors. Moreover, when the thickness of the monolayer membrane is reduced to the approximate length of two valinomycin molecules, the permeation mechanism changes from carrier behaviour (shuttle from one side to another) to channel behaviour (fixed in the membrane) due to the formation of a dimer permeation pore [46].

Finally, the organization of the lipids within the monolayer can be influenced by interactions with proteins. The S-layer protein recrystallization on natural tetraether lipid films has led to a new concept of biomimetic membrane [47]. The electrophysical features of S-layer-supported-GDNT-monolayer were compared with plain GDNT-monolayer ones by valinomycin mediated conductance measurement or by gramicidin incorporation (See Fig. 1 for the lipid structures). The protein association as porous supports exhibited an enhanced stability of the monolayer with highly isolating properties. Indeed, the S-layer supported lipid membranes provide an interesting biomimetic matrix with adequate flexibility and stability for the study of lipid/protein interactions.

4.2. Applications of archaeal liposomes (archaeosomes)

Archaeosomes are liposomes which are made from natural lipids found in Archaea or from synthetically derivated compounds that have the unique structure features of archaeal lipids [9,48]. The lipid membrane of archaeosomes can result in a bilayer, a monolayer, or a combination of mono- and bilayers made from bipolar and monopolar archaeal lipids or conventional phospholipids. The membrane-spanning properties of archaeal tetraethers provide rigidity of the vesicle membrane making these archaeosomes more stable and less permeable than conventional liposomes that frequently need up to 33% of cholesterol to improve their stability [49,50]. Despite this physical constraint, the membrane fusion is still possible if the bipolar lipids are monosubstituted by a complex polar head. Otherwise, vesicles formed by bisubstituted molecules are unable to fuse without adding a very low amount of a monopolar lipid [51].

The archaeal lipids formulations demonstrate relatively higher stabilities to oxidative stress, high temperature, alkaline pH, action of phospholipases, bile salts and serum media [33,34,52,53]. Thanks to archaeal lipid properties, archaeosomes can be formed at any temperature in the physiological range or lower, thus making it possible to encapsulate thermally labile compounds. Moreover, they can be prepared and stored in the presence of air/oxygen without any degradation [53,54]. The *in vitro* and *in vivo* studies indicate that archaeosomes are safe and do not elicit toxicity in mice [53,55]. Thus, the biocompatibility and the superior stability properties of archaeosomes in several conditions offer advantages over conventional liposomes in the manufacture and the use in biotechnology including vaccine and drug/gene delivery.

Since the discovery of lipofection, numerous lipid-DNA complexes, manufactured with cationic lipids and neutral co-lipids such as DOPE or cholesterol, were developed for transfection [56] but their use in clinical gene therapy trials was relatively unsatisfactory [57]. Archaeosomes have a high degree of physical rigidity and chemical/enzymatic stability [53] and represent a new approach in non viral gene delivery. The poor availability of pure natural archaeal lipids has encouraged the synthesis of hemimacrocyclic and macrocyclic tetraethers related to archaeal membrane. We have shown that synthetic archaeal lipids could significantly increase the stability of drug delivery systems even under oral administration conditions. Moreover, they are also good "helper" lipids for *in vitro* gene delivery applications (when compared with DOPE and cholesterol [33,34,58]). To achieve efficient *in vivo* transfection it is known that vectors should both increase their stability, by incorporation of high amount of cholesterol or/and by coating the lipoplexe surface with poly (ethylene) glycol (PEG), and make effective cell targeting via a recognition ligand incorporation [58,59]. Indeed, it seems to be promising to combine these stabilizing effects and targeting abilities in a new generation of archaeosomes for *in vivo* transfection. In that purpose, archaeal lipid analogues bearing a PEG chain (10 units of ethylene glycol) that was further equipped with a folic acid group have been prepared [60]. The first *in vitro* transfection experiments were performed with these synthetic folate-equipped pegylated archaeal lipids and led to clear ligand/receptor internalizations.

Because liposomes and archaeosomes naturally target to the cells of the mononuclear phagocytic system [61–63], they would be ideally suited for the delivery of antigens, as carrier systems and/or directly as adjuvants that stimulate the immune system. Uptake of archaeosomes by phagocytic cells is several times greater than that of liposomes made from ester lipids [63], and co-delivery of other immunostimulants is not necessary unlike most conventional liposomes [64,65]. For example, in murine models, administration of archaeosomes containing encapsulated antigen caused antibody responses comparable to those obtained by the effective but highly toxic Freund's complete adjuvant [64,66]. Archaeosomes are mixed adjuvants that promote potent humoral and cell-mediated immunities as well as robust memory response [65]. Studies of toxicity in mice indicated that archaeal liposomes have a good safe profile after oral or intravenous administration [55,65,67]. The efficiency of archaeosome adjuvants has been validated in murine models of infections and cancer [66,68–70]. Archaeosomes pose a rational choice for induction of the desirable quantity and quality of immune responses for protecting against intracellular infections and cancers [66,69].

To date, relatively little research has been conducted on archaeal proteoliposomes. Tetraether monolayer forms a suitable matrix for the reconstitution of exogenous membrane proteins originating from eukaryotic, bacterial or archaeal sources. Elferink's works represent the first *in vitro* evidence for reconstitution of a protein originating from a regular lipid bilayer into archaeal liposome [71–73]. Both a beef heart cytochrome-c-oxidase (eukaryotic origin) [71] and a leucine transport system (eubacterial origin) [72] were active after incorporation into the archaeal liposome. The investigated membrane protein carried out transport functions, generating transmembrane potentials. Furthermore, the generation of a proton gradient by a bacteriorhodopsin in MPL (Main phospholipid from *Thermoplasma acidophilum*) liposome and its coupling to ATP synthesis were investigated [74]. Co-reconstituted MPL proteoliposomes containing an ATP synthase were capable of light-driven ATP synthesis demonstrating the functional coupling of proton transport and nucleotide generation. Since then, Konings et al. have shown that the tetraether lipid-based formulations form a competent matrix for several primary proton pumps although the activities are restricted at high tetraether lipid concentration, certainly due to the rigidity of the membrane [73]. The structure of the liposomal membrane plays important roles in the protein orientation and activity. For example, the orientation of the light-harvesting polypeptide (LH)/bacteriorchlorophyll *a* (BChl *a*) complex is crucial to optimize energy transfer [75]. More recently, membrane proteins have been reconstituted in synthetic bipolar lipid vesicles with cyclic or hemimacrocyclic C_{20} and C_{32} archaeal lipid analogues [22,76]. It appears that a good Ste14p membrane protein activity (Isoprenylcysteine Carbonyl Methyl transferase from *Saccharomyces cerevisiae*) was recovered in membranes composed of $\leq$50 mol% of C_{20}BAS (See Fig. 3, lipid **11**) [22]. The

Ste14p activity appears to be not affected by the gel-to-liquid-crystal phase transition temperature. However, the thickness of the hydrophobic region of the membrane has a significant impact on the methyl transferase activity, with the thinner C_{20}BAS membrane producing a less active proteoliposome.

5. Conclusion

Since the discovery of archaeal lipids in the late 1970s, several research laboratories around the world have focussed specifically their research topics on the structural and physicochemical characterizations of tetraether-type lipids as well as on their biotechnological applications. Over the last few years, a considerable effort was also devoted to the development of novel bipolar lipids, namely through the synthesis of several cyclic and acyclic lipid analogues that retain some of the essential structural features of archaeal membrane tetraether lipids. These studies allowed the establishment of relationships between the chemical structure and the physicochemical behaviour, particularly in the case of acyclic analogues. These synthetic lipids could represent an elective material for the construction of a new generation of stable liposomes and artificial membranes of technological interest. Nevertheless, further research is still necessary to improve our knowledge of these strange lipids, both to understand more precisely their function in Archaea and to enlarge the scope of their biotechnical applications.

Acknowledgments

We thank our collaborators, whose names are listed in the references, for the remarkable work they have done on this subject. Financial support from the Région Bretagne (grant to A.J.) and the Direction générale des Entreprises (grant to J.B.) is gratefully acknowledged.

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