Archaeosomes based on novel synthetic tetraether-type lipids for the development of oral delivery systems†

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The *in vitro* stability of archaeosomes made from novel synthetic membrane-spanning tetraether lipids was evaluated in conditions mimicking those of oral route application in terms of bile salts, serum and low pH.

Liposomes, or phospholipid vesicles, are self-assembled colloidal particles that were introduced as potential carriers of pharmaceuticals, including vaccines, drugs and diagnostic agents in the 1970s.[1] Although there are various liposomal formulations already on the market or in clinical trials, a major limitation to the use of conventional phospholipid bilayer products (conventional liposomes) is instability.[2] Archaeosomes made with one or more of the fully saturated bipolar tetraether lipids unique to Archaea constitute a novel family of liposomes that demonstrate higher stability to several conditions in comparison with conventional lipids.[3] This presents a significant advancement for the delivery of medicines, allowing for more varied routes of administration including the oral route.

Several general surveys of the structural features of archaeal lipids have reviewed the properties and potential applications of novel liposomes made from the membrane lipids of Archaebacteria.[3,4a] However, in spite of the growing attention to archaeosomes, very few studies have been performed so far to optimize the properties of archaeal lipid-containing liposomes *via* the modulation of lipid polar head groups, in part due to the difficulty of obtaining sufficient amounts of chemically pure tetraether-type compounds from natural sources[4a] or by synthetic methods.[4b,5a–f] Within this context, we report the synthesis of novel archaeal bipolar lipid mimics; the preparation of archaeosomes made exclusively from these synthetic tetraether compounds or resulting from mixtures of natural phospholipids and synthetic tetraethers; the evaluation of *in vitro* archaeosome stability under conditions encountered in the human gastrointestinal (GI) tract. This approach aims at improving the properties of conventional liposomes *via* the controlled incorporation of synthetic tetraether lipids into the bilayer membranes (Fig. 1) with a view to developing applications as an oral delivery system.

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† Electronic supplementary information (ESI) available: Percent leakage of encapsulated CF from liposomes prepared from increasing proportions of lipids **1** or **5**, in a 0.4% sodium cholate KRB buffer solution or upon exposure (37 °C) to fetal bovine serum. See DOI: 10.1039/b511440c

The tetraether lipids **1–5** (Fig. 2) designed in this work contained: (1) two glycerol units linked together by two C15 saturated polymethylene chains connected *via* a central cyclopentane group, allowing the entire lipophilic moiety to span a bilayer membrane and to exhibit a higher aqueous dispersion;[5f] (2) two chiral phytanyl chains having a combined length equivalent to that of the bridging chain, ether linked to the glycerol residues; and (3) two similar or different polar head groups including hydroxyl, lactose or phosphatidylcholine (PC) units.

The strategic plan for the preparation of diol **1** (Scheme 1) involved the successive alkylation of the (*S*)-glycerol derivative **12** with ditriflate **10** containing a *cis*-1,3 cyclopentane unit within the polymethylene chain, and the removal of the benzyl groups. The construction of the bridging chain was performed *via* a double Wittig reaction between 14-benzyloxy-tetradecan-1-al **7** and the bisphosphonium salt **6** obtained from *cis*-1,3-diformylcyclopentane followed by hydrogenation of the double bonds and hydrogenolysis of the benzyloxy groups.[5f] The reaction of the resulting diol **9**

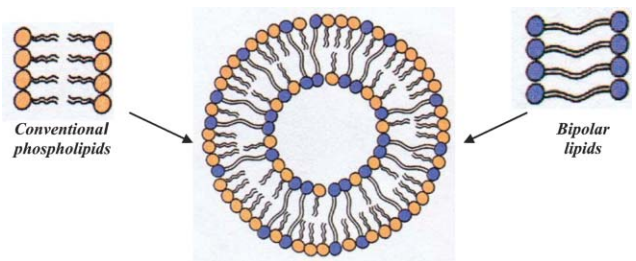


Fig. 1 Structure of an archaeosome.

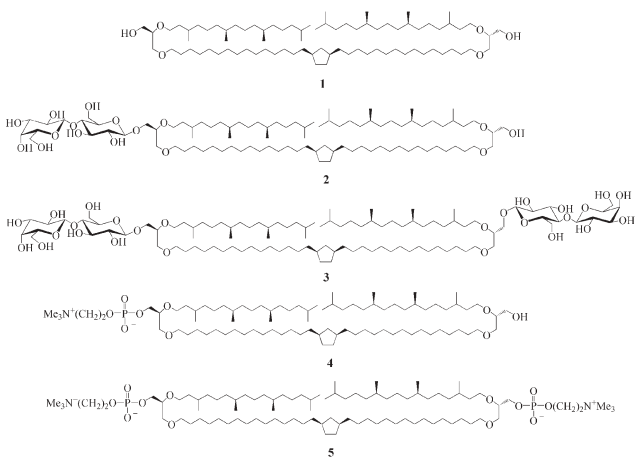
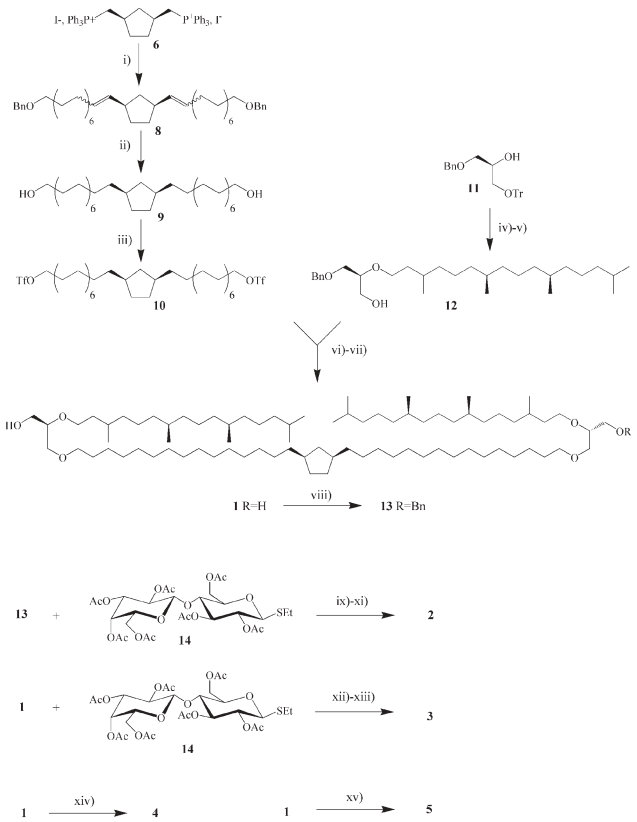


Fig. 2 Synthetic archaebacteria tetraether lipid analogues **1–5**.



Scheme 1 ***Reagents and conditions***: (i) *n*-BuLi, THF, 0 °C, then BnO–$(CH_2)_{13}$–CHO **7**, 63%; (ii) H_2, Pd/C (10%), EtOH, 93%; (iii) Tf_2O, 2,6-lutidine, CH_2Cl_2, 95%; (iv) phytanyl bromide, NaH, THF, 130 °C, 90%; (v) $FeCl_3$,$6H_2O$, CH_2Cl_2, 82%; (vi) **12**, KH, THF, 0 °C, then 10, 69%; (vii) H_2, Pd/C (10%), AcOEt, 80%; (viii) NaH, 15-5 crown-ether, BnBr, THF, 50%; (ix) NIS, Et_3SiOTf, CH_2Cl_2, 0 °C, 60%; (x) H_2, Pd/C (10%), EtOH, 96%; (xi) CH_3ONa, CH_3OH, 60%; (xii) NIS, Et_3SiOTf, CH_2Cl_2, 0 °C, 46%; (xiii) CH_3ONa, CH_3OH, 88%; (xiv) a) 2-chloro-2-oxo-1,2,3-dioxaphospholane, benzene, Et_3N, rt, 36 h: (b) Me_3N, CH_3CN, 65 °C, 48 h, 46%; (xv) (a) 2-bromoethyl dichlorophosphate, CH_2Cl_2, Et_3N, rt, 3 d, and then H_2O: (b) Me_3N (in EtOH), $CHCl_3$/i-PrOH/H_2O (2.7/2.25/0.75, v/v/v), rt, 4 d, 45%. Bn = benzyl, Tr = trityl, NIS = *N*-iodosuccinimide.

with Tf_2O in the presence of the hindered base 2,6-lutidine efficiently furnished the corresponding ditriflate **10**. The preparation of the monophytanylated glycerol unit **12** was achieved *via* the reaction of the sodium salt of 1-*O*-benzyl-3-*O*-trityl-*sn*-glycerol **11**[5e] with phytanyl bromide. These synthetic routes were carried out on a scale such that multigram quantities of diol **1** were obtained.

At this stage, the next step involved the introduction of β-lactosyl or phosphocholine head groups at one or two terminal ends of the tetraether backbone. Mono- or diglycosylation of monobenzylated tetraether **13** and unprotected diol **1**, respectively, were performed stereospecifically from lactosyl thioglycoside **14**.[5e–f] Surprisingly, phosphorylation of diol **1** *via* the established 2-chloro-2-oxo-1,3,2-dioxaphospholane/trimethylamine route[6] only furnished the monophosphocholine **4** (49% yield) which was fully substantiated by NMR spectroscopy and high-resolution mass spectrometry. Finally, bisphosphorylation of **1** was best performed by melting a mixture of the diol and excess (2-bromoethyl)phosphorodichloridate[5d] in CH_2Cl_2 followed by replacement of bromine with trimethylamine to afford the desired product **5** after Sephadex LH-20 and silica gel column chromatography in 45% yield for the two steps.

Archaeosomes were then prepared by hydrating the lipids in a 1X Krebs-Ringer bicarbonate (1X KBR) buffer, pH 7.4 containing 5(6)-carboxyfluorescein, CF (2.5%) under stirring for 12 h at rt. Multilamellar archaeosomes, so formed, were converted by pressure extrusion through filters of 400 nm and then 200 nm pore size using a Liposofast system to achieve a vesicle size range between 145 and 250 nm, measured by light scattering (3000 Zetasizer Malvern Instrument). Just before each experiment, vesicles were separated from nonencapsulated CF by using Sephadex G75 column chromatography. Various formulations based upon exclusively synthetic tetraethers or mixed Egg-PC-bipolar lipid **1–5** compositions were developed and named xLX lipid **1–5**, x representing the molar ratio of the synthetic tetraethers used. The exact composition of formulations was quantified by high performance thin layer chromatography (HPTLC)[7] on Silica gel 60 (Merck) using the Camag Linomat IV.

In vitro liposome stability was evaluated by following the leakage of the encapsulated aqueous dye 5(6)-carboxyfluorescein. The samples were placed at 37 °C under varying condition, were excited at 485 nm and emission was taken at 538 nm. The archaeosome stability in a 0.4% sodium cholate 1 X KBR buffer solution reflecting the bile salt-containing media was evaluated within 1 to 2 min of incubation. Increasing incorporation of neutral diol **1** or bislactosyl lipid **3** into Egg-PC liposomes lowered the leakage of the marker from 100% after 5 s with conventional liposomes to 60, 40 and 30% with 10 LX, 20 LX and 30 LX lipid **1** or **3** formulations, respectively. Conversely, phosphocholine lipids **4** and **5** did not exhibit any liposome stabilization when incorporated into bilayers or even in the absence of Egg-PC lipids. Exposure of liposome formulations to fetal bovine serum showed that the leakage of CF after 75 h was 70% for EPC vesicles and 80% or higher percentages for 10 LX, 20 LX and 30 LX lipid **1** archaeosomes, except those made with 100% bisPC lipid **5** and 40% bislactosylated lipid **3** which lost only 40% of the marker within the same period.

Archaeosome stability at low pH was determined *via* a modified procedure because the fluorescence of CF is pH sensitive: each vesicle preparation was exposed at 37 °C to a buffer at pH 2, and after 5 and 10 min of incubation the pH of the aqueous dispersions was quickly increased to 7.4 by adding a saline NaOH buffer at pH 11.9. After dialysis of the released CF, liposomes were lysed and the retention of encapsulated CF at pH 2 was then monitored by fluorescence measurements. Control experiments to assay marker release at pH 7.4 did not reveal a CF leakage at sufficient rates to influence significantly the values determined for the retention of encapsulated marker at pH 2. Liposomes made from neutral lipids **1** and **3** lost nearly all of the encapsulated marker within the first 5 min of incubation at pH 2 as for conventional Egg-PC formulation. A dramatic improvement in stability was noted with 100 LX lipid **5** archaeosomes which were quite stable at pH 2, leaking less than 10 and 30% of CF after 5 and 10 min of incubation, respectively. This formulation is comparatively as stable as archaeosomes made with natural *Thermoplasma acidophilium* tetraether lipids which lost 20 to 30% of the marker in the same conditions.[3,4a] The different behaviour observed for PC tetraether-containing liposomes may be associated with a higher

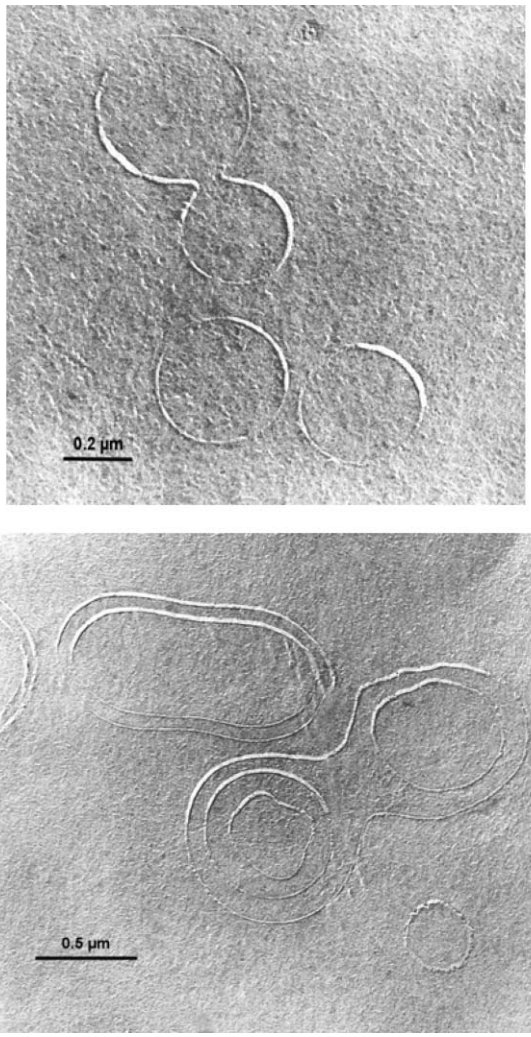


Fig. 3 FFEM of bisPC lipid **5**: cross-fracture behaviour of unilamellar and oligolamellar vesicles.

chemical and/or physical stability towards acidification, probably inhibiting structural changes in lipid layers and aggregation processes.[8] The membrane-spanning organization of bisPC lipid **5** was shown by freeze-fracture electron microscopy (FFEM) from the observation of circles (Fig. 3a,b) instead of convex and concave fracture faces visualized in the case of bilayered systems. The fractured samples were etched before shadowing in order to visualize the propagation path much more clearly. No membrane splitting along the mid-planes of the membranes was found, and only fractures across the membranes were observed as in the case of natural tetraether lipids.[9]

The results reported here clearly reveal the importance of the tetraether lipid polar heads for *in vitro* archaeosome stability; neutral hydroxyl or lactosyl groups are efficient stabilizing agents in the presence of detergents; lactose and phosphatidylcholine moieties enhance stability towards serum lipoproteins; and finally phosphocholine residues introduced at opposite ends of the tetraether lipid backbone exhibit vesicle membrane integrity at low pH. Therefore it is likely that much of the ability of archaebacterial bipolar lipids to enhance stability properties of liposomes can be attributed not only to the orientation of tetraethers which span the lipid layer but also to their bipolar headgroups. It is also noteworthy that the stabilization effect caused by the synthetic tetraether incorporation into Egg-PC liposomes compares favourably to the stability of archaeosomes made with natural thermoacidophilic tetraether lipids. This study should contribute to helping select archaeosome formulations and, in particular, tetraether polar lipids for further oral delivery development.

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