Archaeosomes based on synthetic tetraether-like lipids as novel versatile gene delivery systems†

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Novel cationic liposomes, termed "archaeosomes", based on mixtures of neutral/cationic bilayer-forming lipids and archaeobacterial synthetic tetraether-type bipolar lipids show efficient *in vitro* gene transfection properties and represent a new approach for modulating the lipidic membrane fluidity of the complexes they form with DNA.

Since the discovery of lipofection,[1] cationic lipid–DNA complexes (lipoplexes) have been widely used for gene transfection *in vitro* and *in vivo*.[2] A typical cationic lipid (CL) is basically composed of a cationic headgroup and a lipophilic moiety connected *via* a linker. Current cationic lipids include double-chained amphiphiles and cholesterol derivatives which are generally formulated as cationic liposomes characterized by a bilayered membrane (Fig. 1a).[3] Numerous combinations of DNA with suspensions of vesicles composed of cationic lipids and neutral "helper" lipids such as DOPE (dioleoylphosphatidylethanolamine) or cholesterol, were developed for transfection[3] but their use in clinical gene therapy trials was relatively unsatisfactory.[4] This was in particular due to the inability of conventional bilayered lipidic systems to meet two conflicting requirements: (1) ensure a sufficient rigidity of the lipoplexes *in vivo* and (2) maintain the membrane fluidity required at various cellular stages of the transfection process.[5] Taking into account the importance of membrane stability, we have developed in the present work liposomes, termed "archaeosomes", characterized by membranes which don't exhibit a bilayer structure as in conventional liposomes but a monolayer (or a combination of monolayer and bilayer) arrangement (Fig. 1).[6] The monolayer membrane organization results here from the presence of archaeobacterial-like bipolar lipids that span the membrane from polar headgroups on one side to headgroups on the other side. In contrast to the double-layer cell membranes of eukaryotes, the single-layer membranes formed by these unusual lipids have a high degree of physical rigidity and chemical/enzymatic stability.[7] Fine-tuning of the membrane rigidity *via* modulation of its lipid composition (bilayer/monolayer-forming lipids) as in natural archaebacteria[7] represents a new approach in nonviral gene delivery.

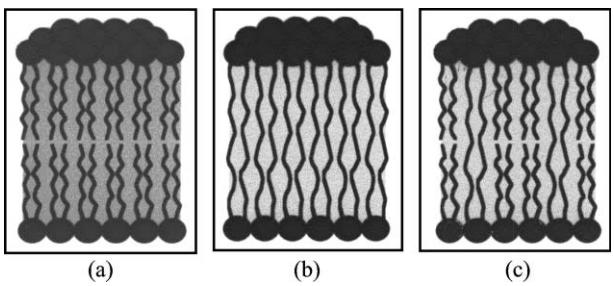

Fig. 1 Membrane models of conventional bilayered liposomes (a) and monolayered (b) or mixed bilayered–monolayered (c) archaeosomes.

The archaeosomes studied in this work are based on a set of synthetic lipids that include the cationic tetraether lipid GRcat and the neutral tetraether GR (Fig. 2) whose structures are typical of natural archaeobacterial membrane lipids. These artificial bipolar lipids are actually characterized by 1) a hemicyclic lipid core composed of two phytanyl chains and a 31 atom-long bridging chain containing a cyclopentane ring (supposed to increase the lipid dispersion into water)[8] linked together by 2 glycerol moieties to which they are attached *via* ether bonds and 2) two neutral hydroxyl groups (GR) or two positive quaternary ammonium groups provided by glycine betaïne residues linked, *via* an amide bond, to each end of the backbone (GRcat). The presence of oxygen atoms within the spanning chain of lipids GRcat and GR results from our aim to simplify the synthetic pathway initially

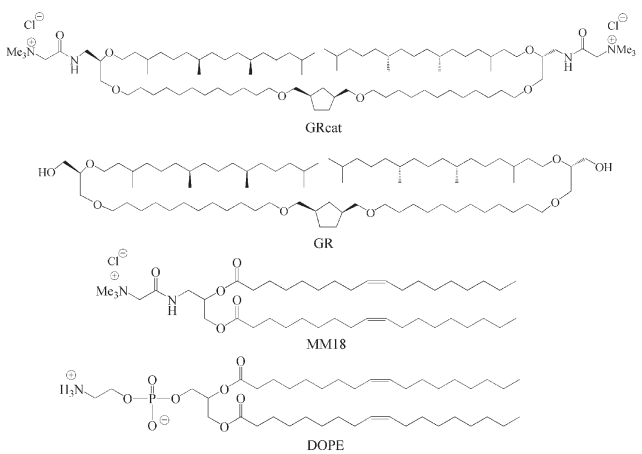

Fig. 2 Structures of the synthetic dicationic tetraether lipid GRcat, neutral tetraether lipid GR, monocationic double-chained lipid MM18[10] and standard helper lipid DOPE.

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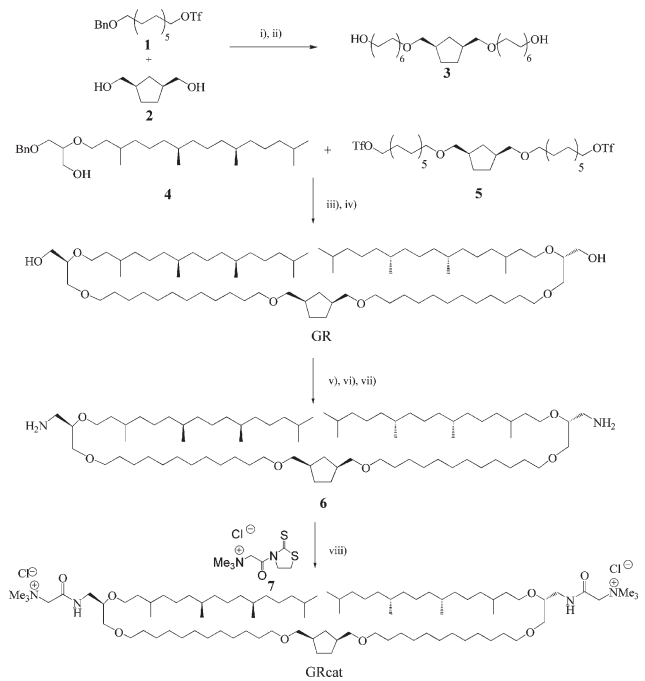
developed for tetraethers including exclusively methylene groups within the bridging chain.[9]

Thus, we herein describe the synthesis of dicationic tetraether GRcat and neutral tetraether GR and report the transfection activity of the complexes (termed "archaeoplexes") they form with plasmid DNA when used either alone or in combination with 1) the neutral colipid DOPE in the case of the cationic lipid GRcat or 2) the typical bilayer-forming monocationic aliphatic lipid MM18[10] (Fig. 2) in the case of the neutral tetraether GR. The aim of our approach was therefore to get a first insight into the transfection properties of these highly innovative formulations and thereby ascertain the potential of such monolayer-forming reagents as cationic lipid GRcat or co-lipid GR.

First, synthesis of bipolar cationic tetraether GRcat involved alkylation of the (*S*)-glycerol derivative **4**[9] containing a phytanyl chain with ditriflate **5** and subsequent hydrogenolysis of the benzyloxy groups to provide the corresponding neutral tetraether GR (Scheme 1). A double *O*-alkylation of 12-*O*-benzyl-dodecane-1-triflate **1** with *cis*-1,3-bis(hydroxymethyl)cyclopentane **2** using Proton-Sponge® as the base allowed an efficient and rapid generation of the corresponding bridging chain **3** (75% yield). Finally, introduction of the cationic glycine betaïne head groups was performed by simultaneous *N*-acylation of diamine **6** resulting from azidation of diol GR and subsequent hydrogenolysis, with the *N*-acyl thiazolidine-2-thione derivative **7**[10] of glycine betaïne (for detailed spectral data, see ESI).

Liposomes and archaeosomes were prepared by hydrating the lipids with sterilized water during 12 h at 4 °C followed by vortexing for 2 min and sonication for 10 min. Thus, various



Scheme 1 *Reagents and conditions*: (i) Proton Sponge, CH_2Cl_2, reflux, 75%; (ii) H_2, Pd/C, cyclohexane, 90%; (iii) KH, THF, 0 °C, 50–55%; (iv) H_2, Pd/C, AcOEt, quantitative; (v) MsCl, NEt_3, CH_2Cl_2, 90–95%; (vi) NaN_3, TBABr, DMF, reflux, 85–90%; (vii) Pd/C, H_2, THF–EtOH (1/1 v/v), 90–95%; (viii) **7**, NEt_3, DMF, 75–80%. TBABr: tetrabutylammonium bromide; MsCl: methanesulfonyl chloride.

formulations containing exclusively a cationic lipid (GRcat or MM18) or a combination of a co-lipid (DOPE or GR) and an aforementioned cationic lipid were prepared, the quantity of co-lipid ranging from 0% to 15% (w/w). Low percentages of co-lipids were used preferentially in the liposomal formulations since addition of large amounts of a co-lipid like DOPE may result in some cytotoxicity.[10b] Liposome and archaeosome sizes were determined as mean diameter using a 3000 Zetasizer Malvern Instrument. Clearly, MM18 liposomes were smaller (80 nm) than the GRcat archaeosomes (240 nm), whereas the sizes of co-lipid containing vesicles ranged from 180 nm (MM18/DOPE 95/5) to 470 nm (GRcat/DOPE 95/5) with a polydispersity index between 0.5 and 0.9 (Table 1). Thus, archaeolipids led generally to larger vesicles than conventional lipids. It is noteworthy that the incorporation of co-lipids also resulted in a significant size increase. For both MM18-based and GRcat-based vesicles, addition of a co-lipid resulted in a significant increase in their surface charge, the zeta potential of GRcat vesicles being however generally more positive than that of MM18 liposomes (Table 1). The precise composition of the formulations was assessed by high performance thin layer chromatography (HPTLC) on Silica gel 60 (Merck) using the Camag ATS4 for automatic sample application. After plate development in an adapted mobile phase, lipids were quantified by fluorescence at 366 nm (primuline derivatization of plates) using the Camag scanner 3. The results showed that the observed archaeosome lipid composition was only slightly different from its expected theoretical composition (see ESI).

Cationic lipid–DNA complexes were prepared by mixing the appropriate amount of aqueous liposome or archaeosome suspensions with plasmid DNA (pTG11033, 9.7 kb) expressing the luciferase reporter gene and analyzed after 30 min of incubation at room temperature. Practically, to a fixed amount of DNA (4 μg), we added increasing amounts of cationic MM18, MM18/GR, MM18/DOPE, GRCat/DOPE or GRCat formulations to obtain lipoplexes with increasing (±) mean theoretical charge ratios R. We next measured the size and the zeta potential of the resulting complexes in the culture medium (OptiMem). Schematically, the size of the lipoplexes was not dramatically modified in comparison with the corresponding unreacted liposomes and, as expected, the zeta potential values varied in agreement with the charge ratios of the complex (data not shown). In addition to these physicochemical studies, we also performed *in vitro* transfection experiments to get some insight into the potential of archaeosome-containing formulations for gene transfection. Practically, transfection experiments were carried out with the human alveolar epithelial cell line A549, a model

Table 1 Mean diameters and zeta potentials of liposomes and archaeosomes measured by using a 3000 Zetasizer Malvern Instrument at 20 °C after an appropriate dilution of the formulations

	Average diameter (nm)	Polydispersity index	Zeta potential (mV)
MM18	80	0.5	16
MM18/DOPE 5%	180	0.6	45
MM18/DOPE 15%	300	0.9	45
MM18/GR 5%	250	0.7	43
MM18/GR 15%	190	0.6	58
GRcat	240	0.4	42
GRcat/DOPE 5%	470	0.7	61
GRcat/DOPE 15%	310	0.5	60

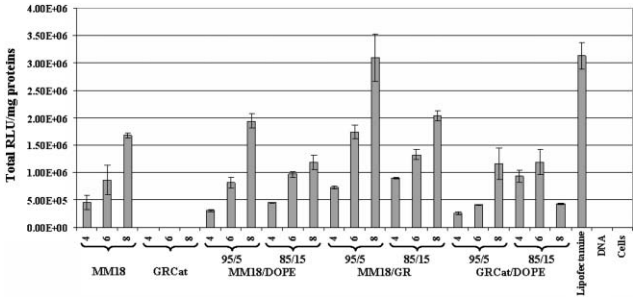


Fig. 3 Transfection efficiency for formulations containing 0, 5 or 15 wt% of neutral co-lipid on A549 cells for 4 μg DNA delivered (charge ratio R = 4 to 8).

system for lung gene therapy.[11] The commercially available cationic lipid Lipofectamine was used as positive control whereas unreacted ("naked") plasmid DNA and untreated cells were used as negative controls. Expression of the transfected luciferase reporter gene was quantified by luminometry (MLX Dynex), the results being expressed as Relative Light Units (RLU) per mg of total protein (Fig. 3).

The results showed that archaeobacterial-like lipids can be used for lipofection either as co-lipids or cationic lipids, the transfection activity being dependent on the nature of the lipids and archaeolipids present in the formulation, the cationic lipid/neutral co-lipid (w/w) ratio, and the (±) charge ratio, clearly positive charge ratios being assumed to allow efficient interaction of the complexes with negative cell surface residues (Fig. 3). First, liposomal formulations of MM18 with small amounts of the neutral tetraether GR were more efficient than pure MM18 liposomes and MM18 formulations containing the classical co-lipid DOPE. The MM18/GR 95/5 (w/w) formulation was actually as efficient as the widely used reagent Lipofectamine (at a +8 charge ratio *i.e.* at a high amount of cationic lipids). These data demonstrate that the neutral lipid GR can be used as a co-lipid and it represents the most favourable helper lipid in these *in vitro* transfection experiments. Here, although the addition of DOPE or GR to MM18 led to similar increases in zeta potential values and consequently possibly of transfection activity, the fact that the highest transfection activity was observed with the GR-containing formulations suggests that addition of GR may provide MM18-based complexes with additional properties beneficial for transfection. These results were quite unexpected since this bipolar lipid should have a lower tendency to form an inverted hexagonal phase (H_{II}) at acidic pH, which is usually indicative of an efficient escape from lysosomal degradation and cytoplasmatic release of DNA.[12]

Next, as also shown in Fig. 3, the cationic tetraether GRcat could mediate significant gene transfection when used as a cationic liposome formulation with DOPE. GRcat was, however, ineffective alone, although it could efficiently bind plasmid DNA as shown by gel retardation (see ESI) and although GRcat archaeoplexes exhibited a clearly positive zeta potential (Table 1). Interestingly, these data suggest that the addition of DOPE provides the GRcat archaeosomes with physicochemical properties (such as membrane fluidity) required for efficient gene transfection. Taken altogether, these results strongly suggest that an optimal lipofection activity may require the reaching of a compromise between fluidity and stability of the lipoplex membranes and that archaeobacterial-like lipids may constitute interesting tools for reaching such a compromise. Accordingly, we are at present studying GRcat formulations containing a variety of archaeobacterial-like neutral co-lipids in order to assess more precisely the transfection activity of archaeosomes characterized by highly rigid membranes. More generally, a better understanding of the mechanisms underlying the transfection activity of such systems is clearly necessary and will probably require sophisticated physicochemical experiments as well as additional biological investigations, such as studies on the interaction between archaeoplexes and the cellular machinery.

In summary, we have developed archaeobacterial-like lipids which can be used as cationic lipids or co-lipids for *in vitro* gene transfection. Our work demonstrates the potential of combining conventional bilayer-forming lipids with monolayer-forming lipids as a new strategy to modulate the membrane properties of CL–DNA complexes. In ongoing studies, we are at present evaluating the potential of our novel archaeoplexes for *in vivo* gene transfection into the airway epithelium by nasal instillation or aerosolization, with a view to lung-directed gene therapy for cystic fibrosis.

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