Stability of Liposomes Prepared from Archaeobacterical Lipids and Phosphatidylcholine Mixtures

G. D. Sprott
Institute for Biological Sciences, National Research Council of Canada

C. J. Dicaire
Institute for Biological Sciences, National Research Council of Canada

L. P. Fleming
Institute for Biological Sciences, National Research Council of Canada

G. B. Patel
Institute for Biological Sciences, National Research Council of Canada

Follow this and additional works at: https://digitalcommons.usu.edu/cellsandmaterials

Part of the Biomedical Engineering and Bioengineering Commons

Recommended Citation

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Cells and Materials by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.
STABILITY OF LIPOSOMES PREPARED FROM ARCHAEOBACTERIAL LIPIDS AND PHOSPHATIDYLCHOLINE MIXTURES

G.D. Sprott*, C.J. Dicaire, L.P. Fleming and G.B. Patel

Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada

(Received for publication March 27, 1996 and in revised form October 1, 1996)

Abstract

In vitro stabilities of liposomes prepared from the total polar lipids (TPL) of various species of Archaeobacteria were compared with those made from egg phosphatidylcholine (EPC) and from mixtures of TPL/EPC. Stability was monitored by measuring the extent of leakage of entrapped dyes. Liposomes prepared from exclusively archaeobacterial lipids (coined archaeosomes), particularly those containing the bilayer spanning tetraether lipids, were generally stable to conditions such as storage at 37°C for greater than 7 days, exposure to phospholipase A₂, incubation with serum, and, to some extent, exposure to bile salts. In contrast, EPC liposomes were generally unstable to these conditions, releasing, for example, 100% of the entrapped dye within 1.5 h of incubation at 37°C. Mixing EPC with archaeobacterial TPL modified stability of the liposomes to varying degrees, depending on the types of the archaeobacterial lipids, the mixing ratio, and the stress factor being tested. For some applications, incorporation of an ester lipid into the liposome layer may be used to modulate the stability of archaeosomes, and decrease the cost of production without necessarily compromising utility.

Key Words: Archaeosomes, stability, autoclaving, serum, bile salts, phospholipase, egg phosphatidylcholine, Archaeobacteria.

Introduction

Archaeobacteria ("Archaea") are microorganisms phylogenetically distinct from eukaryotic cells and true bacteria (Boone et al., 1993). These organisms often flourish in harsh environmental conditions, such as extremes of pH from 2 to 10.5, high temperatures sometimes exceeding 100°C, saturated salt, and sulfurous and/or methanogenic (extremely anaerobic) conditions. A feature distinguishing Archaeobacteria as a third major line of descent (Woese et al., 1978), and germane here, is the structural properties of the polar lipids which comprise most of the total lipid portion of their cytoplasmic membranes.

The head groups of archaeobacterial polar lipids can be removed by chemical methods and separated by solvent partitioning from the lipid moiety (core lipid). Pioneering structural studies on the core lipid from Halobacterium cutirubrum eventually led to its identification as a diether lipid and to its complete structural elucidation. The diether was characterized by constant chain lengths of 20 carbon atoms to form regularly branched and fully saturated phytanyl chains, and chain linkage via ether bonds to a glycerol backbone with sn-2,3 stereochemistry, as opposed to the sn-1,2 stereochemistry found in ester-linked phospholipids typical of other organisms (Kates, 1978). Later studies expanded on this discovery to show the presence in archaeobacterial membrane lipids of diether analogs including macrocyclic diethers (Comita et al., 1984), hydroxydiethers (Ferrante et al., 1988; Sprott et al., 1990), tetraethers (Langworthy, 1978), and tetraethers containing cyclopentane rings (De Rosa and Gambacorta, 1988) (Fig. 1). Structural studies on archaeobacterial ether lipids is incomplete, but sufficient is known to document the occurrence of many different combinations of core lipids and head groups within the Archaeobacteria, and the synthesis by each organism of a series of ether lipids often unique to the genus level of classification (Kates, 1993; Sprott, 1992). In Table 1, the reported data illustrate the amounts of the various core lipids present in the archaeobacteria relevant to this study.

Liposomes are artificial, spherical, closed vesicles consisting of one or more phospholipid bilayers, each
bilayer being separated from the adjacent one by aqueous spaces. Liposomes may be unilamellar (one bilayer) or multilamellar. Water soluble compounds can be encapsulated in the aqueous compartments enclosed by entrapped/associated within the hydrophobic region of the bilayer. Liposomes have been used extensively as biological membrane systems for the study of transmembrane transport, lipid bilayer permeability, membrane fusion, lipid-protein interaction, etc. Recently, there has been considerable interest in the use of liposomes for biotechnological and other applications, such as the delivery of drugs/pharmaceuticals/antigens, gene therapy, cancer imaging, and diagnostics (Alving, 1995; Gregoriadis, 1985; Kersten and Crommelin, 1995; New, 1990; Sato and Sunamoto, 1992). Almost all of the liposome research to-date has been conducted with conventional phospholipids. However, the susceptibility of their ester bonds to enzymatic and chemical hydrolysis, and of the unsaturated fatty acyl side chains to atmospheric oxidation, result in poor stability of these liposomes. Cholesterol and/or special storage conditions may alleviate some of the stability problems; however, cholesterol itself is unsaturated and consequently is subject to oxidation.

It was initially not obvious that liposomes could be made from the total polar lipids extracted from an archaeobacterium. First, in the case of the membrane polar lipids extracted from Sulfolobus sulfataricus and S. acidocaldarius, liposomes did not form and this was explained by determining that the packing parameter was greater than one, which would allow insufficient curvature of the membrane for formation of closed vesicles (Cavagnetto et al., 1992). Liposomes could be formed from a polar lipid fraction isolated from Sulfolobus only after either the addition of at least 25 mol% egg phosphatidylcholine (EPC) (Lelkes et al., 1983), or after extensive purification to obtain a polar lipid sub-fraction called ‘E’ which accounted for 4% of the crude lipids (Lo and Chang, 1990). Second, the total polar lipids of Halobacterium cutirubrum are highly negatively charged and formed a stable bilayer arrangement only at salt concentrations much lower than is optimal for growth. Indeed, stability of the membrane in high salt (4M) required a strong interaction between the lipids and the cytoplasmic membrane proteins (Chen et al., 1974). Further studies with binary mixtures of lipids purified from H. cutirubrum established that the glycolipid sulfate tended to form bilayers, whereas the major phosphatidylglyceromethylphosphate lipid (Kates et al., 1993) formed a non-bilayer arrangement in the presence of salt (Quinn et al., 1986). Finally, the main polar lipid (MPL) sub-fraction isolated from the total polar lipids of Thermoplasma acidophilum was found to form only large liposomes with an average diameter of 600 ± 40 nm, by detergent dialysis or sonication methods (Ring et al., 1986).

The complete structure of the MPL lipid of Thermoplasmatacidaeum has been elucidated recently (Swain et al., 1997). It has been known for some time to be a tetraether with sn-3-glycerophosphate as one head group and an unknown hexose as the other (Langworthy et al., 1982). A detailed analysis revealed that the sugar moiety is a rare β-L-gulopyranoside, and not glucose as widely assumed, and that the MPL lipid is a series of lipids differing only in the number of cyclopentane rings, with one ring per chain dominant (Swain et al., 1997).

Because of the added cost of purifying the lipids from biological extracts, and with industrial applications in mind, we explored whether the total polar lipids from various archaeobacteria could readily form liposomes without the need to sub-fractionate. Indeed, liposomes, which we describe here as archaeosomes, could be formed as multilamellar structures from most archaeobacterial lipid extracts by simple hydration, or as unilamellar vesicles by various techniques such as detergent dialysis (Choquet et al., 1992) or pressure extrusion (Choquet et al., 1993). Also, these liposomes were comparatively more stable to various physical and chemical challenges than conventional lipid liposomes (Choquet et al., 1993). Recent studies with Sulfolobus sulfataricus have shown further that liposomes can be formed by sonication at 60°C from a polar lipid extract fraction called ‘PLE’ (Relini et al., 1994), apparently equivalent to total polar lipids.

Diether analogs of phosphatidylcholine have not been found in archaeobacteria, but can be synthesized chemically. Liposomes prepared by sonication of this synthetic lipid were sufficiently stable to allow the incorporation of a 50% molar ratio of a phosphatidylethanolamine lipid analog designed to bind large amounts of a protein antigen to the liposome surface (Tomoka et al., 1994).

Some archaeobacterial polar lipids have been shown to form stable common phases with bilayer forming ester-phospholipids (Blocher et al., 1985). For example, the MPL lipid sub-fraction from the total polar lipids of T. acidophilum also forms liposomes when mixed with phosphatidylinositol or phosphatidylcholine (Ring et al., 1986). On the contrary, liposomes from lipids of Sulfolobus species, total polar or sub-fractions thereof, could be formed only if mixed at specific molar ratios with conventional phospholipids such as EPC or dipalmitoylphosphatidylcholine (Lelkes et al., 1983, Glozzi et al., 1993). The structural features of the lipids of Sulfolobus are unique, consisting primarily of tetraether and nonitol-tetraether lipids with various
numbers of cyclopentane rings (Fig. 1), and a variety of
different head groups (Gulik et al., 1988). Data on the
stability of liposomes from archaeobacterial polar
lipids/ester lipids mixtures are limited to the use of polar
lipid sub-fractions in these mixtures, and concentrate on
the effect of temperature (Lelkes et al., 1983, Ring et al.,
1986, Gliozi et al., 1993). In general, increasing
the proportion of ester lipids in these mixtures increased
the permeability (i.e., decreased stability) of the result­
lant liposomes.

The present study was designed to evaluate whether
the stability properties of archaeosomes, prepared from
the total polar lipids of various methanogenic archaeo-
bacteria and one thermoacidophile, could be altered in a
predictable manner by the inclusion of various propor­
tions of a bilayer-forming ester lipid. Exposure was to
a variety of biologically relevant conditions including
body temperature, phospholipase and serum. We also
explore the stability properties of archaeosomes exposed to bile
salts.

Materials and Methods

Materials

Egg phosphatidylcholine, phospholipase A2 (from
bee venum), calcein, 5(6)-carboxyfluorescein (CF), and
bile salts were supplied by Sigma-Aldrich Canada Ltd.,
Mississauga, Ont. Fetal bovine serum was purchased
from Gibco, Burlington, Ont. Polycarbonate membranes
for liposome formation were supplied by Avestin,
Ottawa, Ont.

Archaeobacteria

The cultures used were Methanosarcina mazei S-6
(Deutsche Sammlung von Mikroorganismen und Zell-
kulturen [DSM] 2053), Methanospirillum hungatæ GP1
(DSM 1101), Methanobrevibacter smithii (DSM 2375),
Methanococcus jannaschii JAL-1 (DSM 2661),
Methanobacterium espanolae GP9 (DSM 5982),
Methanosphaera stadtmannae MCB-3 (DSM 3091), and
Thermoplasma acidophilum 122-1B3 (American Type
Culture Collection 27658). Cells were grown to the late
exponential growth phase in a 75-L Chemap fermentor
(Choquet et al., 1994) and were harvested using a
Pellicon cell harvester. Cell pastes were frozen at -
20°C prior to lipid extractions.

Total polar lipids (TPL)

Total lipid extract (TLE) was extracted from frozen-
thawed biomass by the method of Bligh and Dyer
(1959), as described in detail before (Sprott et al.,
1995). Neutral lipids were removed from the TLE by
precipitating the total polar lipids (TPL) thrice with cold
acetone (Sprott et al., 1995). The TPL ether lipids
dissolved in CHCl3:CH3OH (2:1, v/v) were stored at
ambient temperature under an atmosphere of air.

Liposome formation

About 20 mg of archaeobacterial TPL, EPC, or
mixtures thereof (wt/wt), dissolved in CHCl3:CH3OH
(2:1, v/v) were dried in a glass scintillation vial with a
stream of N2, followed by a 1 hour period (in vacuo) in
a lyophilizer. Five-3 mm diameter glass beads (CHCl3-
washed) were added along with 1.0 ml of aqueous
buffer. Multilamellar liposomes formed during a 16 h
incubation at 35°C (150 rpm, model G24 Environmental
Incubator Shaker, New Brunswick Scientific Co.
Inc., NJ, USA). Unilamellar liposomes were formed with a
LiposoFast Apparatus (Avestin, Ottawa, Ont.) by
pressure extruding the multilamellar liposomes through
a series of polycarbonate membrane filters beginning
with 400 nm pore size, followed by 200 nm, and finally
100 nm.

CF loaded liposomes were prepared by hydrating and
extruding the lipids in 10 mM potassium phosphate
buffer (pH 7.1) containing 160 mM NaCl (PBS) and 100
mM CF. Encapsulation of the osmotically active, self-
quenching fluorescent dye, calcein, was done by hydrat­
ing and extruding the lipids in water containing 175 mM
calcein (pH 7.1) (Allen and Cleland, 1980).

Unentrapped CF, or calcein, was removed from the
liposome suspension by passage through Sephadex G-50
hydrated with PBS, using the microcolumn centrifuga­
tion method described by New (1990).

Dynamic light scattering

The mean diameters of liposome suspensions were
determined using a NICOMP model 370 particle sizer
equipped with a 5 mwatt helium-neon laser source
(Nicomp, Santa Barbara, CA).

Stability tests

Reaction mixtures in glass screw cap vials consisted of
10 µl of concentrated liposomes (about 0.1 mg dry
weight) diluted into 740 µl of diluent. Diluent was
either PBS, or 100% fetal bovine serum, or in the case of
phospholipase activity, 50 mM Tris-HCl buffer (pH
8.9) containing 160 mM NaCl and 5 mM CaCl2. Stability to phospholipase activity was determined using
calcein-liposomes and 100 Units of phospholipase A2 per
reaction.

To evaluate the stability of these liposomes in bile
salts, calcein-liposomes were diluted into a solution of
six bile salts prepared to simulate human intestinal bile
(Hofmann, 1963), as detailed below. Solutions, 20 mM
each in PBS at pH 6.2, of sodium glycocholate, sodium
glycochenodeoxycholate, sodium glycodeoxycholate,
sodium taurocholate, sodium taurochenodeoxycholate,
and sodium taurodeoxycholate were mixed 30:30:15:10:
10:5 (v/v/v), respectively. This bile salts solution was

Stability of archaeosomes
G.D. Sprott et al.

Figure 1. Predominant core lipids of Archaeobacteria. Diether and tetraether lipids are often referred to as archaeol and caldarchaeol, respectively (Nishihara et al., 1987). In the case of core lipids X, X₁, and X₂ are protons. For polar lipids, X₁, X₂, and X₃ represent polar head groups which can vary widely among archaeobacteria, including sugars, phosphopolyols, phosphoamino groups, or phosphoserine groups.

mixed with PBS 1:1 at pH 6.2 to obtain a final bile salts concentration of 10 mM, and was used as the liposome diluent.

Unless otherwise indicated, all incubations were carried out at body temperature (37°C). As an indication of liposome stability, retention of encapsulated CF or calcein from the liposomes was monitored by fluorescence measurements with a spectrofluorometer (Model Mark I, Farrand Optical Co., Inc., NY, USA) set at excitation and emission wavelengths of 470 nm and 520 nm, respectively (Fugman et al., 1984). To determine the extent of dye retention the fluorescence of an aliquot of the reaction mixture was measured (amount of leakage), and then re-measured after adding 1% Triton X-100 to cause lysis of the liposomes. In all cases, liposome controls were assayed at time zero to correct the data for any small fluorescence readings found prior to initiating the incubations.

Results

Stability upon autoclaving

The relative stabilities of various archaeosomes to sterilization by autoclaving (121°C at 15 p.s.i. for 15
Stability of archaeosomes


Min) under air was determined by Choquet *et al.* (1996), and is illustrated in Fig. 2. Leakage of the entrapped dye was minimal in the case of archaeosomes having a high content of tetraether polar lipids. However, exposure to 121°C caused pronounced leakage when tetraether lipids in the archaeosomes were less than about 25%. The variability observed among repeated experiments was pronounced only for the archaeosomes exhibiting the least stability, especially those prepared from the lipids from *Methanosphaera stadtmanae* which have low amounts of tetraether lipids. Light scattering data indicated no aggregation or significant changes in vesicle size following autoclaving of archaeosomes (Choquet *et al.*, 1996). Indeed, aggregation, or fusion, of these archaeosomes seemed not to occur after 10 to 18 months of storage at 4°C or 25°C, and at different pH values (Choquet *et al.*, 1994). The TPL lipids of the archaeobacteria which generated the most stable archaeosomes were chosen to mix with egg phosphatidylcholine (EPC) to further investigate liposome stability.

Stability upon storage at 37°C

The TPL lipids of *T. acidophilum*, *M. hungatei*, and *M. espanolae* could be mixed with egg PC in wt/wt ratios of 1:1 and 1:3 to form liposomes. The encapsulation of marker dyes such as CF or calcein, indicated that closed vesicles were obtained following pressure extrusion of the lipid mixtures. In contrast to the unstable...
Table 1. Distribution and abundance (%) of the main core lipids found in selected *Archaeobacteria*.

<table>
<thead>
<tr>
<th>Archaeobacteria</th>
<th>Tetraether</th>
<th>Diether</th>
<th>Hydroxydiether</th>
<th>Macrocyclic-diether</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanosarcina mazei</td>
<td></td>
<td>43</td>
<td>57 (sn-2)</td>
<td>-</td>
<td>Sprott <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>Halobacterium cutirubrum</td>
<td></td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>Kates, 1993</td>
</tr>
<tr>
<td>Methanosphaera stadtmanae</td>
<td>13</td>
<td>79</td>
<td>8</td>
<td>-</td>
<td>Choquet <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>Methanobrevibacter smithii</td>
<td>23</td>
<td>77</td>
<td>-</td>
<td>-</td>
<td>Choquet <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>Methanococcus jannaschii (65°C)</td>
<td>42</td>
<td>15</td>
<td>-</td>
<td>43</td>
<td>Sprott <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Methanospirillum hungatei</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>Sprott <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>Methanobacterium espanolae</td>
<td>65</td>
<td>35</td>
<td>-</td>
<td>-</td>
<td>Choquet <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Thermoplasma acidophilum</td>
<td>90¹</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>Langworthy, 1978</td>
</tr>
<tr>
<td>Sulfolobus acidocaldarius</td>
<td>90¹,²</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>De Rosa and Gambacorta, 1988</td>
</tr>
</tbody>
</table>

¹May contain cyclopentane rings. ²Nonitol Caldarchaeol plus Caldarchaeol.

EPC liposomes which lost all the encapsulated dye within 24 h incubation at 37°C, the archaeosomes (100 % TPL) were stable and retained the dye over the one week incubation period (Fig. 3). Liposomes prepared from TPL/EPC mixtures had intermediate stabilities compared to those made with TPL or EPC on its own. Increasing the proportion of EPC resulted in greater leakiness, but those prepared with mixtures containing *T. acidophilum* TPL were more stable than those containing TPL from the other archaeobacteria.

**Stability to phospholipase at alkaline pH**

The archaeosomes were stable during the four hours exposure to pH 8.9 and the action of phospholipase A₂, except for some pH-related loss of calcein from *M. smithii* archaeosomes (Fig. 4). Ester lipid liposomes were very sensitive to attack by the phospholipase, leaking 100% of encapsulated calcein within an hour. The EPC liposomes were also unstable at the alkaline pH of 8.9, where leakage of over 60% of the calcein occurred in four hours. Supplementing EPC with 25 % (wt/wt) TPL resulted in liposomes that were still sensi-
Stability of archaeosomes

Figure 4. Effect of phospholipase A₂ on the leakage of calcein from liposomes prepared from archaeobacterial lipids mixed with varying proportions of EPC. Archaeobacterial lipids consisted of TPL from T. acidophilum, M. hungatei, and M. smithii. Controls with no enzyme added (open bars) are shown, to account for leakage at the pH of the enzyme reaction (pH 8.9). Incubation was at 37°C. Liposome diameters were recorded as described in the legend of Fig. 3. Leakage data are the average of assays in duplicate.

tive to phospholipase activity, but at 50% (wt/wt) ratio the liposomes were of intermediate stability. Of the archaeobacterial lipids tested in mixtures with EPC, those using the TPL from M. smithii were the least stable.

Serum

Archaeosomes prepared from the TPL of T. acidophilum and M. hungatei were stable in fetal bovine serum for at least 5 h (Fig. 5). A trend to decreasing stabilities was evident as the proportion of TPL in the TPL/EPC mixtures was lowered. M. smithii archaeosomes were the least resistant to leakage in serum, and were not dramatically different than the EPC liposomes.

Bile salts

An artificial bile salts solution, consisting of a mixture of six bile salts prepared at 10 mM final concentration to approximate the proportions and concentration of bile salts in the duodenum, was used to determine the stabilities of archaeosomes. The assays were conducted at pH 6.2 (37°C) to simulate conditions in the duodenum. This harsh treatment resulted in a rapid and complete release of the encapsulated calcein from both, EPC liposomes, and archaeosomes prepared from M. mazei TPL (Fig. 6), the latter containing exclusively diether lipids. Although archaeosomes from TPL with about 40 - 50% tetraether lipids showed some resistance to leakage, only the T. acidophilum archaeosomes remained reasonably stable for at least 20 min under these conditions.

Discussion

Liposomes can be formed from the natural mix of the total polar lipids extracted from various archaeobacteria (Choquet et al., 1992). Early extensive studies on the formation of liposomes from the tetraether lipids of Sulfolobus species had shown the opposite, that the packing order of the total polar lipids from Sulfolobus was inconsistent with formation of closed vesicles (Cavegnetto et al., 1992). However, liposome formation could be achieved either through extensive purification to obtain a liposome forming subfraction P2 (Lo and Chang, 1990), by mixing at least 25 mol% of EPC with the lipids (Lelkes et al., 1983), or, as recently discovered, by extensive sonication at 60°C (Relini et al., 1994). In the latter case, large lipid aggregates formed in addition to liposomes of about 90 nm diameter, indicating a need to determine whether the vesicles contained the same mix of lipids as in the starting polar lipid extract PLE, which appears to be the total polar lipids.

For pharmaceutical applications, particularly for injectable liposome preparations, it is important to have sterile formulations. Filtration techniques are time consuming and may not remove virus contaminants. Heat sterilization may cause damaging artifacts such as liposome aggregation, leakage of water-soluble entrapped compounds (Kikuchi et al., 1991) and hydrolysis
of ester phospholipids (Zuidam et al., 1993). Our results show that archaeobacterial ether lipids can either be heat sterilized before entrapment of heat-sensitive drugs/compounds or thermostable compounds can be entrapped into archaeosomes before autoclaving, without causing heat-associated stability problems.

In liposomal applications where a faster release of encapsulated compounds is desirable, or where cost of production can be lowered without loss of essential properties, it may be desirable to prepare liposomes from mixtures consisting of archaeobacterial total polar lipids and ester lipids. Leakage rates of fluorescent dyes from liposomes indicated that incorporation of ether lipids from tetraether containing strains of archaeobacteria into EPC liposomes resulted in the formation of liposomes with improved stabilities upon storage at 37°C, exposure to alkaline pH, to phospholipase A2, or to serum. Conversely, dilution of the ether lipids with egg phosphatidylcholine resulted in decreasing the stability of the resultant liposomes. In contrast to our results, vesicles composed of mixtures of EPC/polar lipids extract of *S. solfataricus* (at 1:2 molar ratio) were more stable than liposomes made from the polar lipids alone (Fan et al., 1995).

Our data showing rates of leakage of internal contents from liposomes made from EPC/TPL mixtures argue against the possibility of having formed two different populations of liposomes, one composed largely

---

**Figure 5.** Effect of serum on the leakage of calcein from liposomes prepared from archaeobacterial lipids mixed with varying proportions of EPC. Archaeobacterial lipids consisted of TPL from *T. acidophilum*, *M. hungatei*, and *M. smithii*. Liposome diameters were recorded, as in Fig. 3 legend. Leakage data are shown as the mean and standard error of the means for two experiments, each performed in duplicate. Liposomes of each type, incubated in parallel at 37°C with no serum, lost less than 3% of internal calcein over 5 h.

---

**Figure 6.** Stability of archaeosomes and EPC liposomes exposed to an artificial bile salts solution (pH 6.2) at 37°C. Archaeosomes were prepared from TPL of the archaeobacteria shown above the respective data bars. Liposome diameters were recorded, as described in Fig. 3 legend.
of EPC and the other of archaeobacterial TPL. Such a possibility would not explain the observed prolonged life span of the liposome population as the content of archaeobacterial lipids increased. The miscibility of archaeobacterial ether lipids with ester lipids such as EPC as well as incorporation of both lipid types into the liposome membrane, have also been indicated by others (Lelkes et al., 1983; Ring et al., 1986; Blocher et al., 1985).

In general, the stability of archaeosomes correlated with the content of tetraether lipids, a conclusion consistent with results obtained comparing stability of archaeosomes exposed to sterilization temperature (Fig. 2; Choquet et al. 1996), or during long term storage (Choquet et al., 1994). This stability may be explained, in part, by the orientation of bipolar tetraethers which span the lipid layer in both natural membranes and liposomes (Beveridge et al., 1993; Gliozzi et al., 1993). Indeed, a tetraether lipid sub-fraction from the lipids of Sulfolobus acidocaldarius formed liposomes which were remarkably heat-stable (Chang, 1994). The total polar lipids from extreme halophiles Natronobacterium magadii and Halobacterium cutirubrum are exclusively diethers, and highly negatively charged (Kates, 1993). Archaeosomes could be formed from the TPL of these archaeobacteria, but were found to be leaky and dramatically so in the case of Natronobacterium magadii (Choquet et al., 1994).

In addition to stability properties, other factors must also be considered when choosing a lipid source for application, such as the ease of liposome formation. For example, TPL high in tetraether content is, in general, more difficult to hydrate and may require either more time and/or rigorous hydration conditions in order to avoid substantial losses of lipid material during archaeosome formation.

Liposomes having some degree of resistance against the harsh conditions of the gastrointestinal tract may have applications in the oral delivery of pharmaceuticals. Some success has been reported in enhancement of mucosal immune responses to antigens upon oral co-administration with cholera toxin or its B subunit coupled to ester lipid liposomes (Harokopakis et al., 1995). Archaeosomes are quite stable to storage at pH 3 (Choquet et al., 1994), but information was not available on the effect of bile salts. Stability against effects of an artificial bile salts solution was achieved in vitro, over a 20 min period, with archaeosomes prepared from TPL containing about 90% tetraether lipids. This contrasted sharply to the instabilities of EPC liposomes and archaeosomes made from TPL of M. mazei which contains only diether lipids. Liposomes prepared from mixtures of various ester lipids, with or without cholesterol, were highly unstable at 37°C in the presence of 10 mM bile salts (similar composition to that in the current study) at pH 6.2 - 7.1, with the exception of dipalmitoyl phosphatidylethanolamine (DPPE) and distearoyl phosphatidylcholine (Richards and Gardiner, 1978; Rowland and Woodley, 1980). However, the DPPE liposomes were unstable at low pH (Rowland and Woodley, 1980). In vivo tests are required to evaluate whether the enhanced stability properties of archaeosomes can be used to advantage to provide an effective oral delivery system.

The comparative stability of archaeosomes to heat sterilization, extremes of pH, serum, phospholipase attack, oxidation (Choquet et al., 1993, 1994, 1996), bile salts, and the modulation of the stability by incorporation of ester lipids such as egg phosphatidylcholine, provide an alternative to overcome the limitations of the use of ester lipids in liposomal applications. However, fundamental studies on the fate of archaeobacterial ether lipids and any potential toxicity need to be conducted using animal models to allow a closer evaluation of archaeosomes for in-vivo applications.

References


Ring K, Henkel B, Valenteijn A, Gutermann R (1986) Studies on the permeability and stability of lipo-
Stability of archaeosomes


Discussion with Reviewers

M. Kates: The stability of various archaeosomes to autoclaving at 121°C (Fig. 2) appears to be directly related to the proportion of tetraether lipids in the TPL samples. However, it may be argued that the tetraether lipid content is not the only variable in these systems; there are variations in the polar head groups from among the various archaea, and the Thermoplasma polar lipids contain tetraethers with cyclopentane rings; also M. jannaschii polar lipids have a high content of C40-macrocyclic lipid core. Can you give an assessment of what effect these other variables might have on the observed stability of the archaeosomes?

Authors: When assessing the reasons for the relative stability of archaeosomes each produced from the total polar lipids of different archaeobacteria, there are very few factors for which sufficient quantitative data is available. One of these factors which is available, and is expected to be dominant in determining stability properties is the percentage of tetraether lipid in the extract. Unfortunately, the purified lipids are usually unavailable in sufficient quantity to prepare archaeosomes from very defined mixtures of diether and tetraether lipids, thus minimizing the number of variables. However, the same correlation between temperature stability of archaeosomes and tetraether content (Fig. 2) is obtained by mixing varying proportions of diether lipids of Methanosarcina mazei with the tetraether lipids of Thermoplasma acidophilum (Choquet et al., 1996). Other variables which alter general properties, such as surface charge, may be expected to alter archaeosome stability properties, and these should be measured and assessed. Of course, it is known that the macrocyclic diether and tetraether content of Methanococcus jannaschii increase as the growth temperature increases (Sprott et al., 1991), as does the number of cyclopentane rings in the tetraether lipid chains of thermoacidophiles (De Rosa and Gambacorta, 1988), implying that a stabilizing effect of these lipid modifications may be expected in pure-lipid archaeosomes.

M. Kates: Can you discuss what is the effect of liposome size on liposome stability with reference to the data in Fig. 3? Can the large decrease in stability of the liposomes with 25% TPL of M. hungatei and M. espanolae be related to the decreased liposome diameters?

Authors: In our studies the sizing data show that liposomes with increasing proportions of EPC tend to decrease in size, but this decrease is not dramatic and is within the range of standard deviations from the means. Although vesicles of pure dipalmitoylphosphatidylcholine were found to be unstable below their transition temperature when smaller than 40 nm (Lichtenberg et al., 1981), our liposomes were always considerably larger than this and we used a temperature of 37°C, well above the transition temperature of the ester lipid (EPC). In addition, fusion was not detected in these experiments. We believe it unlikely, therefore, that the dramatic changes in stability which we observed could be explained by differences in size.

M. Kates: Can you give any explanation why the liposomes of EPC and TPL from M. smithii are so much
more unstable than those from the other archaea toward phospholipase A2 (Fig. 4) and towards serum (Fig. 5)?

**Authors:** In the case of serum, stability correlates with the tetraether content. EPC liposomes with no tetraether lipids and *M. smithii* containing only 23% tetraethers are relatively unstable compared to those liposomes containing 50%, or more, tetraether lipids. Removal of the outer leaflet of the bilayer by serum high-density lipoproteins (Scherphof et al., 1984) may be countered by the presence of membrane spanning tetraether lipids. In the case of phospholipase A2, any instability of archaeosomes (no EPC) is caused by exposure to the alkaline pH of the reaction mixture, and not to enzymatic activity *per se* (Fig. 4).

**M. Foldvari:** Can you comment on the biocompatibility and biodegradability of tetraether lipids?

**T.A. Langworthy:** Tetraether based liposomes are shown to be very stable. Would you expect the release of entrapped materials to be hindered by this stability?

**Authors:** Most anabolic studies have contributed information on pathways for synthesis of archaeobacterial lipids, rather than their catabolism. When considering biocompatibility and degradability it is encouraging that the phytanyl chains of archaeobacterial lipids are composed of the same C5 units as are phytanyl chain vitamins and coenzyme Q10, so essential to mammalian physiology, that vinyl ether lipids are found in the brain, and that archaeobacterial lipids are synthesized in the human intestine by methanogenic archaeobacteria. Whether mammalian enzymes exist to degrade tetraether lipids is presently unknown, but of obvious importance to determine. Our unpublished observations are that following uptake of archaeosomes into macrophages in vitro, the internal content of the archaeosomes are released by a mechanism that can be blocked by inhibitors of metabolism. Further, as shown in this study archaeosomes are degraded by treatments such as bile salts, and stability may be controlled in a predictable way by including conventional phospholipid.

**M. Foldvari:** Did you encapsulate lipid soluble drugs in liposomes constructed from diether or tetraether lipids or lipid mixtures with phospholipids?

**Authors:** Yes, we have encapsulated coenzyme Q10, a drug being tested for anti-apoptosis effects. The loading appears best with diether versus tetraether-type archaeosomes.

**M. Foldvari:** Could you comment on the difficulty of liposome formation from tetraether lipids, e.g. high temperature, great shear forces required?

**Authors:** Tetraether lipids appear, in general, to be more difficult to hydrate, and require a higher pressure to extrude through filters of defined pore-size than are diether lipids. However, hydration of TPL of *Thermoplasma acidophilum* occurs overnight by gentle shaking in PBS at 37°C. Pressure extrusion at ambient temperature occurs well using 400 nm pore-size filters when the lipid is about 20 mg/ml.

**T.A. Langworthy:** Is there any direct evidence that a mixture of ether lipids and ester lipids in fact form an integrated liposomal structure, rather than a mixture of ester-based liposomes and ether-based liposomes?

**Authors:** Yes, beyond the stability data shown here and the arguments presented, there are direct results from differential scanning calorimetry showing that a number of ester-phospholipids including EPC and dipalmitylo-phosphatidylcholine form stable mixed phases with the MPL lipid of *Thermoplasma acidophilum* (Blocher et al., 1985) and with the ether lipids from *Sulfolobus solfataricus* (Lelkes et al., 1983).

**T.A. Langworthy:** Archaeosomes are stable to storage at pH 3 (Choquet et al., 1994). What is the predicted stability of archaeosomes at pH 1 and 37°C, e.g. conditions within the stomach?

**Authors:** Our unpublished results show that archaeosomes prepared from the TPL of *Thermoplasma acidophilum* are stable at 37°C, retaining encapsulated ^14^C-sucrose, during 90 min exposure to pH 2.0. If the pH is decreased to 1.5 dramatic instability was observed. To achieve stability, a small increase in stomach pH may be necessary by taking a neutralizing buffer just prior to ingestion of the liposomes.

**A.K. Dash:** The authors have used leakage of marker molecules from the liposomes as the only parameter to assess the liposome stability. Is it possible to use other parameters to evaluate the stability of the archaeosomes? If so, please explain concisely.

**Authors:** Leakage is a sensitive measure of stability in which permeability is assessed. Leakage could occur, without necessarily affecting overall shape or size of the vesicles. Indeed, in cases where the active agent is attached to, or within, the lipid bilayer stability may be adequately evaluated by directly viewing with an electron microscope.

**A.K. Dash:** In general, what type of toxicity studies should be performed on these archaeobacterial ether lipids to evaluate their suitability for in vivo applications?

**Authors:** Because archaeosomes are composed of a substance or mixture of substances, it is expected that for regulatory purposes they would be considered as a drug, rather than a medical device. Attempts have been made to standardize internationally the toxicity testing of drugs, the result being publication of the International Conference on Harmonization Toxicology Guidelines.
Stability of archaeosomes

These guidelines should be consulted in the answer to this question, which applies to acceptance of any drug. The types of tests required may also depend on the application route; namely, topical, oral, intravenous etc. For cosmetic applications relative to internal drug delivery very little testing would be necessary.

Additional References
