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In vitro assessment of archaeosome stability for developing oral delivery systems☆

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Abstract

The in vitro stability of archaeosomes made from the total polar lipids of *Methanosarcina mazei*, *Methanobacterium espanolae* or *Thermoplasma acidophilum*, was evaluated under conditions encountered in the human gastrointestinal tract. At acidic pH, multilamellar vesicles (MLV) prepared from *T. acidophilum* lipids were the most stable, releasing ~80, 20, 10 and 5% of encapsulated ^{14}C-sucrose at pH 1.5, 2.0, 2.5 and 6.2, respectively, after 90 min at 37°C. Archaeosomes from *M. mazei* lipids were the least stable. For each type of total polar lipid, unilamellar vesicles (ULV) were less stable than the corresponding MLV vesicles. Pancreatic lipase had relatively minor effect on the stability of archaeosomes made from either of the three types of total polar lipids, causing the release of 12–27% of the encapsulated 5(6)-carboxyfluorescein (CF) from ULV and MLV after 90 min at 37°C. In simulated human bile at pH 6.2, MLV from *M. mazei* total polar lipids lost 100% of the encapsulated CF after 90 min at 37°C, whereas those from the polar lipids of *M. espanolae* or *T. acidophilum* lost ~85% of the marker. Pancreatic lipase and simulated human bile had no synergistic effect on the release of carboxyfluorescein from ULV or MLV prepared from any of the total polar lipids. After 90 min in the combined presence of these two stressors at pH 6.2, the leakage of fluorescein conjugated bovine serum albumin from MLV prepared from *T. acidophilum* lipids was similar to that of CF, and 13% of the initially present vesicles appeared to be intact. These results indicate that archaeosomes show stability properties indicative of potential advantages in developing applications as an oral delivery system.

Keywords: Archaeal lipid liposomes; Archaeosomes; Stability; Oral delivery system; GI tract

Abbreviations: GI, gastrointestinal; TPL, total polar lipids; MLV, multilamellar vesicles; ULV, unilamellar vesicles; CF, 5(6)-carboxyfluorescein; SHB, simulated human bile; fc-BSA, fluorescein conjugated bovine serum albumin.

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1. Introduction

Liposomes constitute one of several delivery systems that are currently being developed for the oral delivery of drugs, vaccines and genes (O'Hagan, 1992; Couvreur and Puisieux, 1993; Lasic, 1998; Rogers and Anderson, 1998). One of the requirements of a successful oral delivery system is reasonable stability in the environment of the gastrointestinal (GI) tract, so as to offer some protection to the encapsulated compound during transit to the site of action, especially for proteins that can be easily degraded (Woodley, 1984; Reilly et al., 1997). In this context, a major drawback of liposome formulations that have been studied extensively to date is their poor stability at low pH, susceptibility to attack by bile salts and GI tract enzymes such as lipases, particularly when the latter two stressors are present together (Rowland and Woodley, 1980; Chiang and Weiner, 1987; Lasic, 1998). Attempts to improve the stability of liposomes either by incorporation of high amounts of cholesterol in the bilayer, by coating the liposome surface with other polymers, by polymerizing the liposomes, or by using fluorinated phospholipids to make vesicles have had limited success (Okada et al., 1995; Ravily et al., 1996; Han et al., 1997; Iwanaga et al., 1997).

Archaeosomes are liposomes which are made from lipid compositions that include lipids that are structurally typical of archaeobacterial membrane lipids. Archaeobacterial lipids consist of archaeol (diether) and/or caldarchaeol (tetraether) core structures wherein regularly branched and usually fully saturated phytanyl chains (20–40 carbons in length), are attached via ether bonds to the *sn*-2,3 carbons of the glycerol backbone. The variety of polar head groups may include phospho-, glyco-, phosphoglyco-, polyol- or hydroxy groups similar to those found in the conventional ester lipids present in eukaryotes and eubacteria, but phosphatidylcholine is rarely present (Sprott, 1992; Kates, 1993). Unilamellar archaeosomes prepared from the total polar lipids (TPL) of some archaeobacteria were relatively more stable when exposed to various physical/biochemical stressors, than were some conventional, ester phospholipid, liposome formulations (Choquet et al., 1994; Sprott et al., 1996). Some archaeosome formulations can also act as potent enhancers of immune response to systemically administered, encapsulated protein antigens (Sprott et al., 1997; Makabi-Panzu et al., 1998). The objective of the current work was to conduct an in vitro evaluation of the stability of unilamellar and multilamellar archaeosomes under conditions that would be encountered in the human GI tract environment, and that have been used by others to identify stability problems in conventional liposomes. This study would help select archaeosome formulations for further research to develop oral vaccine and drug delivery systems in mammals.

2. Materials and methods

2.1. Growth of archaeobacteria and lipid extraction

Methanosarcina mazei S-6 (DSM 2053), *Methanobacterium espanolae* GP9 (DSM 5982) and *Thermoplasma acidophilum* 122-1B3 (ATCC 27658) were cultivated in fermenters, as described earlier (Choquet et al., 1994). The cells were frozen at -20°C (overnight) and thawed at room temperature. The freeze–thaw cycle was repeated two more times and the total lipids from ~ 30 g of cell dry weight were extracted by the Bligh and Dyer (1959) method. TPL were precipitated from the total lipids dissolved in chloroform/methanol, using cold acetone (Sprott et al., 1995). The TPL from *M. mazei* was passaged through a column of silica gel G (Sprott et al., 1995), because a previous study had indicated that this improved the ability of vesicles prepared therefrom to retain encapsulated low molecular weight marker such as 5(6)-carboxyfluorescein (CF) (Patel et al., 1999). The silica 'treatment' was not required for the TPL from *M. espanolae* and *T. acidophilum*. The TPL in chloroform/methanol was stored in air at 5°C until required.

2.2. Preparation of archaeosomes

About 20 mg of the dried TPL was hydrated in 1 ml of phosphate buffered saline (PBS, 10 mM

potassium phosphate buffer at pH 7.14, containing 160 mM NaCl) that was supplemented with either 100 mM CF (catalogue no. C-7153, Sigma Chemical Co., St. Louis, MO) or 150 mM sucrose and 12.5 μCi of ^{14}C-sucrose (New England Nuclear Research Products, Mississauga, Ont., Canada; 155 Mbq/mmol). The hydration was at 35°C for 16 h. To obtain multilamellar vesicles (MLV), the hydrated lipids were bath sonicated to achieve an average vesicle diameter of about 1000 nm, measured with a NICOMP model 370 particle sizer (NICOMP, Santa Barbara, CA). To obtain unilamellar vesicles (ULV) from the TPL of *M. mazei*, the hydrated lipids were pressure extruded through 400 nm polycarbonate filters (19 times, at room temperature) using a LiposoFast apparatus (Avestin, Ottawa, Ont., Canada). To obtain ULV from the TPL of *T. acidophilum* or *M. espanolae*, the hydrated lipids were bath sonicated first, to obtain vesicles of ~ 500 nm average diameter, and then pressure extruded through 100 nm polycarbonate filters as described for *M. mazei* TPL. The ULV and MLV vesicles were annealed for 20 h at 5°C, and the unencapsulated ^{14}C-sucrose from MLV formulations was removed by ultracentrifugation (89 000 × g for 5 min) and washing the vesicles twice, with PBS supplemented with 150 mM glucose. The unencapsulated CF was removed from MLV formulations by centrifugation (5600 × *g*, 5 min) and washing twice with PBS. The washed MLV preparations with sucrose marker were resuspended in PBS containing 150 mM glucose, and those with CF marker were resuspended in PBS. The unencapsulated ^{14}C-sucrose from ULV was removed by minicolumn centrifugation through Sephadex G-50 (New, 1990), the column being equilibrated and eluted with PBS containing 150 mM glucose. Unencapsulated CF in ULV was also removed by the minicolumn centrifugation method, using PBS for column equilibration and elution. Unless specified otherwise, the typical average diameters of ULV from *M. mazei* TPL, *M. espanolae* TPL and *T. acidophilum* TPL were 130 ± 30, 138 ± 58 and 141 ± 66 nm, and that of MLV were 697 ± 85, 870 ± 135 and 996 ± 189 nm, respectively.

2.3. Archaeosome stability at low pH

The stability of ULV and MLV at low pH (1.5–6.2) was determined using vesicles containing ^{14}C-sucrose as the aqueous marker because the fluorescence of CF is pH sensitive. Aliquots of MLV or ULV (50 μl) were added to 450 μl of PBS containing 150 mM glucose, the pH of the latter being pre-adjusted to 1.5, 2.0, 2.5 or 6.2 with 1 N HCl. After 10, 30 and 90 min of incubation (37°C water bath), 100-μl aliqouts were removed and centrifuged (89 000 × *g*, for 5 min). The supernatant was transferred to 5 ml of Eco Lite scintillant (ICN Pharmaceuticals, Montreal, PQ, Canada) and radioactivity determined in a liquid scintillation counter (LKB Wallack 1217 Rackbeta, Pharmacia Canada, Inc.). For each vesicle preparation, the initial amount of encapsulated radioactive sucrose was determined and used to calculate the percentage leakage. Each assay was done in duplicate and repeated at least once. All percentage leakage data are presented as mean ± S.D.

In some experiments with MLV, the percentage of vesicles that still appeared to be intact after 1.5, 5.0 and 24.0 h in PBS buffers at pH 1.5, 2.0 and 6.2 was determined by direct microscopic count (in triplicate assays). The MLV were visualized by phase contrast optics and counted using a Petroff–Hausser counting chamber (A.H. Thomas and Co., Philadelphia, PA). These MLV were prepared without any encapsulated marker. The vesicle count in PBS at pH 6.2, at 0 h, was taken as the 100% intact vesicle value, for each respective MLV type. The results are expressed as a percentage of the initial MLV that were visually intact (mean ± S.D.).

2.4. Archaeosome stability against lipase, simulated human bile, and sodium taurocholate

These assays were done with vesicles containing encapsulated CF as the aqueous marker. ULV or MLV were diluted in 1.5 ml final volume of the appropriate reactant buffer. The amount of liposomes added was such that 100% release of CF, caused by lysing the vesicles with octyl-β-D-glucopyranoside (60 μl of 10%, w/v, to 1.5 ml reac-

tant buffer, 0.5 h at room temperature), would keep the fluorescence reading (490 nm excitation, 515 nm emission wavelength) within the linear range for the fluorometer (Turner Digital Fluorometer, model 450, Barnstead/Thermolynne Corp., Dubuque, IA).

Stability of ULV and MLV against attack by pancreatic lipase (Type VI-S from Porcine pancreas, Sigma Chemical Co., St. Louis, MO) was conducted in 1.5 ml final volume of PBS (pH 7.14) supplemented with 1 mM calcium and 5000 U of the enzyme. At 2, 20, 60 and 90 min of incubation after addition of the vesicles (~ 5 μg liposomal lipid), the test tube (12 mm diameter × 75 mm) containing the reactants was removed for fluorescence measurement and quickly re-incubated (37°C, reciprocal shaking water bath). Corresponding control samples in the absence of the phospholipase were also similarly analyzed to determine the effect of pH per se on the observed leakage. At the end of the incubation, the vesicles in each tube were lysed with octyl-β-D-glucopyranoside as described above, to determine the fluorescence reading equivalent to 100% leakage of CF in each respective tube. The assays in duplicate were repeated at least once, and the percentage leakage was presented as mean ± S.D.

The stability in the presence of bile salts was done as above for pancreatic lipase but at pH 6.2, using simulated human bile (SHB) as the reactant. The SHB, reflecting the bile acids present in the human intestine, was prepared as described earlier (Rowland and Woodley, 1980). Briefly, 20 mM solutions of six different bile salts were prepared in PBS at pH 6.2 and were then mixed as follows (%, v/v): sodium glycocholate, 30%; sodium glycochenodeoxycholate, 30%; sodium glycodeoxycholate, 15%; sodium taurocholate, 10%; sodium taurochenodeoxycholate, 10%; sodium taurodeoxycholate, 5%. This solution was diluted 1:1 with pH 6.2 PBS to give 10 mM final concentration of bile salts (SHB) in the stability assay. The incubation, assay intervals, and data compilation were as for the pancreatic lipase assays above.

The combined effect of pancreatic lipase and SHB on the stability of ULV and MLV was studied as above, using 1.5 ml final volume of reactant consisting of SHB (pH 6.2) that was supplemented with 5000 U of pancreatic lipase and 1 mM calcium.

The CF-containing MLV prepared from *T. acidophilum* TPL were assayed also under conditions similar to that used recently by Okada et al., (1995) for evaluating the stability of polymerized liposomes. This consisted of 5 U phospholipase A_2/ml (from bovine pancreas, Sigma, St. Louis, MO), 10 mg/ml sodium taurocholate, 6 mg/ml liposomal lipid, assayed at pH 7.4 in PBS containing 3 mM calcium. Aliquots were removed at various times during incubation at 37°C, and fluorescence measured to determine the percentage leakage.

2.5. SHB-induced release of bovine serum albumin from archaeosomes

Leakage of a large molecular weight compound from MLV prepared from *T. acidophilum* TPL, upon exposure to SHB, was conducted under the assay conditions described above, but using fluorescein conjugated bovine serum albumin (fc-BSA, cat. no. A-841, Molecular Probes, Santa Barbara, CA) as the encapsulated marker. The dried TPL (20 mg) were hydrated in 2 ml of PBS (pH 6.2) supplemented with 5.0 mg of fc-BSA, and MLV prepared as described above. After annealing, the unencapsulated fc-BSA was removed by ultracentrifugation (49 000 × *g* for 10 min) and washing three times in PBS at pH 6.2. To start the stability assay, 0.5 ml aliquots of MLV were added to 0.25 ml of PBS (pH 6.2), and this was diluted 1:1 with 20 mM bile salts solution to give a final volume of 1.5 ml of SHB (10 mM bile salts). The control consisted of MLV made to 1.5 ml with PBS alone. At the indicated period of incubation at 37°C, 50-μl aliquots were removed, made to 1.5 ml with PBS and ultracentrifuged (49 000 × *g* for 10 min) to separate the leaked fc-BSA from the vesicles. The fluorescence in the supernatant was measured at the wavelengths used for CF. The vesicle pellet was washed twice, resuspended in 1.5 ml PBS and the fluorescence was determined after adding 60 μl of octyl-β-D-glucopyranoside solution (10%, w/v). The fluorescence in the supernatant divided by the total fluorescence (that measured in supernatant and in

vesicles) was used to determine the percentage leakage of fc-BSA. The fluorescence of fc-BSA was not affected by the SHB, as judged from the similar, concentration dependent, linear responses obtained when fluorescence standard curves were prepared with fc-BSA dissolved in SHB and in PBS at pH 6.2 (data not shown). The leakage data are presented as the mean ± S.D., from triplicate analyses. At each time period the percentage of the initially present MLV that appeared visually as intact vesicles was determined by direct microscopic count as described earlier, using the 0 h count of vesicles in the PBS control as the initial, 100% intact vesicle value.

3. Results

3.1. Archaeosome stability at low pH

The leakage of ^{14}C-sucrose from ULV and MLV vesicles made from the TPL of *M. mazei*, *M. espanolae* and *T. acidophilum* upon exposure at pH 1.5, 2.0, 2.5 and 6.2, was determined over 90 min incubation at 37°C. The ULV from *M. mazei* TPL lost all of the encapsulated marker within the first 10 min of incubation at pH 1.5 and 2.0 (Fig. 1A). The ULV from *M. espanolae* TPL were relatively more stable, but still lost ~100% of the marker by the 90-min incubation at pH 1.5 (Fig. 1B). At pH 1.5, MLV from *T. acidophilum* TPL were the most stable, losing ~80% of the marker after 90 min (Fig. 1C). The vesicles from *M. mazei* TPL were the least stable at each tested pH. A dramatic improvement in stability was noted, especially for vesicles made with *T. acidophilum* TPL, when the test pH was increased from 1.5 to 2.0. Except for vesicles from *M. mazei* TPL which lost 34–22% of sucrose after 90 min, all other archaeosome types were quite stable at pH 6.2, leaking less than 8% of the sucrose. For each TPL type, at each pH, the MLV were comparatively more stable than the corresponding ULV.

The percentage of the initial MLV from *M. mazei* TPL, *M. espanolae* TPL and *T. acidophilum* TPL, respectively, that were intact after 1.5, 5.0 and 24 h exposure at pH 1.5, 2.0 and 6.2, is shown in Fig. 2A–C. Although between 80 and 100% of the sucrose marker leaked out of the MLV after 1.5 h at pH 1.5 (Fig. 1A–C), ~33% of *M. mazei* TPL and *M. espanolae* TPL, and 48% of *T. acidophilum* TPL vesicles appeared to be intact (Fig. 2A–C). The results showed also that MLV from *T. acidophilum* TPL, the most stable preparation, still had 29 and 59% of the vesicles intact after 24 h incubation at pH 1.5 and 2.0, respectively. The microscopic count data on MLV confirmed that *M. espanolae* TPL vesicles were of intermediate stability and those of *M. mazei* TPL were the least stable.

3.2. Archaeosome stability against lipase and in simulated human bile

Although ULV from *M. mazei* TPL were relatively stable to attack by pancreatic lipase, releasing less than 5% of the encapsulated CF after 2

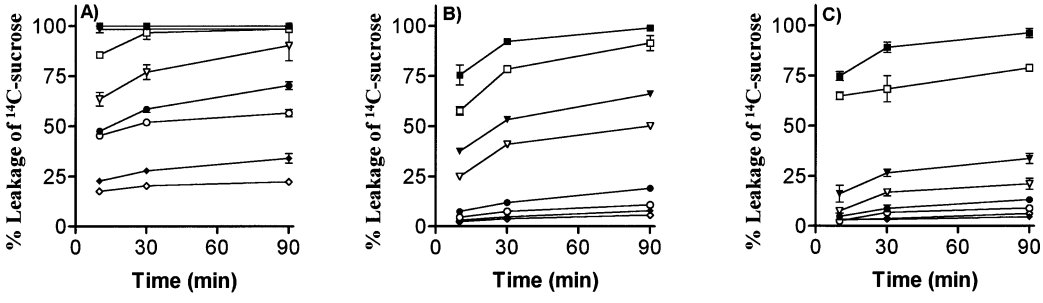

Fig. 1. Percent leakage of encapsulated ^{14}C-sucrose from ULV (closed symbols) and MLV (open symbols) prepared from (A) *M. mazei* TPL, (B) *M. espanolae* TPL, and (C) *T. acidophilum* TPL, upon incubation (37°C) in buffer at pH 1.5 (■, □), pH 2.0 (▼, ▽), pH 2.5 (●, ○), and pH 6.2 (◆, ◇).

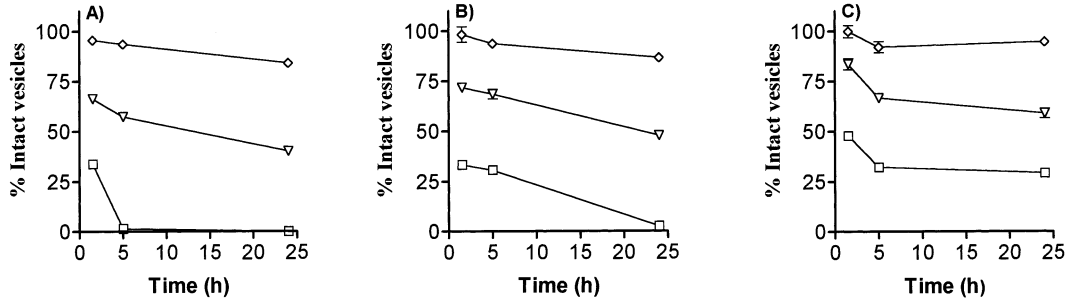

Fig. 2. Percent of initial MLV, prepared from (A) *M. mazei* TPL, (B) *M. espanolae* TPL, and (C) *T. acidophilum* TPL, that were visually intact after 1.5, 5.0 and 24.0 h exposure (37°C) in buffer at pH 1.5 (□), pH 2.0 (▽), and pH 6.2 (◇).

min of incubation, all of the CF leaked out within 2 min of exposure to SHB (data not shown). However, MLV were comparatively more stable to attack by SHB, leaking ~30% of the CF in the first 2 min and 84–100% between 60 and 90 min, respectively (Fig. 3). At 90 min, pancreatic lipase alone caused ~25% of the CF to leak out.

The ULV from *M. espanolae* TPL were stable compared to those from *M. mazei* TPL, losing ~67 and 96% of the CF, respectively, after 2 and 90 min exposure to SHB (Fig. 4A). The MLV were much more stable than the corresponding ULV, with ~15 and 88% CF leakage, respectively, after 2 and 90 min incubations in SHB (Fig. 4B). In contrast to the ULV where SHB caused release of the bulk of the CF in the first 2 min, the leakage of CF from the MLV was more gradual.

Compared with the ULV from *M. mazei* TPL and *M. espanolae* TPL, those from *T. acidophilum* TPL were the most stable in SHB (Fig. 5A), losing only ~15% of the CF after 2 min. The stability of MLV (Fig. 5B) from *T. acidophilum* TPL appeared to be similar to that of the corresponding ULV. This is in contrast to the observations with *M. mazei* and *M. espanolae* TPL, where the MLV were significantly more stable than their respective ULV. Pancreatic lipase alone caused <20% CF release from *T. acidophilum* TPL vesicles, after 90 min incubation. Amongst the MLV preparations, those from *M. espanolae* TPL and *T. acidophilum* TPL appear more stable to SHB than those from *M. mazei* TPL.

The effect of a combination of SHB and pancreatic lipase on stability was similar to that of SHB alone, for the respective type of TPL used, in all three types of archaeobacterial TPL vesicles (Figs. 3–5). This illustrates that there was no synergistic effect of SHB and pancreatic lipase, on the leakage of CF from these vesicles.

The effect of 5 U phospholipase A_2/ml, in combination with either sodium taurocholate (10 mg/ml) or SHB, at pH 7.4, on the leakage of CF from MLV (6 mg lipid/ml) made from *T. acidophilum* TPL is illustrated in Fig. 6. It appears that the effect of sodium taurocholate is similar to that of the SHB, on the vesicle stability in the presence of phospholipase A_2. However, com-

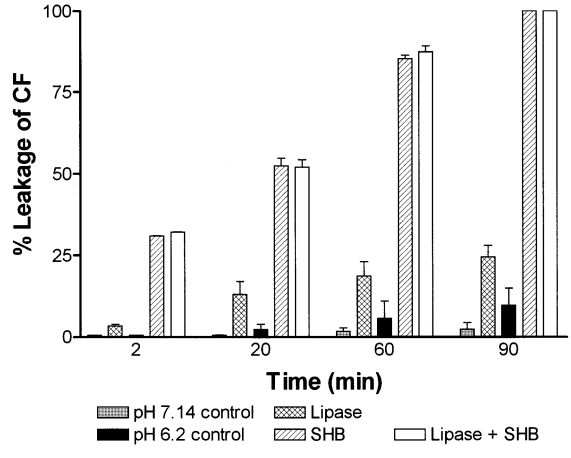

Fig. 3. Percent leakage of encapsulated CF from MLV prepared from *M. mazei* TPL, upon exposure (37°C) to pancreatic lipase (3333 U/ml) at pH 7.14, simulated human bile (SHB) at pH 6.2, or a combination of both at pH 6.2.

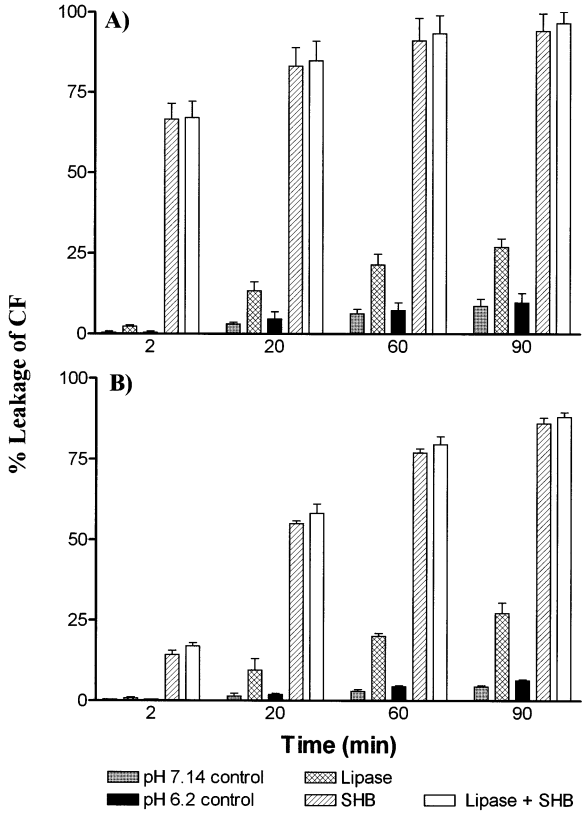

Fig. 4. Percent leakage of encapsulated CF from (A) ULV, and (B) MLV prepared from *M. espanolae* TPL, upon exposure (37°C) to pancreatic lipase (3333 U/ml) at pH 7.14, simulated human bile (SHB) at pH 6.2, or a combination of both at pH 6.2.

pared to the combined effect of pancreatic lipase (3333 U/ml) and SHB at pH 6.2 (Fig. 5B), the vesicles were relatively more stable in the presence of phospholipase A_2 in combination with SHB or sodium taurocholate at pH 7.4. Under the latter combination (Fig. 6), less than 50% of CF leaked out after 1.5 h, and $\sim$90% after 5 h.

3.3. SHB-induced release of bovine serum albumin from MLV made from T. acidophilum TPL

Since CF is a relatively small molecular weight compound (M_W of 376.3), we wanted to see if the leakage of an encapsulated, larger M_W compound such as BSA (66 000 Da) was similar when vesicles were exposed to SHB. The use of fluorescein conjugated BSA allowed for convenient monitoring of the stability of the vesicles after 1.5, 5.0 and 24.0 h of exposure. Only MLV from *T. acidophilum* TPL were evaluated since they were also the most stable at low pH. The results indicated that similar to the leakage of CF, $\sim$89% of fc-BSA leaked out from the MLV after 1.5 h (Table 1). About 13, 7 and 3% of the MLV appeared to be intact at 1.5, 5, and 24 h incubation, respectively.

4. Discussion

The archaeobacterial total polar lipids used in this study were selected due to the differences in

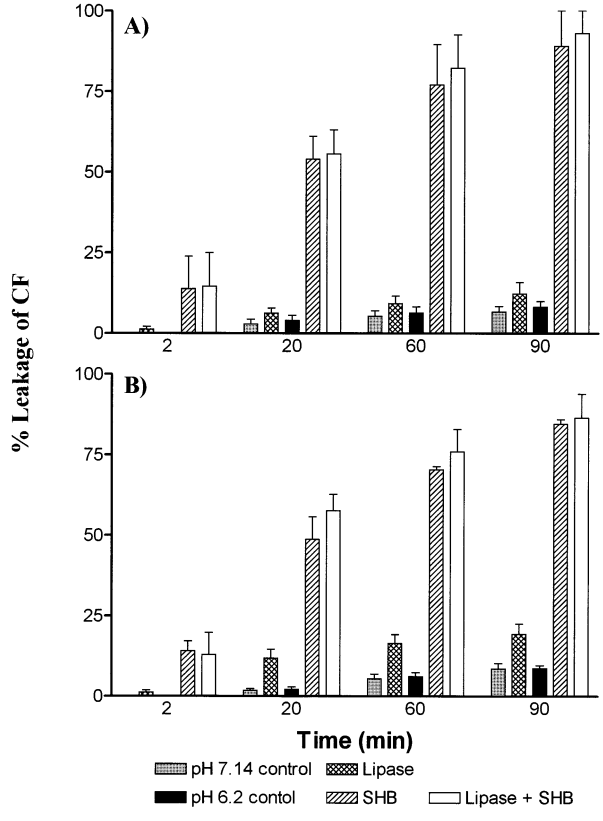

Fig. 5. Percent leakage of encapsulated CF from (A) ULV, and (B) MLV prepared from *T. acidophilum* TPL, upon exposure (37°C) to pancreatic lipase (3333 U/ml) at pH 7.14, simulated human bile (SHB) at pH 6.2, or a combination of both at pH 6.2.

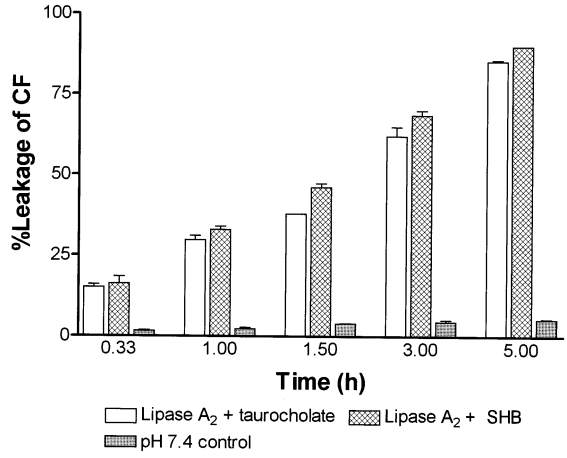

Fig. 6. Percent leakage of encapsulated CF from MLV prepared from *T. acidophilum* TPL, upon exposure (pH 7.4, 37°C) to a combination of phospholipase A_2 (5 U/ml) with either sodium taurocholate at 10 mg/ml or with simulated human bile (SHB).

their core lipid structures and implications on stability. The TPL of *M. mazei* consists entirely of archaeol and hydroxyarchaeol core lipid structures, those of *M. espanolae* of 35% archaeol and 65% caldarchaeol, and those of *T. acidophilum* of primarily (90%) caldarchaeols (Sprott et al., 1996). Unlike ester phospholipids and archaeol lipids, the caldarchaeol lipids are membrane spanning (Lo and Chang, 1990; Beveridge et al., 1993), and hence are likely to result in a more rigid and stable membrane.

Archaeosomes were evaluated at pH 1.5–6.2 to reflect the GI tract environment, where the stomach pH can be as low as 1.4, and intestinal pH can vary between 5.5 and 7.0 (Kararli, 1995). Of the three TPL compositions evaluated, the stability of vesicles at pH as low as 1.5 correlated with the caldarchaeol lipid content. Although published stability data at pH 1.5 are not available for ester phospholipid liposomes, the leakage of CF at pH 2.0, from various multilamellar formulations was reported to vary from 20–60% after 2 h exposure (Aramaki et al., 1993). However, MLV prepared from distearoylphosphatidylcholine:cholesterol mixtures (2:1 or 7:2 molar ratio) were quite stable at pH 2.0, leaking less than 10% of entrapped glucose, CF or polyvinylpyrrolidone marker over 60–120 min incubation (Rowland and Woodley, 1980; Chiang and Weiner, 1987; Han et al., 1997). This compares favourably to $\sim$20% release of sucrose after 90 min at pH 2.0, from MLV made with *T. acidophilum* TPL. However, an important observation with the archaeosomes in the current study was that the leakage of sucrose at low pH did not appear to be due to the total disruption of the MLV, because a large proportion of the MLV were visually intact (Fig. 2), when a significant amount of the marker had leaked out (Fig. 1). This suggests that the leakage of sucrose at low pH was partly caused by subtle changes resulting in increased permeability of the archaeosome vesicle membrane, at least to low molecular weight compounds.

Pancreatic juices contain 1.5–16.0 U of phospholipase A_2 activity per ml (Senior, 1987; Kozumplik et al., 1989) and a mixture of other

Table 1
Percent leakage of encapsulated fluorescein conjugated BSA from MLV prepared from *T. acidophilum* TPL and percentage of initial vesicles that were visually intact, after incubation at 37°C in SHB at pH 6.2[a]

Time (h)	pH 6.2 control		SHB at pH 6.2	
	% BSA leakage	% Intact vesicles	% BSA leakage	% Intact vesicles
1.5	4.3 ± 1.9	98.5 ± 2.1	88.7 ± 1.3	13.3 ± 3.5
5.0	4.1 ± 0.4	93.0 ± 7.1	92.4 ± 1.0	7.6 ± 2.6
24.0	3.0 ± 1.7	95.0 ± 4.2	94.3 ± 1.8	3.4 ± 1.8

[a] Data are presented as mean $\pm$ S.D.

lipases with undefined activity for archaeobacterial ether lipids. Pancreatic lipase can cause significant hydrolysis of conventional liposomal phospholipids, which can lead to a rapid release of encapsulated compounds in the intestines. Unilamellar vesicles prepared from dipalmitoylphosphatidylcholine leaked >90% of the encapsulated marker in 3 h, compared with 7–18% leakage from various unilamellar archaeosomes following a 5-h exposure to very high pancreatic lipase activity (Choquet et al., 1994). The current data confirm that archaeosomes are relatively resistant to the action of pancreatic lipase. Of all the conventional liposomes tested, MLV from distearoylphosphatidylcholine/cholesterol appeared to be the most stable to attack by pancreatic lipase, leaking less than 20% of the marker after a 120-min exposure at 37°C (Rowland and Woodley, 1980; Chiang and Weiner, 1987).

Human bile consists of six major bile acids, in the proportions indicated in the methods section. The average concentration of bile in the human duodenum is 10.8 mM, while the range in the GI tract is 3–45 mM (Richards and Gardner, 1978; Kararli, 1995). Specific bile salts are expected to cause varying degrees of destabilization of liposomes, depending on the ability of the bile salts to permeate into the lipid membrane and form soluble, mixed micelles with the membrane lipids. Permeation of bile salts into the liposomal bilayer membrane makes the membranes more fluid (Richards and Gardner, 1978; O'Connor et al., 1985), and ultimately may lead to complete disintegration of the vesicles.

Unilamellar and multilamellar liposomes made from conventional ester phospholipids are rapidly disrupted by bile salts (10 mM or lower), resulting in the loss of the major portion of an encapsulated aqueous marker such as sucrose, glucose, polyvinylpyrrolidone, chromate, or CF within 5–30 min of incubation at 37°C (Richards and Gardner, 1978; Rowland and Woodley, 1980; O'Connor et al., 1985; Nagata et al., 1988). Multilamellar vesicles from egg lecithin/cholesterol were highly unstable in 10 mM taurocholic acid (pH 7.0, 37°C), releasing greater than 95% of entrapped CF within 5 min of incubation (Chiang and Weiner, 1987). Although MLV made with distearoylphosphatidylcholine/cholesterol (7:2 or 2:1 molar ratio) were one of the most stable conventional liposomes in the presence of 10 mM bile salts (at 37°C), these vesicles released 24% of encapsulated glucose (2 h) or 100% of CF (1 h) at pH 7.0 (Chiang and Weiner 1987); or 16% of ^{125}I-labelled polyvinylpyrrolidone at pH 6.2 (Rowland and Woodley, 1980). It has been pointed out previously that the observed stability of liposomes is dependent on the marker used for the assay (Chiang and Weiner, 1987). Our results with SHB revealed similar leakage of either CF or fluorescein conjugated BSA from MLV made with *T. acidophilum* TPL, suggesting vesicle disruption rather than subtle increases in permeability. The close correlation between the % intact MLV and observed leakage of the bovine serum albumin in presence of SHB, further supports the suggestion that the release of the marker is due to vesicle disruption. Of the various strategies employed to improve stability in the presence of bile, surface coating of vesicles with distearoylphosphatidylethanolamine–polyethylene glycol or cetylmucin, was one of those which showed promise (Iwanaga et al., 1997). These authors reported that the leakage of encapsulated insulin declined from 20 to 50% in control liposomes, to less than 10% from the surface-coated liposomes, after 6 h exposure to 10 mM sodium glycocholate or taurocholate.

In some aspects, archaeosomes are similar to conventional liposomes, in that of all the possible stressors that could be encountered in the GI tract, bile salts are the most potent destabilizers. Our results demonstrate that archaeosomes made with archaeol core lipids, such as *M. mazei* TPL, were unstable in SHB, while those made from caldarchaeol-rich core lipids, such as TPL from *T. acidophilum* or *M. espanolae*, were relatively more stable.

Most of the in vitro stability studies have evaluated the individual effects of stressors such as phospholipase and bile salts on vesicle stability. In the GI tract however, a combination of both stressors are encountered together, and hence this assay condition would be more relevant in predicting in vivo stability. Although MLV composed of distearoylphosphatidylcholine /

cholesterol lost $< 20\%$ of the encapsulated CF when exposed to either pancreatic lipase or sodium taurocholate alone (5 min at 37°C), the leakage was significantly greater (80% in 5 min) in the presence of both stressors, with almost 100% leakage occurring in 30 min (Chiang and Weiner, 1987). In contrast to this, there was no synergistic effect of pancreatic lipase and simulated human bile on the stability of ULV or MLV prepared from any of the three archaeobacterial TPL. This observation was not unexpected, considering the relative tolerance of archaeobacterial ether lipid vesicles to attack by phospholipase A_2. Polymerized multilamellar vesicles, made from the phospholipid 1,2-di (2,4-octadecadienoyl)phosphatidylcholine (DODPC), were reported to retain up to 75% of encapsulated sucrose in the presence of 10 mg/ml sodium taurocholate and phospholipase A_2 (5 U/ml, with 6 mg/ml of liposomal lipid) after 24 h incubation at 37°C at pH 7.4 (Okada et al., 1995). These vesicles were reported to be twice as stable as those made from hydrogenated egg PC/cholesterol (1:1 molar ratio). In the current study, when the stability of multilamellar archaeosomes prepared from *T. acidophilum* TPL was assessed under the conditions used to evaluate DODPC polymerized liposomes above, 50% of the CF was retained after 90 min, and about 15% after 5 h. There was no difference in these observations when taurocholate was replaced by SHB. The substantial improvement in stability (Fig. 6 vs. Fig. 5B) could be due to the much lower amount of phospholipase (5 U/ml of A_2 in place of 3333 U/ml of pancreatic lipase), and much higher amount of liposomal lipid (6 mg/ml in place of ~ 3.5 μg/ml) in the assay, illustrating the importance of these parameters in making comparisons. The differences in assay pH may also have an impact.

The toxicity and the extent of biodegradability of polymerized liposomes, as well as the release of entrapped drugs in vivo, needs to be examined (Ausborn et al., 1992; Okada et al., 1995; Chen et al., 1996). Also, concerns regarding biocompatibility have been raised due to interactions between certain methacryloyl-based polymerizeable lipids and blood components (Bonté et al., 1987). Detailed in vivo and in vitro toxicological studies on the main polar lipid, which constitutes 80% of the TPL from *T. acidophilum*, have indicated no biocompatibility related concerns (Freisleben et al., 1993, 1995). Work from our own laboratories, involving archaeosomes prepared from the TPL of many different archaeobacteria, indicate that so far there are no observable toxicity related concerns (Tolson et al., 1996; Makabi-Panzu et al., 1998).

The in vitro stability of archaeosomes indicate that some formulations are more stable in the GI tract environment than many of the liposomes made from conventional ester phospholipids and cholesterol. The current study suggests that archaeosomes merit detailed in vivo evaluation for developing oral delivery applications.

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