Calcium-induced aggregation of archael bipolar tetraether liposomes derived from the thermoacidophilic archaeon *Sulfolobus acidocaldarius*

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**Summary** Previously, we showed that the proton permeability of small unilamellar vesicles (SUVs) composed of polar lipid fraction E (PLFE) from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* was remarkably low and insensitive to temperature (Komatsu and Chong 1998). In this study, we used photon correlation spectroscopy to investigate the time dependence of PLFE SUV size as a function of Ca²⁺ concentration. In the absence of Ca²⁺, vesicle diameter changed little over 6 months. Addition of Ca²⁺, however, immediately induced formation of vesicle aggregates with an irregular shape, as revealed by confocal fluorescence microscopy. Aggregation was reversible upon addition of EDTA; however, the reversibility varied with temperature as well as incubation time with Ca²⁺. Freeze-fracture electron microscopy showed that, after a long period of incubation (2 weeks) with Ca²⁺, the PLFE vesicles had not just aggregated, but had fused or coalesced. The initial rate of vesicle aggregation varied sigmoidally with Ca²⁺ concentration. At pH 6.6, the threshold calcium concentration (Cₚ) for vesicle aggregation at 25 and 40 °C was 11 and 17 mM, respectively. At pH 3.0, the Cₚ at 25 °C increased to 25 mM. The temperature dependence of Cₚ may be attributable to changes in membrane surface potential, which was −22.0 and −13.2 mV at 25 and 40 °C, respectively, at pH 6.6, as determined by 2-(p-toluidinyl)naphthalene-6-sulfonic acid fluorescence. The variation in surface potential with temperature is discussed in terms of changes in lipid conformation and membrane organization.

**Keywords:** fluorescence, light scattering, membranes, microscopy, pH, surface potential, temperature, vesicle size.

**Introduction**

The major lipid components of the plasma membrane of the thermoacidophilic archaeon *Sulfolobus acidocaldarius* are tetraether lipids (De Rosa et al. 1986, Langworthy and Pond 1986, Kates 1992), among which the polar lipid fraction E (PLFE) is the main constituent (Lo and Chang 1990). The PLFE contains a mixture of bipolar tetraether lipids with either a glycerol dialkyl calditol tetraether (GDNT; also called calditoglycerocaldarchaeol) or a glycerol dialkyl glycerol tetraether (GDGT; also called caldarchaeol) skeleton (Figure 1) (Lo and Chang 1990, Sugai et al. 1995, Gliozzi et al. 2002). Both GDGT and GDNT have bisubstituted polar head groups and are thus designated bipolar tetraether lipids. Glycerol dialkyl calditol tetraether (~90% of total PLFE) has phospho-myo-inositol on the glycerol end and β-glucose on the calditol end, whereas GDGT (~10% of total PLFE) has phospho-myo-inositol attached to one glycerol and β-D-galactosyl-D-glucose to the other glycerol skeleton. The phospho-myo-inositol groups of PLFE lipids are oriented toward the cytoplasmic side of the cell’s plasma membrane (De Rosa et al. 1983). The nonpolar regions of these lipids consist of a pair of 40-carbon biphytanyl chains, each of which contains up to four cyclopentane rings. The number of cyclopentane rings increases with increasing growth temperature (De Rosa et al. 1980).

In aqueous solution, PLFE lipids form stable multilamellar and unilamellar liposomes (Lo and Chang 1990, Elferink et al. 1992, Bagatolli et al. 2000) in which the lipids span the entire lamellar structure, forming monomolecular membranes (Elferink et al. 1992). Liposomes composed of PLFE lipids exhibit rates of proton permeation and dye leakage that are unusually low and insensitive to temperature (In’t Veld et al. 1992, Chang 1994, Elferink et al. 1994, van de Vossenberg et al. 1995, Komatsu and Chong 1998). These traits have been attributed to the unique chemical structure of bipolar tetraether lipids and their organization within membranes, particularly the network of hydrogen bonds between polar head groups, the rigid and tight packing of lipids within the membrane, and the negative charges on the membrane surface (Elferink et al. 1994, Relini et al. 1994, van de Vossenberg et al. 1995, Komatsu and Chong 1998). The remarkable thermostability of proton permeation across PLFE membranes has provided a partial explanation of how *S. acidocaldarius* can grow at high temperatures (65–83 °C) (van de Vossenberg et al. 1995) in...
acidic environments (pH 2–3) and yet maintain the intracellular compartment at pH 6.5 (Brock et al. 1972).

In addition to thermal and acid stability, PLFE and other archaeal bipolar tetraether liposomes exhibit remarkable resistance to mechanical stress and the actions of phospholipases, bile salts and serum proteins (reviewed in Gliozzi and Relini 1996). Because of their extraordinary stability, which permits sterilization (Choquet et al. 1994) and filtration (Bauer et al. 1983), bipolar tetraether liposomes have found wide application in immunoassays (Tomioka et al. 1994) and in vaccine and drug delivery (Ring et al. 1986, Sprott 1992, Elferink et al. 1994, Freisleben et al. 1995, Gliozzi and Relini 1996, Sprott et al. 1997, Krishnan et al. 2000, Patel et al. 2000). Although stability against macrophages and other bioactive species is desirable in liposomes used as drug delivery vehicles, high membrane stability also hinders the release of entrapped drugs. Therefore, a compromise between membrane stability and drug release is required. It is known that drug release from liposomes composed of typical phospholipid bilayers can be facilitated via fusion and lipid mixing, both of which are preceded by vesicle aggregation (Boni et al. 1984, Bentz and Duzgunes 1985). Hence, it is of interest to investigate the mechanisms underlying the aggregation and fusion processes of PLFE liposomes.

Previous studies (Relini et al. 1994, 1996) reported that fusogenic agents such as calcium chloride and polyethylene glycol induced fusion between vesicles derived from total lipid extracts of the thermoacidophilic archaeon Sulfolobus solfataricus. However, with vesicles made from the P2 bipolar tetraether lipid fraction of the same archaeon, only vesicle aggregation, not fusion, was observed. The total lipid extracts of S. solfataricus contain both bisubstituted (e.g., GDGT and GDNT) and monosubstituted tetraether lipids, whereas the P2 fraction contains only bisubstituted tetraether lipids (reviewed in Gliozzi et al. 2002). Liposomal membranes made from the P2 fraction have a strict lamellar structure (Gulik et al. 1988), and fusion occurs only when this lamellar structure is disturbed (Relini et al. 1994, 1996). Furthermore, Ca²⁺-induced aggregation or fusion of bipolar tetraether liposomes occurs on the time scale of tens of minutes (Relini et al. 1994), which is much slower than that of monopolar diester liposomes (Wilschut et al. 1980, Hui et al. 1988). Moreover, unlike most monopolar diester lipid membranes, bipolar tetraether lipid monolayers show no detectable changes in surface tension even when fusion occurs (Relini et al. 1994). As such, bipolar tetraether liposomes possess many unusual properties related to membrane aggregation and fusion that warrant further study.

In this study, we have focused on the calcium-induced aggregation of PLFE liposomes derived from the thermoacidophilic archaeon S. acidocaldarius. The PLFE fraction of S. acidocaldarius is equivalent to the P2 fraction of S. solfataricus (reviewed in Gliozzi et al. 2002). Aggregation was monitored in this study because it is the first step involved in
membrane fusion and lipid mixing and because aggregation of bipolar tetraether liposomes has not been studied extensively. In the present study, dynamic light scattering revealed that, in the absence of Ca\(^{2+}\), there was little change in PLFE vesicle diameter over 6 months. Addition of sufficient amounts of Ca\(^{2+}\), however, induced immediate vesicle aggregation, which was largely reversible by EDTA. The initial rate of Ca\(^{2+}\)-induced vesicle aggregation and the reversal of this aggregation by EDTA were examined at different temperatures, pH, and Ca\(^{2+}\) concentrations. The morphology of the aggregates was studied by freeze-fracture and confocal fluorescence microscopy. The temperature and pH dependencies of the threshold Ca\(^{2+}\) concentration for vesicle aggregation are discussed in relation to changes in membrane surface potential and lipid organization and conformation.

Materials and methods

Materials

* Sulfolobus acidocaldarius* cells (strain DSM639, ATCC, Rockville, MD) were grown aerobically and heterotrophically at 69–70 °C and pH 2.5–3.0. Growth was monitored by absorbance at 420 and 540 nm. Cells were harvested just before the stationary phase. Polar lipid fraction E lipids were isolated from dry cells as previously described (Lo and Chang 1990).

1-Palmitoyl-2-oleoyl-L-α-phosphatidylcholine (POPC) and N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (N-NBD-PE) were purchased from Avanti Polar Lipids (Alabaster, AL) and 2-(p-toluidinyl)naphthalene-6-sulfonate (TNS) was obtained from Molecular Probes (Eugene, OR). Cholesterol (Sigma, St. Louis, MO) was recrystallized from ethanol.

Liposome preparation

For vesicles without fluorescent probe, PLFE was dispersed in 64:25:4 chloroform:methanol:water (v/v). The solvents were evaporated under nitrogen and the lipid was dried under high vacuum for ~12 h. Vesicles with the fluorescent probe N-NBD-PE were generated in two steps (Khan and Chong 2000). First, PLFE and N-NBD-PE were mixed in 64:25:4 chloroform:methanol:water (v/v), then dried as described above. Second, the dried lipid film was resuspended in 65:25:10 chloroform:methanol:water (v/v) and again dried under nitrogen followed by high vacuum for ~12 h.

To the dried PLFE film (with or without N-NBD-PE), appropriate amounts of either 100 mM citric acid/sodium citrate buffer (pH 3.0) or 50 mM KCl (pH 6.6) containing 0.02% NaN\(_3\) were added. The mixture was vigorously vortexed at 65 °C for 12 min to generate multilamellar vesicles (MLVs). Unilamellar vesicles were made from MLVs by the freeze-thaw and extrusion method (Hope et al. 1985). Briefly, MLVs were subjected to at least five cycles of freezing (dry ice/acetone) and thawing (65 °C) and were then extruded at 65 °C in a lipid extruder (Lipex, Vancouver, BC) through two stacked polycarbonate membranes (1 μm pore size for conformational experiments and 50, 100 or 200 nm pore sizes for the other experiments) under N\(_2\) gas pressure (400, 600 and 5000 kPa for pore sizes of 1 μm, 200 nm and < 200 nm, respectively). In N-NBD-PE-labeled PLFE liposomes, the ratio of probe to PLFE was 1:500.

Measurement of surface potential

To determine the surface potential of PLFE liposomes, the steady-state fluorescence intensity of the membrane probe TNS was measured. This probe is an anionic amphiphilic molecule that binds strongly to lipid membranes (Huang and Charlton 1972, Easter et al. 1978, Lakowicz and Hogen 1981), mainly via van der Waals interactions (Seeleg and Ganz 1991). In aqueous medium, TNS has a low quantum yield; however, upon binding to lipid vesicles, the fluorescence intensity of TNS (I\(_f\); dimensionless) is enhanced. Both I\(_f\) and the concentration of TNS in the bulk solution ([TNS]\(_b\); µM) are related to membrane surface potential (Ψ\(_o\); mV) according to Equation 1 (Eisenberg et al. 1979, Cafiso et al. 1989):

\[
I_f / [TNS]_b = \beta [L] e^{F \Psi_o / RT}
\]

where β is a proportionality constant, F is the Faraday constant (9.65 x 10\(^4\) C mol\(^{-1}\)), R is the gas constant (8.3145 J mol\(^{-1}\) K\(^{-1}\)), T is absolute temperature and [L] is lipid concentration (µM).

We measured I\(_f\) as a function of [TNS]\(_b\) at a fixed lipid concentration (16 µM). At constant [L], there is a linear relationship between I\(_f\) and [TNS]\(_b\), and the slope of the plot of I\(_f\) versus [TNS]\(_b\) gives Ψ\(_o\). Appropriate amounts of TNS were incubated with lipid vesicles at the desired temperature for 4–6 h prior to measurement of I\(_f\); thereafter, no further enhancement of fluorescence was observed. This experiment was performed with two lipid systems: PLFE unilamellar vesicles and POPC unilamellar vesicles containing 19 mol% cholesterol (prepared as described in Komatsu and Chong 1998). At neutral pH, the Ψ\(_o\) of the zwitterionic lipid POPC is zero. Then,

\[
I_{PLFE}[TNS]_{b,PLFE} / I_{POPC}[TNS]_{b,POPC} = e^{F \Psi_o,PLFE / RT}
\]

where I\(_{PLFE}\) and I\(_{POPC}\) are the fluorescence intensities of TNS with PLFE and POPC liposomes, respectively, Ψ\(_o,PLFE\) is the surface potential of PLFE liposomes and [TNS]\(_{b,PLFE}\) is the bulk concentration of TNS when the designated lipid is used. The liposomes with probe were stirred while fluorescence intensity was measured with an SLM 8000C fluorometer (SLM Instruments, Urbana, IL). The excitation wavelength was 320 nm (2-nm band-pass) and the emission was observed between 380 and 560 nm (8-nm band-pass). Sample temperatures were maintained with a circulating bath. Blank readings (vesicles or TNS in buffer) were subtracted from the sample I\(_f\) readings.

Photon correlation spectroscopy

The hydrodynamic diameters of the extruded PLFE vesicles were measured by photon correlation spectroscopy using a
Malvern Zetasizer 1000HAS spectrometer (Malvern Instruments, Worcestershire, U.K.). The light source was a 10 mW He-Ne laser (633 nm) and the scattered light was measured at a right angle using an avalanche photodiode detector. The instrument was calibrated with 200-nm latex beads in 10 mM NaCl. The solution was filtered through a 0.22-µm syringe filter and degassed prior to measurements. Because very dilute solutions were used, refractive index and viscosity values for water were used for calculation of hydrodynamic diameters. Specifically, the refractive index was 1.33 at all temperatures examined, and the viscosity was 0.891, 0.653 and 0.488 cP (1 cP = 0.001 Pa s) at 25, 40 and 54 °C, respectively (Weast 1987). Data were analyzed with the Contin algorithm (Malvern Instruments), which calculates the Z-average size and polydispersity. The former is the mean vesicle hydrodynamic diameter and the latter is a measure of the width of the vesicle size distribution. To induce aggregation, various volumes of 204 mM CaCl2 were added to a solution of PLFE vesicles. To reverse aggregation, an appropriate amount of 100 mM EDTA was added.

Freeze-fracture electron microscopy

Samples without cryoprotectant were placed between copper specimen carriers (Balzers, Liechtenstein) and rapidly plunged into liquid propane. Fracturing and replicating was performed at −115 °C in a Balzers BAF 400T Freeze-Etch unit. Replicas were cleaned with NaClO3 solution and viewed on a Philips 300 transmission electron microscope at a typical magnification of 27,000×.

Confocal fluorescence microscopy

Liposomes labeled with N-NBD-PE were incubated with or without Ca2+ and then adsorbed to a glass coverslip. Images were captured with a Model TCS-SP confocal laser scanning microscope (Leica Microsystems, Exton, PA) using an argon/krypton laser for excitation at 488 nm. Fluorescence (around ~534 nm) was detected in the photon counting mode as a single channel through a 100× oil-immersion lens.

Results and discussion

In this study, we investigated the kinetics of the change in size of PLFE SUVs as a function of Ca2+ concentration. The photon correlation spectroscopy data show that, in the absence of Ca2+, the mean diameter of PLFE SUVs in 50 mM KCl is stable for 6 months at room temperature (~24 °C) (Figure 2). Furthermore, the mean size of PLFE SUVs remained constant at temperatures ranging from 25 to 55 °C (data not shown).

The addition of Ca2+, however, had a pronounced effect on particle size. Figure 3 shows the effect of Ca2+ concentration on mean PLFE particle diameter over time in 50 mM KCl at 40 °C. At low Ca2+ concentrations (e.g., < 10.2 mM), PLFE unilamellar vesicles showed no appreciable change in size over time. However, at higher Ca2+ concentrations (e.g., 15.0–25.1 mM), mean particle diameter increased over time (Figure 3). The vesicle size distribution also grew broader over time. The increase in particle size is attributable either to vesicle aggregation or to fusion or coalescence (lipid mixing). In either case, the change in size is presumably triggered by binding of Ca2+ to the negatively charged phospho-myo-inositol groups, followed by charge neutralization, complex formation and membrane structural changes (Wilschut et al. 1985). Figure 3 also shows that the rate of Ca2+-induced aggregation or fusion of PLFE liposomes is low (on the order of tens of minutes) compared with the rate of aggregation of negatively charged monopolar diester liposomes at comparable Ca2+ and lipid concentrations (on the order of seconds) (Sundler and Papahadjopoulos 1981).

Confocal fluorescence microscopy also showed that, in the absence of Ca2+, the diameter of the extruded vesicles was rel-
atively uniform (initially about 500 nm; Figure 4A). However, at high Ca\(^{2+}\) concentrations, vesicles formed aggregates with irregular shapes and heterogeneous sizes (Figure 4B). These observations are supported by the photon correlation spectroscopy data, which revealed an increase in size as well as a broadening of the size distribution. Note that the two studies used different vesicle preparations with different initial vesicle sizes.

As shown in Table 1, the increase in PLFE particle size following incubation with 12.5 mM Ca\(^{2+}\) for 4 h at 25 °C was reversed (~98%) by addition of a twofold excess of EDTA (Wilschut et al. 1980, 1981), suggesting that little vesicle fusion had occurred. In conjunction with the decrease in size, the polydispersity also decreased (data not shown). Furthermore, the data in Table 1 demonstrate that the reversal of particle size change by EDTA is dependent on both temperature and the incubation time of the vesicles with Ca\(^{2+}\). At higher temperatures, and when the incubation time with Ca\(^{2+}\) is increased, the degree of reversal engendered by EDTA is decreased. These results strongly suggest that Ca\(^{2+}\) causes not only PLFE vesicle aggregation, but also some vesicle fusion or coalescence. However, if the Ca\(^{2+}\)-induced size increase had been a result of fusion or coalescence only, the size increase would have been completely nonreversible by a twofold excess of EDTA (Leventis et al. 1986). These results are in agreement with previous findings that, at room temperature, Ca\(^{2+}\) induces aggregation, but not fusion, of vesicles made from the P2 lipid fraction from *S. solfataricus* (Relini et al. 1994, 1996). The PLFE fraction from *S. acidocaldarius* and the P2 fraction from *S. solfataricus* both consist primarily of bisubstituted tetraether lipids carrying negative charges at neutral pH.

A possible explanation for the limited fusion of PLFE li-
posomes at 25 °C after a brief (4-h) incubation with Ca²⁺ is the rigid and tight membrane packing in these liposomes (Kao et al. 1992, Chang 1994, Komatsu and Chong 1998, Bagatolli et al. 2000, Gabriel and Chong 2000, Khan and Chong 2000). Vesicle fusion would require a membrane defect or destabilization (Papahadjopoulos et al. 1977, 1990, Hui et al. 1981). Another possibility is that the bulky hydrated inositol group sterically inhibits fusion by preventing the formation of dehydrated complexes between the apposed PLFE vesicles, as is the case with phosphatidylinositol membranes, which do not undergo fusion following Ca²⁺-induced aggregation (Sundler and Papahadjopoulos 1981). In addition, a greater amount of energy would be expected to be required for the molecular rearrangements necessary for fusion of liposomes composed of bipolar lipids, because one head group would be expected to completely traverse the lipid core. Coalescence of liposomes composed of bipolar lipids would not occur in the manner typically observed for fusion of liposomes composed of monopolar lipids, where single monolayers interact to form hemifusion intermediates (Ellens et al. 1989). Note that, in many monopolar diester liposomes, divalent cation-induced vesicle aggregation is followed by immediate membrane fusion, which cannot be reversed by addition of EDTA.

Fusion or coalescence may occur as a result of impurities in the PLFE (Chang and Lo 1991). An alternative explanation is that calcium ions induce lateral segregation of the bulky phospho-myo-inositol groups in the plane of the membrane. As a result, the less bulky polar head groups (i.e., those with nonitol and glucose) may become more readily susceptible to dehydration, leading to hydrophobic interactions between the apposed vesicles and eventually to fusion or lipid mixing. Lipid lateral reorganization could be a slow process, on the time scale of minutes to days; this may explain why incubation of PLFE vesicles with Ca²⁺ for a longer period of time lowers the reversibility of vesicle size change by EDTA (Table 1).

Table 1. Effect of EDTA on Ca²⁺-induced aggregation of PLFE vesicles.

| Temperature (°C) | Initial particle diameter (nm) | [Ca²⁺] (mM) | Incubation time (h) | Resulting particle diameter (nm) | EDTA (mM) | Incubation time (h) | Final particle diameter (nm) |
|-----------------|-------------------------------|-------------|--------------------|---------------------------------|-----------|--------------------|-----------------------------|
| 25              | 117                           | 12.5        | 4                  | 1245                            | 25        | 12                 | 140                         |
| 25              | 90                            | 10.0        | 154                | 2545                            | 20        | 10                 | 180                         |
| 40              | 89                            | 19.6        | 120                | 2374                            | 40        | 12                 | 352                         |

Figure 5. Electron micrographs of freeze-fracture replicas of 500 µl of 0.4 mg ml⁻¹ PLFE unilamellar vesicles in 50 mM KCl. (A) Vesicles without Ca²⁺; mean vesicle diameter ~84 nm. (B) Vesicles incubated with 15.1 mM CaCl₂ for 2 weeks. Magnification = 46,000×.
electron microscopy data are in agreement with observations made by Lo and Chang (1990), who looked at PLFE multilamellar vesicles. We initially observed unilamellar vesicles (Figure 5A), which coalesced to form multilamellar vesicles (multiple rings) after incubation with Ca$^{2+}$ for about 2 weeks (Figure 5B). If the initial unilamellar PLFE vesicles had merely aggregated, not fused or coalesced, then we would have expected the vesicles in Figure 5B to look like those shown in Figure 5A, but at a higher density.

The threshold Ca$^{2+}$ concentration for PLFE vesicle aggregation can be determined from the photon correlation spectroscopy data. The first 15 min of data from the plot of mean vesicle diameter versus aggregation time (e.g., Figure 3) were fitted to a linear line, the slope of which gives the initial rate of vesicle aggregation. The initial rate of PLFE vesicle aggregation varied sigmoidally with Ca$^{2+}$ concentration (Figure 6). The Ca$^{2+}$ concentration corresponding to the midpoint of the abrupt change (50% change) in vesicle size is the threshold calcium concentration ($C_r$) for vesicle aggregation. We found that the $C_r$ increased with increasing temperature, but decreased with increasing pH (Table 2).

Because membrane surface charge may affect Ca$^{2+}$ binding and thus change the value of $C_r$, we determined the temperature dependence of the membrane surface potential of PLFE vesicles using TNS fluorescence. The surface potential of PLFE SUVs at pH 6.6 and 25 °C was determined to be –22 mV (Table 2), which is comparable with the published zeta potential (–34.3 mV) for PLFE SUVs of similar sizes at pH 7.6 and 20 °C (Komatsu and Chong 1998). The surface potential of PLFE liposomes decreased (i.e., became less negative) with increasing temperature (Table 2). When the surface potential is less negative, more calcium ions are required to form stable complexes with PLFE vesicles. This explains why $C_r$ increases with increasing temperature (Table 2). The same rationale explains why $C_r$ increases with decreasing pH (Table 2). The negative charge on the phospho-myo-inositol group in PLFE lipids should be significantly lower at pH 3.0 than at pH 6.6, leading to a lower surface potential on PLFE liposomes and a higher $C_r$ at pH 3.0 than at pH 6.6. This interpretation is consistent with the observed decrease in the threshold calcium concentration for lipid mixing with increasing phosphatidic acid (PA) content in PA–phosphatidylcholine mixtures (Leventis et al. 1986), as PA is negatively charged at neutral pH.

Temperature may induce changes in polar head group orientation, leading to changes in dipole potential. However, Cafiso et al. (1989) found that the absorption of TNS to monopolar lipid membranes is independent of the dipole potential of the lipid head group, presumably because the charge of TNS is located such that it is unaffected by a change in dipole potential. Therefore, it is unlikely that a change in lipid dipole potential is responsible for the change in surface potential with temperature (Table 2), unless the location of TNS in PLFE liposomes is different from its location in non-archaeal monopolar lipid membranes.

Another possible explanation for the decrease in surface potential with increasing temperature is that higher temperatures induce more phospho-myo-inositol moieties to turn toward the intravesicular side of the membrane. This explanation would be consistent with the previous finding that, in vivo, the phospho-myo-inositol moieties of bipolar tetraether lipids mainly face the intracellular compartment of thermoacidophilic archaea at the high growth temperatures characteristic of these microorganisms (De Rosa et al. 1983). However, from an energetic point of view, flip-flop of PLFE lipids in tightly packed membranes would be difficult to achieve. Nevertheless, a change in lipid transmembrane asymmetry has previously been proposed to explain why the rate and temperature sensitivity of proton permeation across PLFE liposomal membr-

![Figure 6](http://archaea.ws)
branes are reduced as vesicle diameter decreases (Komatsu and Chong 1998). We cannot exclude the possibility that a temperature-induced change in lipid lateral organization (Bagatolli et al. 2000) (rather than transmembrane asymmetry) in the PLFE membrane affects TNS binding and fluorescence, thus changing the surface potential.

Archaeal bipolar tetraether liposomes have wide technological applications (Ring et al. 1986, Tomioka et al. 1994, Freisleben et al. 1989, Eisenberg et al. 1979, Bentz and Duzgunes 1985). We cannot exclude the possibility that a temperature-induced change in lipid lateral organization (Bagatolli et al. 2000) (rather than transmembrane asymmetry) in the PLFE membrane affects TNS binding and fluorescence, thus changing the surface potential.

Table 2. Effects of pH and temperature on the threshold Ca\(^{2+}\) concentration \((C_t)\) for PLFE vesicle aggregation and on the surface potential \((\Psi_s)\) of PLFE vesicles (117 nm in diameter) prior to aggregation.

| pH   | Temperature (°C) | \(C_t\) (mM) | \(\Psi_s\) (mV) |
|------|------------------|--------------|----------------|
| 6.6  | 25               | 11           | \(-22.00 \pm 0.003\) |
| 6.6  | 40               | 17           | \(-13.20 \pm 5.30\) |
| 6.6  | 54               | 18           | \(-10.28 \pm 3.49\) |
| 3.0  | 25               | 25           | ND\(^1\)          |
| 3.0  | 40               | 60           | ND               |

\(^1\) ND = not determined, because the TNS method works well only at neutral pH.

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