

Thermotropic phase behaviour of mixed liposomes of archaeal diether and conventional diester lipids

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Abstract The phase behaviour and phase stability of mixed liposomes prepared from diether polar lipids isolated from thermophilic Archaea and from conventional diester dipalmitoyl-L- α -phosphatidylcholine (DPPC) at different molar ratios were investigated by differential scanning calorimetry. The permeability of these mixed liposomes was investigated as a function of temperature, by measuring the fluorescence emission intensity for the release of calcein in the temperature range from 20 to 98 °C. Our data show that diether polar lipids isolated from hyperthermophilic Archaea can form mixed liposomes with synthetic α -DPPC. Liposomes prepared from pure archaeal lipids do not show the characteristic gel-to-liquid crystalline phase transition characteristics of DPPC liposomes in the temperature range from 0 to 100 °C. In the presence of 5 mol% archaeal lipids in mixed α -DPPC liposomes, the temperature of the phase transition decreases and the broadness of the peak increases, although the enthalpy change (ΔH) of the phase transition is not significantly influenced. At molar ratios of archaeal lipids greater than 50 mol% in these mixed liposomes, the typical gel-to-liquid crystalline phase transition disappears, which indicates that the lipids are predominantly in the liquid crystalline state. At ratios of archaeal lipids greater than 50 mol% in these mixed liposomes, their permeability for anionic calcein is the same as for liposomes made from

100 mol% archaeal lipids, across the whole temperature range.

Keywords Archaeal lipids · Dipalmitoyl-L- α -phosphatidylcholine · Differential scanning calorimetry · Phase behaviour · Liposome · Calcein release

Introduction

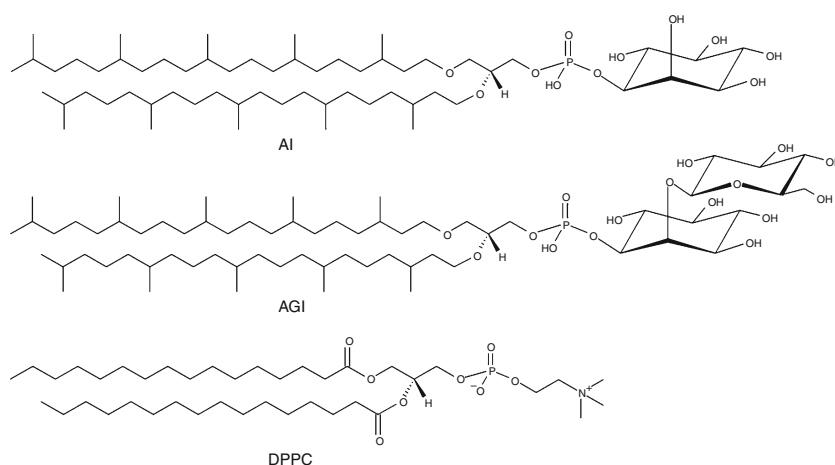
Archaeal lipids have been shown to differ from bacterial phospholipids in four major ways: (i) the glycerophosphate backbone of archaeal lipids is composed of *sn*-glycerol-1-phosphate, which is an enantiomer of the *sn*-glycerol-3-phosphate in bacterial phospholipids; (ii) the hydrocarbon chains in archaeal polar lipids are bonded to the glycerol moiety exclusively by ether linkages, in contrast to bacterial lipids, most of which have ester linkages; (iii) the hydrocarbon chains of polar lipids in Archaea are highly methyl-branched isoprenoids and isoprenoids, whilst the bacterial counterparts are mostly straight-chain fatty acids; and (iv) significant numbers of Archaea species contain bipolar lipids with a tetraether core that spans the membranes (bolaform amphiphilic), which are rarely found in bacteria [1].

The lipids of the aerobic hyperthermophilic archaeon *Aeropyrum pernix* are different from those of the anaerobic sulphur-dependent hyperthermophiles in terms of a lack of tetraether lipids and the direct linkage of inositol and sugar moieties [2]. The core lipid consists solely of C_{25,25}-archaeol (2,3-di-sesterpanyl-*sn*-glycerol). Then, with a glucosyl-inositol polar head-group, C_{25,25}-archaeolyl(glucosyl)inositol (AGI) accounts for 91 mol%, and with a myo-inositol polar head-group, C_{25,25}-archaeolylinositol (AI) accounts for the remaining 9 mol% [2] (Fig. 1). The chain lengths of these C₂₅-isoprenoid hydrocarbons are 20% longer than

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Fig. 1 Structures of 2,3-di-*O*-sesterterpanyl-*sn*-glycerol-1-phospho-*myo*-inositol ($C_{25,25}$ -archaetidylinositol) (*top* AI), 2,3-di-*O*-sesterterpanyl-*sn*-glycerol-1-phospho-1'-(2'-*O*- α -D-glucosyl)-*myo*-inositol ($C_{25,25}$ -archaetidyl(glucosyl)-inositol) (*middle* AGI), and 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (*bottom* DPPC)



those of the C_{20} -isoprenoids and C_{18} straight-chain fatty acids. Therefore, with the membrane of *A. pernix* composed of only $C_{25,25}$ -archaeol-based lipids, its thickness should be 20% greater than membranes of the $C_{20,20}$ -archaeol-based lipids of other Archaea [2].

Recently, we characterised liposomes prepared from total polar lipids isolated from *A. pernix* physicochemically [3]. Our results indicated that liposomes made of $C_{25,25}$ lipids do not show the typical cooperative gel-to-liquid crystalline phase transition in the temperature range from 0 to 100 °C, as determined by differential scanning calorimetry (DSC) and other applied techniques. However, a very broad gradual transition was observed in the temperature range from 0 to 40 °C, which coincided with increasing fluidity of the membrane and its permeability for entrapped calcein. This permeability of $C_{25,25}$ liposomes for entrapped calcein was substantially lower across the whole temperature and pH ranges, in comparison to liposomes prepared from dipalmitoyl-*L*- α -phosphatidylcholine (DPPC).

The role of archaeal lipids as vaccine adjuvants [4] and the possibility to use liposomes formed from archaeal lipids as delivery systems for drugs, genes, and proteins provide incentives to investigate the interactions between archaeal and eukaryotic lipids. Our studies of the physicochemical properties of model membranes formed of archaeal lipids and standard phospholipids, e.g., DPPC, have provided insight into the mechanisms of mixed liposome formation, and have allowed us to create new delivery systems with lower contents of archaeal lipids. In this study, we have continued on from our previous study, and we report here how increasing the mol% of $C_{25,25}$ lipids in mixed $C_{25,25}$ -DPPC liposomes affects the thermal stability and permeability of DPPC-based liposomes. To achieve this, we used a combination of DSC and measurements of the fluorescence emission intensity of calcein released from mixed $C_{25,25}$ -DPPC liposomes, in the temperature range from 20 to 100 °C.

Experimental

Isolation and purification of lipids and vesicle preparation

The polar-lipid methanol fraction from the lyophilised *A. pernix* cells was composed of approximately 91% AGI and 9% AI [2] (average molecular mass of $1181.42 \text{ g mol}^{-1}$), and these were purified as described previously [3]. After isolation, the lipids were fractionated using adsorption chromatography [5]. The polar-lipid methanol fraction was used for further analysis. Organic solvents were removed under a stream of dry nitrogen, followed by removal of the last traces under vacuum. For preparation of mixed liposomes, the appropriate weights of the $C_{25,25}$ lipids and DPPC were dissolved in chloroform and mixed together in glass round-bottomed flasks. The lipid film was prepared by drying the sample on a rotary evaporator. For preparation of pure DPPC lipid film, the solvent used was chloroform:methanol (7:3, v/v). The dried lipid films were then hydrated with warm ($\sim 45 \text{ °C}$) 20 mM HEPES buffer, pH 7.0, for thermal analysis. The mol% of the diether $C_{25,25}$ lipids in the mixed $C_{25,25}$ -DPPC liposomes were: 0 (pure DPPC), 5, 25, 50, 75, and 100 (pure $C_{25,25}$). Multilamellar vesicles (MLVs) were prepared by vortexing the lipid suspensions for 10 min.

DSC

The phase transitions of MLVs prepared from mixed $C_{25,25}$ -DPPC liposomes containing various mol% of $C_{25,25}$ (0, 5, 25, 50, 75, and 100) were measured using a Nano DSC series III (Calorimetry Science, Provo, UT). The lipid concentrations in the MLVs were 1.0 mg mL^{-1} in 20 mM HEPES buffer (pH 7.0). The MLV samples were loaded into the calorimetric cell, and each sample was heated/cooled repeatedly in the temperature range from 0 to

100 °C. The heating/cooling rate was 1 °C min⁻¹. The first DSC scan was used to obtain the phase-transition temperature, T_m , and the calorimetric enthalpy change, ΔH , as previously described [6]. Subsequent scans were used to assess the reversibility of the phase transition.

Fluorescence emission intensity of calcein

Vesicles filled with calcein were prepared by hydrating the dried lipids with 80 mM calcein (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 20 mM HEPES aqueous solution. To decrease the turbidity, the samples were sonicated in ice for a total of 30 min, with 10-s on-off cycles at 50% amplitude using a Vibracell Ultrasonic Disintegrator VCX 750 (Sonics and Materials, Newtown, USA). Gel filtration on Sephadex G-50 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) columns was used to remove the extra-vesicular calcein. Calcein release from liposomes was assayed using a Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave, Australia). The temperature-dependent release of calcein from mixed C_{25,25}-DPPC liposomes of 0, 25, 50, 75, and 100 mol% C_{25,25} was studied at pH 7.0 in the temperature range of 20–98 °C, with a heating rate of 1 °C min⁻¹. The concentration of lipids used in these experiments was 100 μM. The intrinsic emission fluorescence of calcein was measured at 517 nm with an excitation wavelength of 490 nm. A 5-nm excitation and emission slit width was used. The percentage of calcein released was calculated according to Eq. 1:

$$\text{Percentage of released calcein} = (F - F_{\min}/F_{\max} - F_{\min}) \times 100, \quad (1)$$

where F is the fluorescence emission intensity (measured at the different temperatures), F_{\min} (base line) is the minimal fluorescence of free calcein given the same emission intensity as the vesicles filled with calcein at the start of each experiment, and F_{\max} is the maximal fluorescence of calcein obtained after addition of 10 μL 16 mM Triton X-100 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), either in a reference cuvette containing 2.5 mL 100 μM SUVs filled with calcein at the beginning of an experiment or in a sample cuvette at the end of an experiment.

Results and discussion

Characterisation of the phase state of C_{25,25}-DPPC lipid MLVs by DSC

It has been shown that tetraether (bolaform) lipid chains are more ordered and less flexible than normal bilayer

membranes. Only at elevated temperatures (80 °C) does the flexibility of the chain environment in tetraether lipid assemblies approach that of fluid bilayer membranes [7]. Archaeal tetraether lipids are an excellent source for the formation of mixed liposomes with enhanced tightness against solute leakage [8]. In this study, we investigated the phase behaviours and permeabilities of mixed C_{25,25}-DPPC liposomes using DSC and the emission fluorescence intensity of calcein.

These C_{25,25} lipids are archaeal diether lipids that can form bilayer membranes that have a thickness that is 20% greater than membranes of tetraether C_{20,20} archaeal-based lipids [2]. As the chain lengths of the DPPC lipids are shorter compared to those of the C_{25,25} lipids, we used transmission electron microscopy (TEM) to see if mixed C_{25,25}-DPPC liposomes can be formed at all molar ratios of the C_{25,25} lipids. Our data show that mixed C_{25,25}-DPPC liposomes can be formed as small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), and MLVs across all investigated molar ratios of C_{25,25} and DPPC (our unpublished data).

By using the DSC measurements, we followed the lipid thermotropic phase behaviour of the mixed C_{25,25}-DPPC liposomes. Figure 2 shows the DSC melting profiles of the zwitterionic DPPC vesicles at pH 7.0 (20 mM HEPES) in the absence and presence of the C_{25,25} lipids at various molar ratios of C_{25,25} to DPPC. In the absence of C_{25,25}, DPPC shows two endothermic transitions at 35.5 ± 0.3 and 41.2 ± 0.3 °C (Fig. 2, Table 1). The first transition at 35.5 ± 0.3 °C corresponds to the melting of the lipid head groups, and the second transition at 41.2 ± 0.3 °C corresponds to the highly cooperative gel-to-liquid crystalline (chain-melting) transition of the lipid side-chains. The

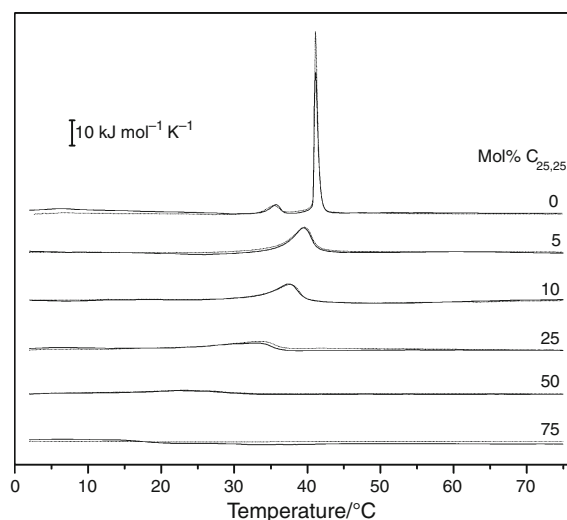


Fig. 2 DSC curves of mixed C_{25,25}-DPPC MLVs at pH 7.0 at different molar ratios of C_{25,25} to DPPC, indicated as mol% C_{25,25} (0, 5, 10, 25, 50, and 75). *Solid lines* first heating scan, *dashed lines* second heating scan

Table 1 Characteristics of the phase state of C_{25,25}-DPPC lipid MLVs shown by DSC

C _{25,25} /mol%	T _m /°C	ΔH/kJ mol ⁻¹	ΔT _{1/2} /°C	T _m (rev)/°C	ΔH (rev)/kJ mol ⁻¹	ΔT _{1/2} (rev)/°C
0	41.2 ± 0.3	35.6 ± 0.4	1.0 ± 0.1	41.5 ± 0.3	36.8 ± 0.4	0.9 ± 0.1
5	39.1 ± 0.9	35.7 ± 0.8	4.5 ± 0.4	39.1 ± 0.8	35.1 ± 0.8	4.7 ± 0.5
10	36.8 ± 1.2	32.3 ± 1.7	7.3 ± 1.2	36.9 ± 0.9	31.8 ± 2.1	7.3 ± 1.0
25	31.2 ± 1.8	23.8 ± 2.1	15.1 ± 2.0	32.0 ± 1.9	22.2 ± 2.1	16.0 ± 2.2
50	21.5 ± 2.4	20.1 ± 2.9	17.8 ± 2.2	21.6 ± 2.1	20.5 ± 3.3	19.2 ± 2.2

ΔH changes in transition enthalpy of the gel-to-liquid crystalline phase, T_m transition temperature, ΔT_{1/2} width of the transition, rev reversibility (for repeated heating scan)

enthalpy change (ΔH) of the gel-to-liquid crystalline transition of DPPC is 35.6 ± 0.4 kJ mol⁻¹, which is in good agreement with the literature values, which range from 35 to 41 kJ mol⁻¹ [9, 10]. At 5 mol% C_{25,25} in the C_{25,25}-DPPC liposomes, the chain-melting transition of DPPC was significantly affected (Fig. 2, Table 1). This suggests that C_{25,25} significantly alters the packing of the hydrocarbon chains in the gel and liquid crystalline states of these zwitterionic DPPC vesicles. At high molar ratios of C_{25,25}, the chain-melting transition of DPPC practically disappears (Fig. 2, Table 1). Previously [3], we showed that C_{25,25} liposomes do not have the typical cooperative gel-to-liquid crystalline phase transition in the temperature range from 0 to 100 °C that is typically observed for DPPC liposomes. The transition peak was very broad (ΔT ~ 30 °C), with the T_m below 20 °C. The height of this transition peak in the C_{25,25} liposomes corresponded to the height of the pre-transition peak in the DPPC curve, whilst ΔH of the transition of C_{25,25} liposomes was comparable to DPPC liposomes [3].

The characteristic physiochemical properties of archaeal tetraether lipids include their phase-transition temperatures, which are far lower than those of fatty acyl ester lipids [11]. DSC has shown that the phase transition point of polar tetraether lipids from *Thermoplasma acidophilum* is between -20 and -15 °C. [12]. Yamauchi et al. [13] reported a phase-transition temperature lower than -20 °C for diphytanyl (diether) liposomes. The phase-transition temperature of fatty acyl ester phospholipids depends on chain length, number of double bonds, and positions of methyl branching. Therefore, archaeal- and caldarchaeol-based polar lipid membranes can be assumed to be in the liquid crystalline phase in the temperature range from 0 to 100 °C, whilst liposomes of fatty acyl diesters are in the gel phase or the liquid crystalline phase across this temperature range, depending on their fatty-acid composition [11]. Gliozzi et al. [14] studied the thermal stability of tetraether lipids isolated from *Caldariella acidophila*. They detected two transitions calorimetrically and reported that the transition at higher temperatures was related to the breaking of hydrogen bonds between the nanitol-containing

polar heads, whilst the lower temperature transition was due to the partial melting of the hydrophobic core. In the excellent study of Chong et al. [15] on differential calorimetry of bipolar tetraether liposomes derived from the thermo-acidophilic archaeon *Sulfolobus acidocaldarius*, they observed that the ΔH in the phase transition of these tetraether liposomes was small [15]. In the case of diether C_{25,25} lipids, the *trans-gouch* conformational transition is likely to be enhanced due to less restriction, which results in a higher ΔH [3] compared to the results of Chong et al. [15]. By studying the effects of increasing molar ratios of C_{25,25} in mixed C_{25,25}-DPPC liposomes, the broadness of the gel-to-liquid crystalline transition increase when compared to DPPC liposomes can be noted (Fig. 2). The ΔH and T_m decrease with increasing mol% C_{25,25} in the mixed C_{25,25}-DPPC liposomes (Fig. 3, Table 1). This broadness of the transition reflects the relatively low cooperativity of the transition, which can be attributed to the liposomes being a mixture of archaeal and DPPC lipids.

Calcein release

One of the main characteristic properties of archaeal lipid membranes is their extremely low permeability to solutes [11]. By incorporating the archaeal lipids into liposomes made of conventional lipids, such as DPPC, we can significantly decrease the permeability of solutes like calcein. Figure 4 shows the temperature-dependent release of calcein at pH 7.0, where the release of calcein from SUVs made solely of C_{25,25} lipids is less temperature sensitive compared to pure DPPC liposomes [3]. With increasing molar ratios of C_{25,25} in these mixed C_{25,25}-DPPC liposomes, the release of calcein decreases compare to the pure DPPC liposomes. Calcein release in the DPPC liposomes rose abruptly at their phase-transition temperature (~42 °C), whilst the release of calcein from the mixed C_{25,25}-DPPC liposomes increased moderately with temperature. At 90 °C, the calcein release from the mixed C_{25,25}-DPPC liposomes at 75 mol% C_{25,25} reached 10% of that of the pure C_{25,25} liposomes (Fig. 4), whilst the calcein released from the mixed liposomes at 50 and 25 mol%

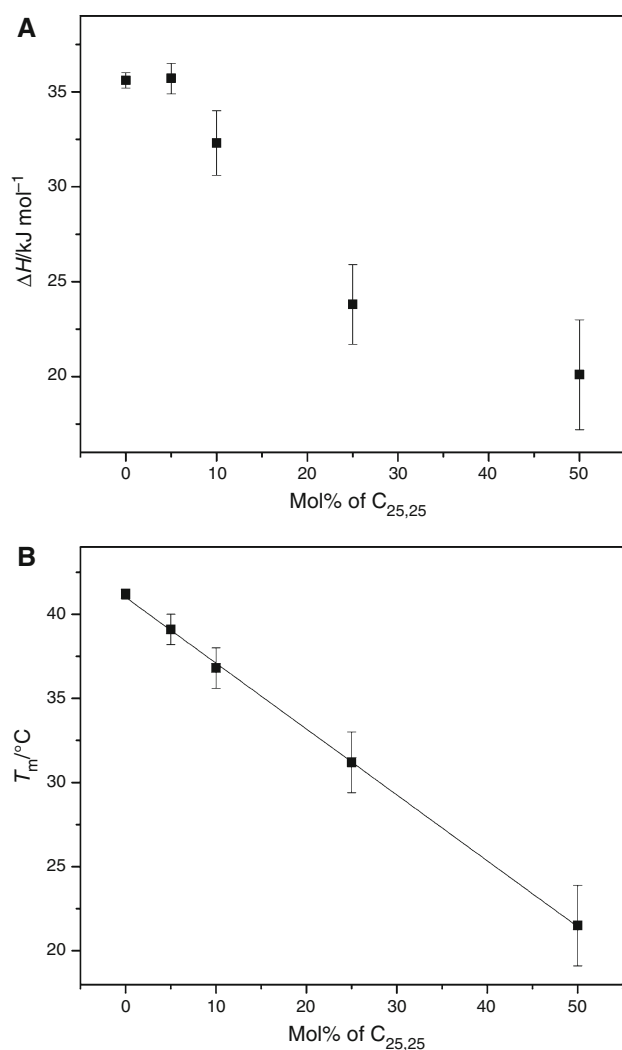


Fig. 3 Effects of increasing the mol% $C_{25,25}$ in mixed $C_{25,25}$ -DPPC liposomes on enthalpy change, ΔH (a) and transition temperature, T_m (b). Data are means \pm SD of three measures

$C_{25,25}$ were 15 and 25%, respectively. Detailed inspection of Fig. 4 reveals that in the temperature range from 0 to 40 $^{\circ}\text{C}$, at 25 and 50 mol% $C_{25,25}$ in these mixed $C_{25,25}$ -DPPC liposomes, the leakage of calcein was higher compared to pure DPPC liposomes (Fig. 4).

Previously, we reported that these chain lengths of the C_{25} -isoprenoid hydrocarbons have lower permeabilities for anionic probes that are due in part to the negative charges of the polar-lipid membrane surface [3]. The unusual stability of $C_{25,25}$ liposomes can be attributed in part to tight and rigid lipid packing, as suggested by our fluorescence measurements. In addition, the release of calcein from $C_{25,25}$ liposomes was less temperature sensitive compared to pure DPPC liposomes in the temperature range from 20 to 98 $^{\circ}\text{C}$ [3]. Recently, Chong et al. [16] showed that low relative volume fluctuations can explain why tetraether liposomes have unusually low levels and low temperature

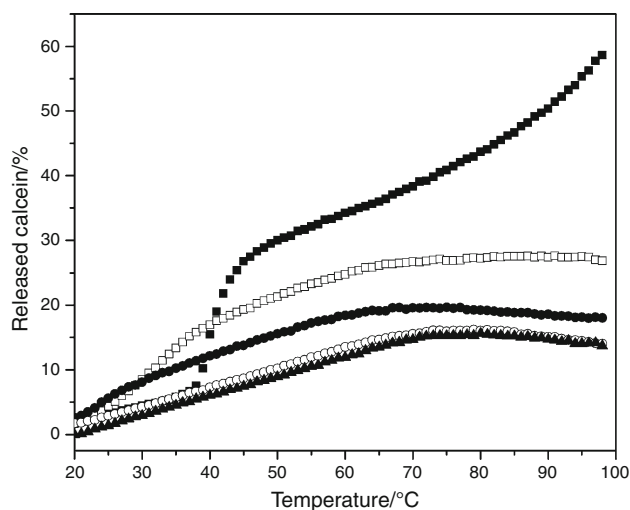


Fig. 4 Temperature-dependant release of calcein from mixed $C_{25,25}$ -DPPC liposomes at pH 7.0 at different molar ratios of $C_{25,25}$ to DPPC, indicated as mol% $C_{25,25}$: 0 (filled square), 25 (open square), 50 (filled circle), 75 (open circle), and 100 (filled triangle)

sensitivities for proton permeation and dye leakage, and limited lateral lipid motion in the membrane. With these tetraether lipids, the phase transition does not involve large changes in the relative volume fluctuation, as might be expected for lamellar phase transitions with minor changes in the chain packing density [16].

Based on our observations here, we suggest that at less than 50 mol% $C_{25,25}$ in these mixed $C_{25,25}$ -DPPC liposomes, the gel structure of the DPPC bilayer is interrupted by the $C_{25,25}$ lipids, and the equilibrium is shifted towards the liquid state, indicating that the $C_{25,25}$ lipids destabilise the gel form. At greater than 50 mol% $C_{25,25}$, the effect is the opposite: the $C_{25,25}$ lipids decrease the permeability of the anionic probe calcein at neutral pH, probably due to an increase in the negative surface charge of the mixed $C_{25,25}$ -DPPC liposomes and to an increase in the tightness and rigidity of the lipid packing. Similar effects have been observed for binary lipid mixtures of DPPC with cholesterol [17], which implies that the isoprenoid alkyl chains of archaeal lipids have a function in preventing pure lipids from forming highly ordered gel structures at low temperatures (below 40 $^{\circ}\text{C}$) and in preventing pure lipids from forming completely unordered structures, and thus taking on a membrane liquid crystalline state.

Conclusions

By using DCS, we have shown that $C_{25,25}$ archaeal lipids have significant effects on the gel-to-liquid crystalline transition of DPPC liposomes. By increasing the mol% of

C_{25,25} in the mixed C_{25,25}-DPPC liposomes, the temperature and cooperativity of the transition decrease. At lower mol% C_{25,25} in the mixed C_{25,25}-DPPC liposomes, ΔH of the transition is not significantly affected compared to the DPPC liposomes, whilst at higher mol% C_{25,25}, the peak in the DSC curve is lower. The incorporation of archaeal lipids into DPPC liposomes significantly decreases the permeability of calcein at temperatures above the phase-transition temperature of DPPC (higher than 40 °C), regardless of the mol% C_{25,25} in the mixed C_{25,25}-DPPC liposomes, whilst at temperatures below the phase-transition temperature of DPPC, the permeability at lower mol% C_{25,25} (less than 50 mol%) increases.

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