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A high sensitivity differential scanning calorimetry study of the interaction between poloxamers and dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine liposomes

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Abstract

High sensitivity differential scanning calorimetry (HSDSC) has been used to measure the thermal behaviour of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) liposomes to which poloxamer surfactants P338 or P407 had been added during or after preparation. The phospholipid pre-transition was more sensitive than the main transition to the association of poloxamers with liposomal bilayers. Poloxamers reduced the enthalpy of the pre-transition of liquid-crystalline state DMPC and DPPC MLVs but not that of gel state DPPC MLVs. Freezing and thawing DMPC and DPPC liposomes in the presence of poloxamers was shown to increase their interaction with the liposomal bilayers.

Keywords: Freeze-thaw; High sensitivity differential scanning calorimetry; Liposome; Phase transition temperature; Phospholipid; Poloxamer; Surfactant

1. Introduction

Poloxamers are non-ionic surface-active ABA block copolymers comprising two hydrophilic polyoxyethylene (POE) moieties enveloping a hydrophobic polyoxypropylene (POP) moiety. When associated with model colloidal drug delivery systems they reduce phagocytosis, leading to an increase in blood circulation time (Illum et al., 1987; Rudt and Müller, 1993).

Poloxamers are known to interact with liposomes, although the nature of the interaction is not well understood. Jamshaid et al. (1988)

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showed that the bilayer permeability of egg phosphatidylcholine (eggPC) liposomes was increased by the presence of poloxamers leading to loss of encapsulated marker. However, a reduction in the loss of aqueous phase markers was observed when hydrogenated soybean PC/cholesterol liposomes (Woodle et al., 1992) or dipalmitoylphosphatidylcholine (DPPC)/cholesterol liposomes (Khattab et al., 1995) were substituted for eggPC liposomes. Jamshaid et al. (1988) suggested that poloxamers associate with the bilayer of relatively fluid eggPC liposomes, in a way which results in their polyoxyethylene groups projecting from the liposome surface, producing a measurable increase in vesicle size. Similar increases could not be detected with more rigid gel state distearoylphosphatidylcholine (DSPC) liposomes (Moghimi et al., 1991), suggesting that the interaction between poloxamers and liposomes may be due to penetration of poloxamer some way into the bilayer, and not through simple adsorption of poloxamer onto the liposome surface.

If the packing of the molecules within the liposome bilayer is disrupted by the presence of an impurity, such as a drug molecule, this will be reflected in the thermal phase transition profile of the bilayer due to interference with the co-operative movements occurring during phase transitions. Differential scanning calorimetry (DSC) analysis has demonstrated that the inclusion of PEG-lipid conjugates within the bilayers of DPPC (Bedu-Addo et al., 1996) or DSPC liposomes (Kenworthy et al., 1995a; Kenworthy et al., 1995b) caused a new peak to appear as a shoulder on the high temperature side of the main gel to liquid crystalline phase transition endotherm. This was ascribed to the gradual solubilization of liposomes from the lamellar state to a mixed micellar state as the concentration of polymer conjugate was increased. Ringsdorf et al. (1993) used DSC to detect the presence of hydrophobically modified copolymers of poly-(*N*-isopropylacrylamide) within the phospholipid bilayers of dimyristoylphosphatidylcholine (DMPC) and DSPC small unilamellar vesicles (SUVs). The unexpected insertion of the copolymer hydrophobic moieties into gel state SUVs was attributed to phospholipid bilayer packing defects.

Baekmark et al. (1997) employed DSC to assess the interaction of DPPC liposomes with POE-containing lipopolymers and POE-polystyrene-POE tri-block copolymers. The half height width (HHW) of the main transition endotherm was broadened as the concentration of POE-containing lipopolymers was increased. For formulations containing POE-polystyrene-POE tri-block copolymers, the temperature (T_c) and enthalpy of the main transition of liposomes went through a minimum at 1 mol% polymer content, with a corresponding maximum HHW value. This is indicative of conventional impurity-induced depression of the transition, and since aqueous solutions of POE molecules do not bind to the surface of liposomes (Evans and Needham, 1988), it was concluded that the copolymer incorporated into the lipid bilayer (Baekmark et al., 1997).

2. Materials and methods

2.1. Preparation of liposomes

Multilamellar vesicles (MLVs) were manufactured by weighing appropriate amounts of dimyristoylphosphatidylcholine (DMPC; approximately 99%, Sigma, Poole, UK) or dipalmitoylphosphatidylcholine (DPPC; approximately 99%, Sigma, Poole, UK) into a round-bottomed flask, and adding chloroform (HiPerSolv, BDH, UK) to dissolve the phospholipid. Chloroform was removed by rotary evaporation under vacuum, in a water bath at 55°C for 15 min. The flask was then flushed with nitrogen for 1–2 min to remove any traces of residual solvent. An appropriate volume of filtered (100 nm pore filter [Poretics, USA]), bi-distilled deionised water (Model WP 700, Whatman, UK), further purified by passing through an Elgastat Ultra High Quality Purification System (Elgastat, UK) was added to the dry film in the flask to give a final phospholipid concentration of 50 mg/ml. Glass beads were added to aid mixing, the flask flushed with nitrogen and gently rotated for 30 min in the water bath, and shaken to produce MLVs. The suspension was annealed for a further 2 h in the water bath before storage under nitrogen in a refrigera-

tor at 2–8°C. Hydration and annealing temperatures were 40°C and 55°C for DMPC and DPPC MLVs, respectively. For some experiments, poloxamers P338 (ICI, France) and P407 (Blagden, UK) were added to MLVs following production to give a final phospholipid concentration of 50 mg/ml and a poloxamer concentration of 0.2% w/v. For other experiments poloxamers were added at the hydration stage of liposome production. Poloxamers P338 and P407 were dissolved in bi-distilled, filtered water to give 0.2% w/v poloxamer solutions. Phospholipid films were hydrated with poloxamer solutions to give a final lipid concentration of 50 mg/ml. The size of liposomes was determined using laser diffraction analysis (Malvern 2600C, Malvern Instruments, UK). All results were the mean of four determinations.

2.2. High sensitivity differential scanning calorimetry analysis of liposome preparations

High sensitivity DSC (HSDSC; Micro DSC III, Seteram, France) was used to study liposome/poloxamer interactions by determining the effect of poloxamers on the phase transition behaviour of liposome bilayers. Poloxamer-free and poloxamer-added (0.2% w/v) samples were prepared. All samples were incubated at 18°C for 24 h (below the poloxamer critical micelle concentrations; Alexandridis et al., 1994) before HSDSC analysis. Each suspension (100 μl) was pipetted into the sample container, weighed and sealed. A reference container containing 100 μl of bi-distilled water was also prepared and the crucibles were placed in the head of the HSDSC apparatus and heated from 3 to 45°C at a rate of 1°C/min.

2.3. The effect of freeze-thawing on the thermal profile of liposome / poloxamer dispersions

Poloxamers P338 and P407 were added to some dispersions of DMPC and DPPC MLVs to give final poloxamer concentrations of 0.2% w/v. Poloxamer-free samples were also prepared. A volume of 1 ml of each replicate was prepared, with a final lipid concentration of 50 mg/ml, and each replicate was incubated at 18°C for 24 h before a 100 μl aliquot was withdrawn for HSDSC analysis. Each replicate was then subjected to five freeze-thaw cycles in which dispersions were frozen for three min at −196°C in liquid nitrogen and then thawed for 3 min at 50°C in a water bath before a second HSDSC analysis. All results were the mean of four determinations (± S.D.).

2.4. The effect of repeated heating and cooling on the thermal profile of liposome/poloxamer dispersions

HSDSC sample cells containing 500 μl aliquots of DMPC MLVs were heated from 3 to 45°C at a rate of 1°C/min and then cooled to 3°C at the same rate alongside a reference cell containing an equal volume of bi-distilled, filtered water. Similarly DPPC MLVs were heated from 15 to 60°C and then cooled to 15°C. A 10 μl aliquot of 10% w/v poloxamer solution was then added to the sample container of some dispersions to give a poloxamer concentration of 0.2% w/v. Bi-distilled water (10 μl) was added to the reference cell to equalise the volume in each cell and the crucibles were repeatedly heated and cooled from 3 to 45 to 3°C (DMPC MLVs) or 15 to 60 to 15°C (DPPC MLVs) at a rate of 1°C/min for 36 h. All results were the mean of three determinations (± S.D.). Three poloxamer-free runs were performed in which 10 μl of bi-distilled water was added to the sample cell in place of poloxamer solution.

3. Results and discussion

3.1. The effect of adding poloxamer to liposomes or inclusion at the hydration stage on the thermal profile of DMPC MLVs

Fig. 1, Fig. 2 and Fig. 3, respectively, show typical HSDSC scans of DMPC MLVs alone, following addition of 0.2% w/v P407 and after hydration with 0.2% w/v P407. The temperature of the pre- and main phospholipid phase transition endotherms and the enthalpy of the main transition of DMPC MLVs was not significantly altered ($P < 0.05$) by poloxamer (either added or included at the hydration stage) (Table 1). How-

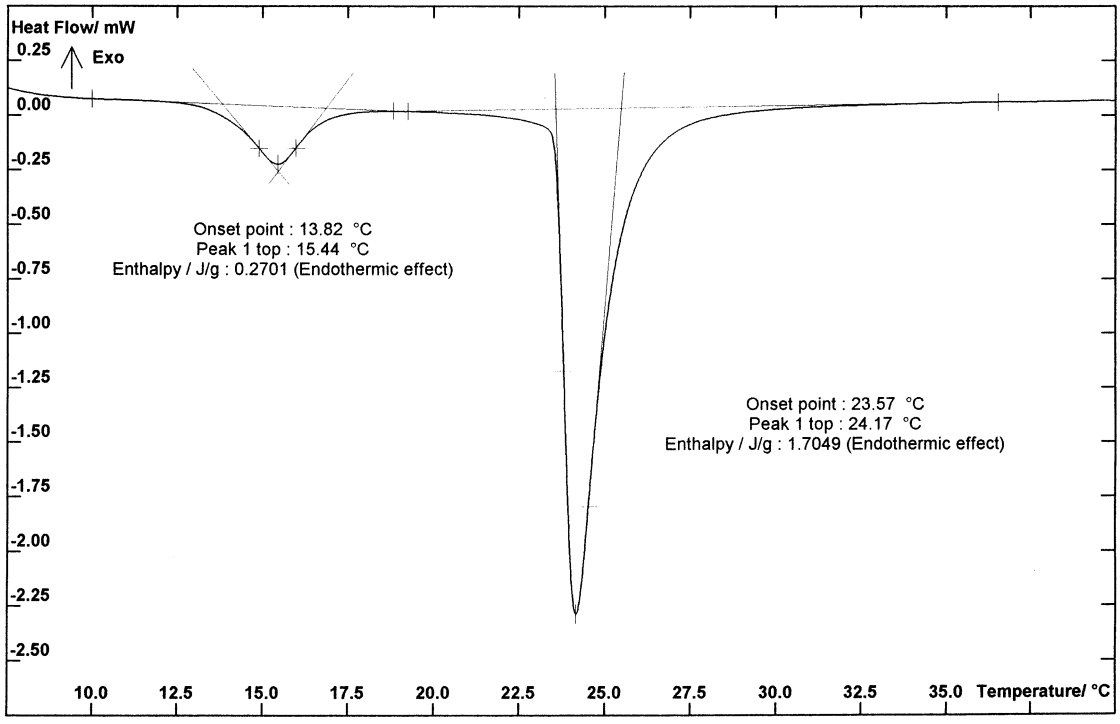

Fig. 1. A typical HSDSC scan of DMPC MLVs incubated at 18°C for 24 h.

ever, pre-transition enthalpies of DMPC MLVs with poloxamer added or included in the hydrating medium were significantly lower ($P < 0.05$) than those of poloxamer-free samples (Table 1). Thus the pre-transition is the more sensitive parameter for detecting the presence of small quantities of the poloxamer surfactant molecules in the phospholipid bilayers. This agrees with previous studies which have indicated that the pre-transition is a sensitive indicator of the presence of extraneous molecules within bilayers, its enthalpy being reduced or eradicated depending on the nature and concentration of the impurity (Cater et al., 1974). Most hydrophobic drugs will modify the pre-transition at concentrations below that required to modify the thermal profile of the main phospholipid phase transition (Posch et al., 1983; Biltonen and Lichtenberg, 1993). The mean pre-transition enthalpy of formulations in which P407 was included at the hydration stage was significantly lower ($P < 0.05$) than when P407 was added after MLV manufacture, suggesting that P407 interacts more with liposomal bilayers when included in the hydrating medium.

The mean pre-transition enthalpy of formulations in which poloxamer P338 was included at the hydration stage was lower than but not significantly different ($P < 0.05$) to the mean pre-transition enthalpy of samples where P338 was added after liposome formation. This may be partly due to the large standard deviations for samples where P338 was included at the hydration stage, obscuring any effects.

The mean volume median diameters (VMDs) of MLVs were determined by laser diffraction particle size analysis following incubation at 18°C for 24 h (Table 2). VMDs of liposomes hydrated in poloxamer, or treated with poloxamer following production were significantly lower ($P < 0.05$) than the poloxamer-free sample, possibly indicating that inclusion of surfactant is preventing vesicle aggregation. However, all measured vesicle sizes are representative of a typical polydispersed population of MLVs, indicating that differences

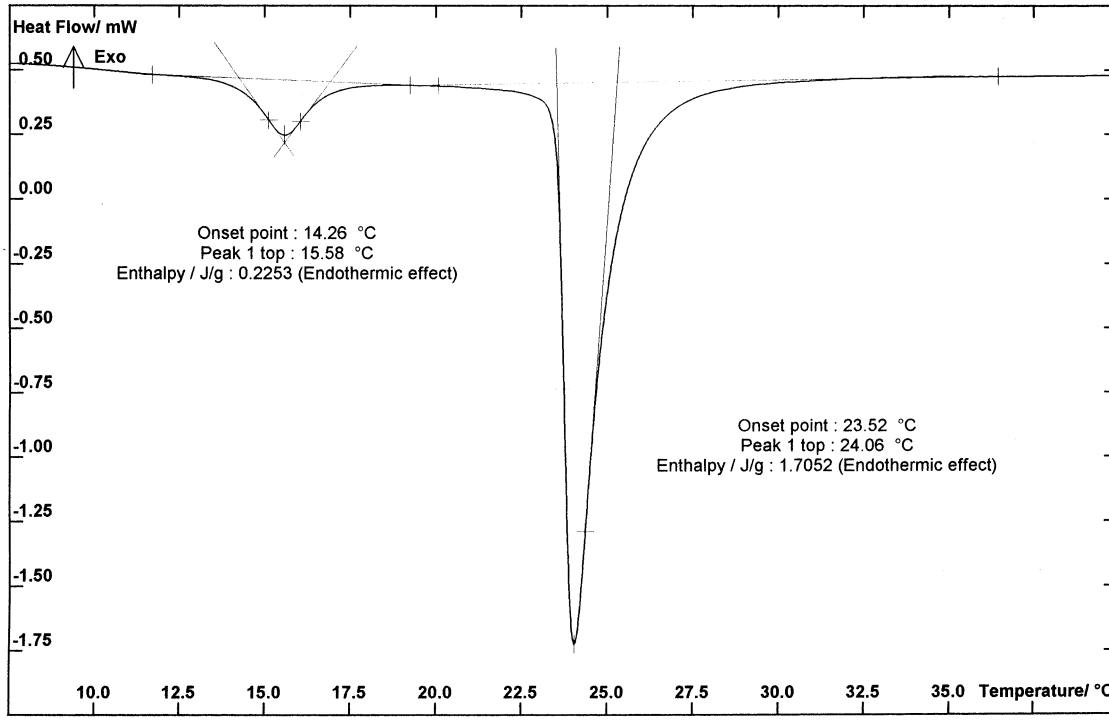

Fig. 2. A typical HSDSC scan of DMPC MLVs + 0.2% w/v P407 incubated at 18°C for 24 h.

in pre-transition enthalpy were not due to large changes in vesicle diameter, inducing strain within the bilayers.

The orientation of the POE groups may affect the strength of the interaction between the poloxamer and the liposome. Kostarelos et al. (1995) hypothesized that if the POP moiety was incorporated into the bilayer, the POE moieties would either extend externally from the vesicle or span the membrane. In the latter case the poloxamer molecule would possibly be more securely attached to the bilayer (for reasons of geometry). It is possible that including poloxamer at the hydration stage enables the poloxamer to interact with the bilayer to a greater extent, possibly by spanning it.

3.2. The effect of freeze-thawing on the thermal profile of liposome/poloxamer dispersions

Fig. 4 shows the pre-transition enthalpies of DMPC MLVs before and after freeze- thawing in the presence of P338 and P407. As shown previously, pre-transition enthalpies of dispersions of DMPC MLVs incubated with 0.2% w/v P338 or P407 were significantly lower ($P < 0.05$) than those incubated without poloxamer. The pre-transition enthalpy of DMPC MLVs, in the absence of poloxamer, was not significantly different before or after freeze-thawing ($P < 0.05$). However, the pre-transition enthalpies of DMPC MLVs incubated with either P338 or P407 were significantly reduced following freeze-thawing ($P < 0.05$). The temperature of the pre- and main transition endotherms and the enthalpy of the main transition were not significantly altered ($P < 0.05$) by poloxamer (either before or after freeze-thawing). Since freeze-thawing of MLVs produces equilibrium distributions of solute (Mayer et al., 1985) it is likely to improve the penetration of poloxamer molecules throughout MLVs. A typical HSDSC scan of DMPC MLVs + 0.2% w/v P407 after freeze-thawing is shown in Fig. 5.

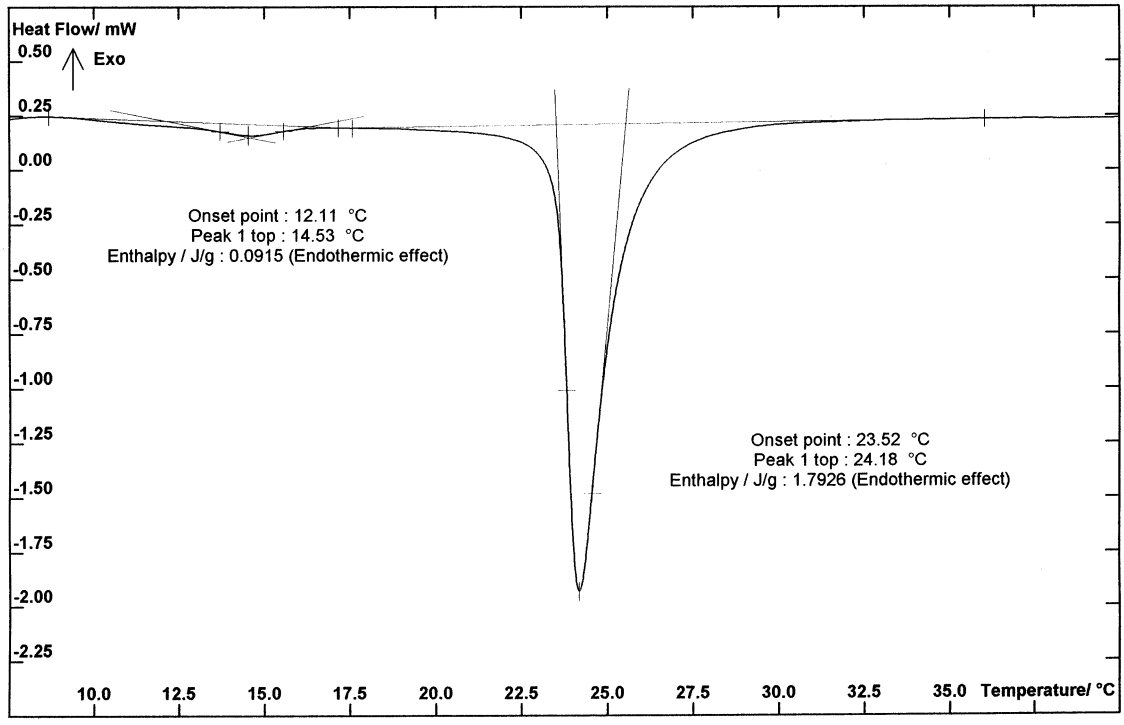

Fig. 3. A typical HSDSC scan of DMPC MLVs hydrated in 0.2% w/v P407, then incubated at 18°C for 24 h.

Table 1
Effect of poloxamer on the pre- and main transition temperatures and the main transition enthalpy of DMPC MLVs ($n = 4$, $\pm$S.D.)

Sample	Pre-transition		Main transition	
	T_c (°C)	ΔH (J/g)	T_c (°C)	ΔH (J/g)
DMPC MLVs (control)	15.47 ± 0.11	0.27 ± 0.006	24.04 ± 0.17	1.83 ± 0.34
DMPC + 0.2% w/v P338	15.61 ± 0.07	0.17 ± 0.01	24.21 ± 0.03	1.68 ± 0.05
DMPC + 0.2% w/v P407	15.64 ± 0.11	0.18 ± 0.01	24.20 ± 0.04	1.83 ± 0.12
DMPC in 0.2% w/v P338	15.46 ± 0.15	0.12 ± 0.06	24.24 ± 0.02	1.73 ± 0.14
DMPC in 0.2% w/v P407	15.33 ± 0.53	0.04 ± 0.03	24.21 ± 0.04	1.78 ± 0.11

Before freeze-thawing, there was no difference between the pre-transitions of DPPC MLVs incubated with poloxamers P338 or P407 and poloxamer-free samples ($P < 0.05$), with all samples producing mean pre-transition enthalpies of approximately 0.28 J/g of suspension (5.6 J/g or 4.21 kJ/mol of phospholipid) (Fig. 6). Freeze-thawing did not affect the mean pre-transition enthalpy of the poloxamer-free DPPC MLVs ($P < 0.05$). However, the pre-transition enthalpies of samples incubated with P338 or P407 were significantly lower following five freeze-thaw cycles ($P < 0.05$). Again no significant differences ($P < 0.05$) in the temperature of the pre- and main transition endotherms and the enthalpy of the main transition were produced by poloxamer (either before or after freeze-thawing).

The mean VMDs of all DMPC and DPPC MLVs were determined before and after freeze-thawing using laser diffraction particle size analy-

Table 2
Mean VMD and mean distribution span of DMPC MLVs before and after freeze-thawing ($n = 4$)

Preparation	Mean VMD, (mean distribution span)	
	Before freeze-thawing	After freeze-thawing
DMPC	7.25 μm (1.40)	9.48 μm (1.57)
DMPC + 0.2% w/v P338	7.14 μm (1.61)	7.50 μm (1.72)
DMPC + 0.2% w/v P407	7.23 μm (1.55)	7.74 μm (1.52)
DMPC hydrated in 0.2% w/v P338	7.23 μm (1.55)	7.74 μm (1.52)
DMPC hydrated in 0.2% w/v P407	7.23 μm (1.55)	7.74 μm (1.52)
DPPC	6.40 μm (1.35)	7.59 μm (1.61)
DPPC + 0.2% w/v P338	5.96 μm (1.54)	5.62 μm (1.42)
DPPC + 0.2% w/v P407	6.46 μm (1.29)	5.69 μm (1.38)

sis (Table 2). All preparations had diameters indicative of a population of large MLVs both before and after freeze-thawing.

The fact that freeze-thawing further reduced the pre-transition enthalpy of DMPC MLVs and reduced the pre-transition enthalpy of DPPC MLVs incubated with poloxamers suggests that freeze-thawing augmented the interaction between poloxamers and these liposomes. The results suggest that freeze-thawing compromised the long-range order of the phospholipid bilayer possibly allowing poloxamer molecules to penetrate into the hydrocarbon region of the bilayer. Freeze-thawing of MLVs produces equilibrium distributions of solute (Mayer et al., 1985). The process damages liposome bilayers by internal ice formation. Damaged bilayers re-anneal and it seems that poloxamer molecules are incorporated into the compromised bilayer structure during this process. In addition, the thaw temperature (50°C) was above the T_c of DPPC MLVs (41°C), and so the permeability of bilayers would be increased once they had reached this temperature, possibly permitting penetration of poloxamer molecules into the bilayer.

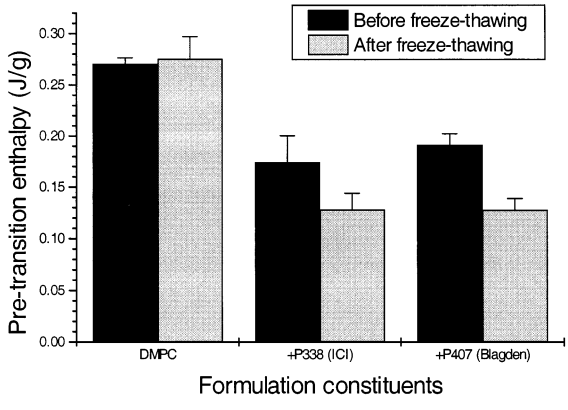

Fig. 4. Pre-transition enthalpy of DMPC MLVs before and after five freeze-thaw cycles in the presence of 0.2% w/v poloxamer ($n = 4$, ± S.D.).

3.3. The effect of repeated heating and cooling on the thermal profile of liposome/poloxamer dispersions

The mean pre-transition enthalpy of DMPC MLVs remained unchanged during repeated heating and cooling, whereas the mean pre-transition enthalpy of all samples to which poloxamer was added reduced incrementally after each heating/cooling cycle (Fig. 7). An approximately exponential model best describes the decrease in pre-transition enthalpy of poloxamer-containing formulations with time suggesting that there was a gradual increase in the cumulative amount of poloxamer associated with DMPC liposomes with time, but a decrease in the rate of association as time progressed. After 36 h (27 heating-cooling cycles), the pre transition enthalpy of P338 was reduced to $60.4 \pm 5.8\%$ of its original value compared to $56.0 \pm 6.1\%$ for P407. There was no significant difference ($P < 0.05$) between the mean pre-transition enthalpy after 24 and 36 h for formulations containing either poloxamer, indicating that there was no further increase in the amount of poloxamer interacting after 24 h (18 heating-cooling cycles).

The pre-transition enthalpy of DPPC MLVs was unaltered by repeated heating and cooling. The pre-transition enthalpy of samples to which poloxamer were added reduced with each heating/cooling cycle for the first 12 h (eight cycles) suggesting that there was a gradual increase in the

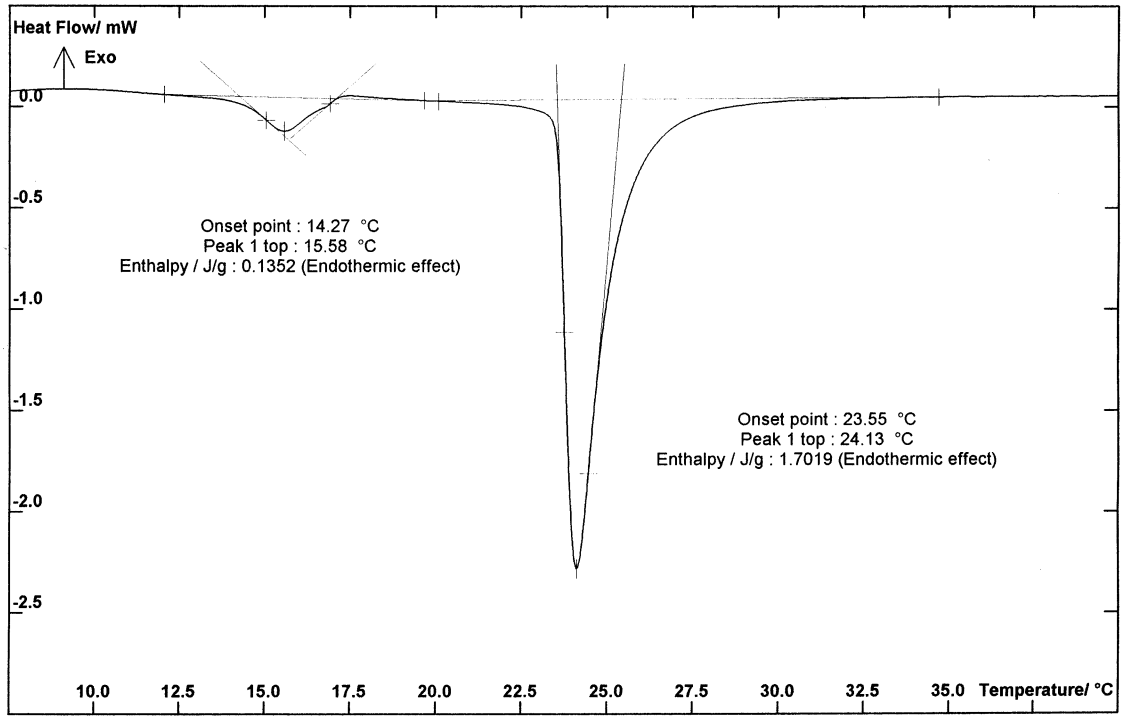

Fig. 5. A typical HSDSC scan of DMPC MLVs + 0.2% w/v P407 incubated at 18°C for 24 h, after freeze-thawing.

cumulative interaction between the two components with time (Fig. 8). After 36 h (22 cycles), the pre transition enthalpy of P338 was reduced to $51.8 \pm 6.3\%$ of its original value compared to $39.3 \pm 9.04\%$ for P407. There was no significant difference ($P < 0.05$) between the enthalpy after 24 and 36 h for formulations containing either poloxamer, indicating that no further increase in the amount of poloxamer interacting occurred after 24 h.

The fact that the pre-transition enthalpy of DPPC MLVs was reduced after heating to temperatures above the gel to liquid-crystalline phase transition indicates that the interaction of liposomes with poloxamers added to the system occurs at temperatures above the phase transition. DPPC MLVs incubated at a temperature below the T_c failed to interact with poloxamers added to the system. However, in this system, as the temperature was increased above the T_c the bilayers would be in a more fluid state and hence the barrier against diffusion of the POP moieties into the hydrophobic region of the bilayer was diminished. This provides evidence that formulating gel state vesicles sterically stabilized with poloxamers may be possible, by heat cycling.

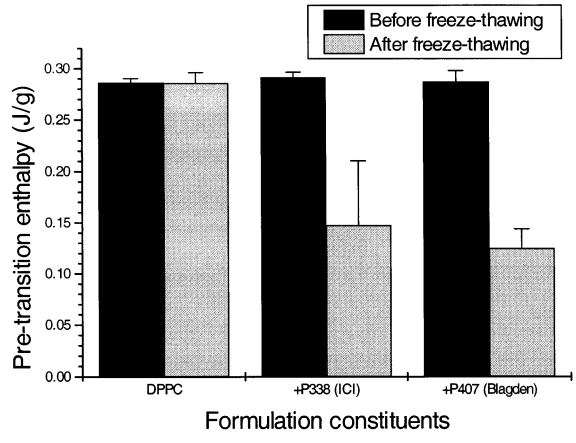

Fig. 6. Pre-transition enthalpy of DPPC MLVs before and after five freeze-thaw cycles in the presence of 0.2% w/v poloxamer ($n = 4$, $\pm$ S.D.).

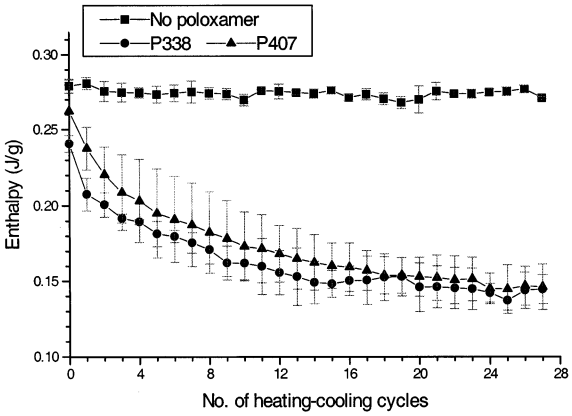

Fig. 7. Pre-transition enthalpy of DMPC MLVs subjected to repeated heating and cooling cycles in the presence of 0.2% w/v poloxamer ($n = 3$, $\pm$S.D.).

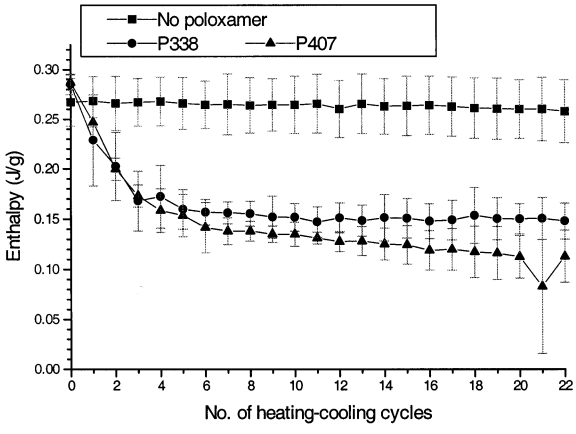

Fig. 8. Pre-transition enthalpy of DPPC MLVs subjected to repeated heating and cooling cycles in the presence of 0.2% w/v poloxamer ($n = 3$, $\pm$S.D.).

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