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Effect of cycloinulohexaose with additives on the freeze-drying of liposome

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Abstract

The ability of additives to reinforce the cryoprotective effects of cycloinulohexaose (CF-6) during the freeze-drying of liposomes was investigated. Using CF-6 with ethylene glycol, propylene glycol, glycerol and proline, the retention of calcein in freeze-dried liposomes was increased compared with the use of CF-6 only. The results of Fourier transform infrared spectroscopy (FTIR) suggested that one of the reasons for this difference was the increase in the ratio of hydrogen bonding between lipid and CF-6 with additives. The results of differential scanning calorimetry (DSC) suggested that critical distance between lipids molecules is thought to be maintained by this interaction. Because of this distance, the intermolecular interaction of lipids may be weakened. Using glycerol as the additive, when the ratio of glycerol to CF-6 increased from 0 to 0.2 (w/w), the retention of calcein in the freeze-dried liposomes increased from $72.9 \pm 5.1\%$ to $85.0 \pm 1.0\%$ and attained a maximum at the ratio of 0.15. © 1998 Elsevier Science B.V.

Keywords: Liposomes; Phase transition temperature; Glass transition temperature; Freeze-drying; Cycloinulohexaose; Vitrification

1. Introduction

Liposomal formulation for many kinds of drugs has been studied as a drug delivery system (Bally et al., 1994; Litzinger et al., 1994), and the lyophilization of liposomes is one of the most promising ways to keep the liposomes stable during long-term storage (Shulkin et al., 1984). The presence of cryoprotectants is necessary for protection of the membrane structure during the freeze-drying of liposomes. Saccharides, especially disaccharides such as trehalose and saccharose, have been extensively investigated as cryoprotectants of liposomes (Crowe et al., 1988; Tanaka et al., 1992; Koster et al., 1994; Mobley and

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Schreier, 1994). The mechanisms of the cryoprotective effect of sugar on freeze-drying of liposomes were also investigated and two hypothesis are proposed.

Crowe et al. (1988) reported that in the case of fluid lipids such as egg phosphatidylcholine, or 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), the damage to liposomes can be ascribed to both their fusion and phase transition from gel to liquid crystalline during the rehydration of freeze-dried liposomes. Trehalose, a disaccharide, especially inhibits fusion and decreases the gel-to-liquid crystalline phase transition temperature (T_m) of the dry lipid caused by a direct interaction between the sugar and the polar head group of the lipid (water replacement hypothesis).

Koster et al. (1994) suggested that the cryoprotective effect of sugars is due to vitrified sugars, which can be ascribed to a change in the surface tension between the sugar solution and lipid. They indicated that the sugar which had a high glass transition temperature (T_g) was the most effective cryoprotectant (vitrification hypothesis).

We reported that cycloinulohexaose (CF-6) has a cryoprotectant effect on the freeze-drying of liposomes (Ozaki and Hayashi, 1996). CF-6, which is produced from inulin, is a cyclohexaose of β-D-fructofranose consisting of a $\beta(2\rightarrow1)$-linked oligofructose chain (Fig. 1) (Kawamura et al., 1989). It was also reported that the cryoprotective effect of CF-6 is reinforced by adding glycerol, compared with using CF-6 only (Ozaki and Hayashi, 1996). In the present study, we examined the effect of additives including CF-6 on the freeze-drying liposomes and we discuss the mechanisms of the cryoprotective effect of CF-6.

2. Materials and methods

2.1. Materials

CF-6 was synthesized in the Research Center of the Mitsubishi Chemical (Yokohama, Japan) (Kushibe et al., 1994). The purity of CF-6 was over 97%. L-α-dipalmitoylphosphatidylcholine (DPPC) was purchased from Nichiyu Liposome (Tokyo, Japan); glycerol, ethylene glycol, propylene glycol, D(+)-proline, L(+)-isoleucine and L-leucine from Wako (Osaka, Japan); bovine serum albumin (BSA) from Sigma (St. Louis, MO); gelatin (nippi high-grade Type B) from

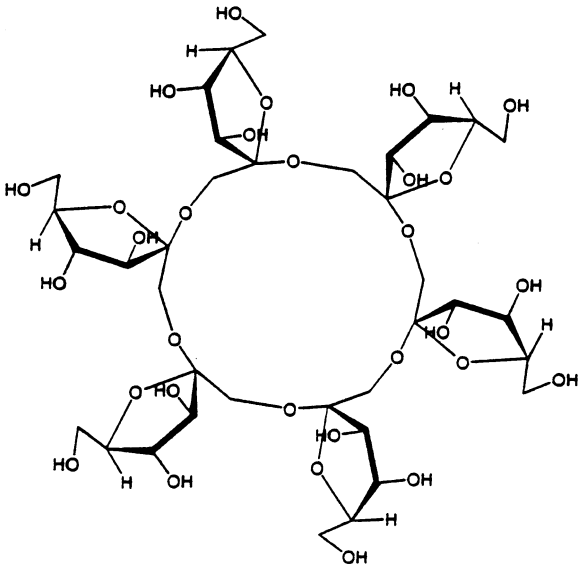

Fig. 1. The structure of CF-6. Formula: $C_{36}H_{60}O_{30}\cdot 3H_20$. Molecular weight: 1026.9.

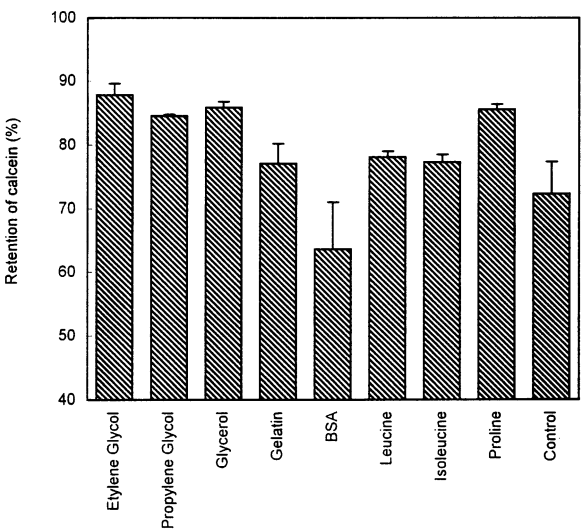

Fig. 2. Effect of CF-6 in the presence of additives on the retention of calcein in freeze-dried liposomes. CF-6 and each additive (100 and 15 mg/ml, respectively) were added to the outside solution of liposomes ($n=3$, $\pm$S.D.). BSA, bovine serum alubumin; control, CF-6 without any additives.

Nippi (Tokyo, Japan) and calcein from Dojindo (Kumamoto, Japan). Polycarbonate membrane filters were purchased from Costar (Cambridge, MA).

2.2. Preparation of liposomes

Multilamellar vesicles (MLV) were prepared by Bangham's method (Bangham et al., 1974). The lipid (DPPC) was dissolved in a small amount of chloroform in a round bottomed flask and was dried by rotary evaporation to make a thin film. The residual solvent was removed in a desiccator. Calcein solution (77–156 mM adjusted to pH 7.0 with 1 M NaOH solution) was added to the round bottomed flask, and the flask was shaken by a vortex mixer for 10 min above 60°C to make the MLV liposomes. The osmotic pressure of the calcein solution was adjusted to the same value as that of the solution outside the liposomes (230–470 mOsmol) to which the cryoprotectants were added. To prepare liposomes of uniform size, the liposome solution (> 60°C) was extruded through two polycarbonate membrane filters (pore diameter 100 nm). The extrusion was repeated 10 times. Untrapped calcein was removed through a Sepharose-6FF column using distilled water as the eluent. During gel filtration with distilled water, the isotonicity was not maintained but only a very small amount of leakage was observed under our experimental conditions (data not shown). The trapping efficacy was about 4–7% of all samples. Subsequently, the cryoprotectants were added to the outside of the liposomes to make them isotonic with respect to the solution inside the liposomes. The concentration of lipids in the liposomal solution was adjusted to 20 mg/ml.

2.3. Particle size

The mean particle size of the liposomes was determined by quasi-elastic laser light scattering measurement using a COULTER N4 sub-micron particle analyzer (Courter Electronics, Hialeah, FL). The mean particle size of all samples before lyophilization, which was analyzed by both unimodal and SDP (size distribution processor) analysis (weight results), was about 90–100 nm.

2.4. Freeze-dried liposomes

Two ml of the liposomal solution was placed into 10 ml glass vials. The vials were partially capped with rubber stoppers and then lyophilized by using an RL-100BSW lyophilizer (Kyowa Vacuum Engineering, Tokyo, Japan). The liposomes were frozen at a final shelf temperature of −40°C and placed under vacuum at a pressure of 0.05 mbar. Subsequently, the shelf temperature was set to −20°C for 20 h, and thereafter to 20°C for 10 h. Dried nitrogen gas was flowed into the drying chamber and the vials were sealed inside the drying chamber. The water content of all samples after freeze-drying was about 0.7–1%. The liposomal size and calcein leakage for each sample was measured after rehydration with 2 ml of distilled water at about 25°C.

2.5. Retention of calcein (%)

Any free calcein due to leakage in each sample was separated from the liposomes using a Sepharose-6FF column. Ten μl of 10% Triton X-100 solution was added to the liposomal fraction and the mixture was heated to 60°C. The amount of free calcein and the amount of calcein retained inside the liposomes was determined by fluorescence measurement using an RF-5000 fluorescence spectrophotometer (Shimadzu, Kyoto, Japan) set to 490 nm for excitation and 520 nm emission wave lengths.

2.6. Differential scanning calorimetry (DSC) measurement

Fifty mg of DPPC was dissolved in chloroform and dried via rotary evaporation. Then 100 mg of CF-6 and 15 mg of each additive in 1 ml of distilled water was added. Freeze-dried liposomes were made by the method described above. The scans were analyzed using a DSC 8230C (Rigaku, Tokyo, Japan). The first scan was performed from −10 to 100°C at a heating rate of 10°C/min. The second scan, after annealing the samples at 100°C for 5 min, was performed from −10 to 100°C at a heating rate of 10°C/min. The T_m was measured as the endotherm peaks.

Table 1
Effect of additives (15 mg/ml) on the T_m and ΔH of DPPC freeze-dried liposomes with CF-6 (100 mg/ml)

	Before annealing			After annealing		
	T_m (°C)	ΔH (J/g)	$\Delta t_{1/2}$	T_m (°C)	ΔH (J/g)	$\Delta t_{1/2}$
Ethylene glycol	51.3 ± 3.4	28.5 ± 4.7	5.1 ± 0.5	41.3 ± 0.7	39.6 ± 9.6	3.7 ± 0.3
Propylene glycol	47.7 ± 2.1	39.6 ± 11.9	5.6 ± 0.3	38.9 ± 2.5	35.3 ± 12.7	4.2 ± 0.9
Glycerol	52.0 ± 0.2	24.3 ± 0.5	5.0 ± 0.7	41.9 ± 0.8	43.3 ± 1.1	3.6 ± 0.5
Gelatin	63.8 ± 2.5	19.3 ± 2.4	7.0 ± 0.2	28.3 ± 4.1	22.7 ± 1.0	10.1 ± 0.9
Albumin	69.1 ± 3.8	21.6 ± 0.4	8.4 ± 1.1	27.2 ± 2.9	24.3 ± 2.2	8.3 ± 0.9
Leucine	67.1 ± 3.2	20.9 ± 1.1	7.7 ± 0.1	29.3 ± 2.7	22.8 ± 1.8	8.8 ± 0.3
Isoleucine	67.2 ± 2.5	22.0 ± 0.5	7.6 ± 1.1	28.8 ± 1.9	22.8 ± 1.2	8.3 ± 0.8
Proline	57.7 ± 1.8	27.4 ± 1.2	5.5 ± 0.5	41.6 ± 3.6	26.8 ± 1.9	7.6 ± 1.1
Control	68.8 ± 4.2	21.7 ± 1.6	7.2 ± 1.3	30.8 ± 2.2	24.4 ± 2.4	6.3 ± 0.2

2.7. Fourier transform infrared spectroscopy (FTIR) analysis

Each sample was prepared in the same way as described for DSC measurement and was applied to the surface of the diamond of a Golden Gate Single Reflection Diamond Attenuated Total Reflectance (ATR) machine (Graseby Specac, Kent, UK) and was compressed between the sapphire and diamond anvil. Then the samples were analyzed with an FIS-165 FTIR spectrophotometer (BIO-RAD Laboratories, Cambridge, MA) running 64 scans with a resolution of 4 cm^{-1}.

3. Results

3.1. The effect of additives on the retention of calcein of freeze-dried liposomes with CF-6.

As shown in Fig. 2 the retention of calcein after rehydration of the freeze-dried liposomes in the presence of only CF-6 (the control) was about 72.9 + 5.1%. Using ethylene glycol, propylene glycol, glycerol or proline with CF-6, the retention of calcein was improved to about 85–88%, almost the same retention as that in the presence of trehalose (88.5 ± 2.9%) or saccharose (92.1 ± 2.0%) (Ozaki and Hayashi, 1996) The use of gelatin, leucine or isoleucine with CF-6 did not improve the retention of calcein, and BSA decreased it.

3.2. The effect of additives on the T_m and ΔH of freeze-dried liposomes with CF-6

The effects of various kinds of additives on the T_m and ΔH of freeze-dried liposomes with CF-6 was investigated by DSC. As shown in Table 1 and Fig. 3a, in the presence of additives which did not improve the retention of calcein, the T_m and ΔH values were almost the same as in the case of CF-6 only (the control). For the control in the first scan, the T_m was observed to be about 68.8 ± 4.2°C, in the second scan, about 30.8 ± 2.2°C. In contrast, in the cases of ethylene glycol, propylene glycol or glycerol, in the first scan, the T_m was observed to be about 50°C and in the second scan, about to be 41°C, and the width of the peak at half height ($\Delta t_{1/2}$) of the second scan (3.6 ± 0.5°C) became narrow compared with the control (6.3 ± 0.2°C). In the case of proline, the T_m values of the first and second scans were 57.7 ± 1.8 and 41.6 ± 3.6°C, respectively, but the $\Delta t_{1/2}$ of the second scan (7.6 ± 1.1°C) did not change compared to the control. In addition the other endotherm peak was observed at about 50°C.

3.3. Effect of additives on the phosphate asymmetric stretching band of freeze-dried liposomes with CF-6

The effect of additives on the phosphate asymmetric stretching band of freeze-dried liposomes

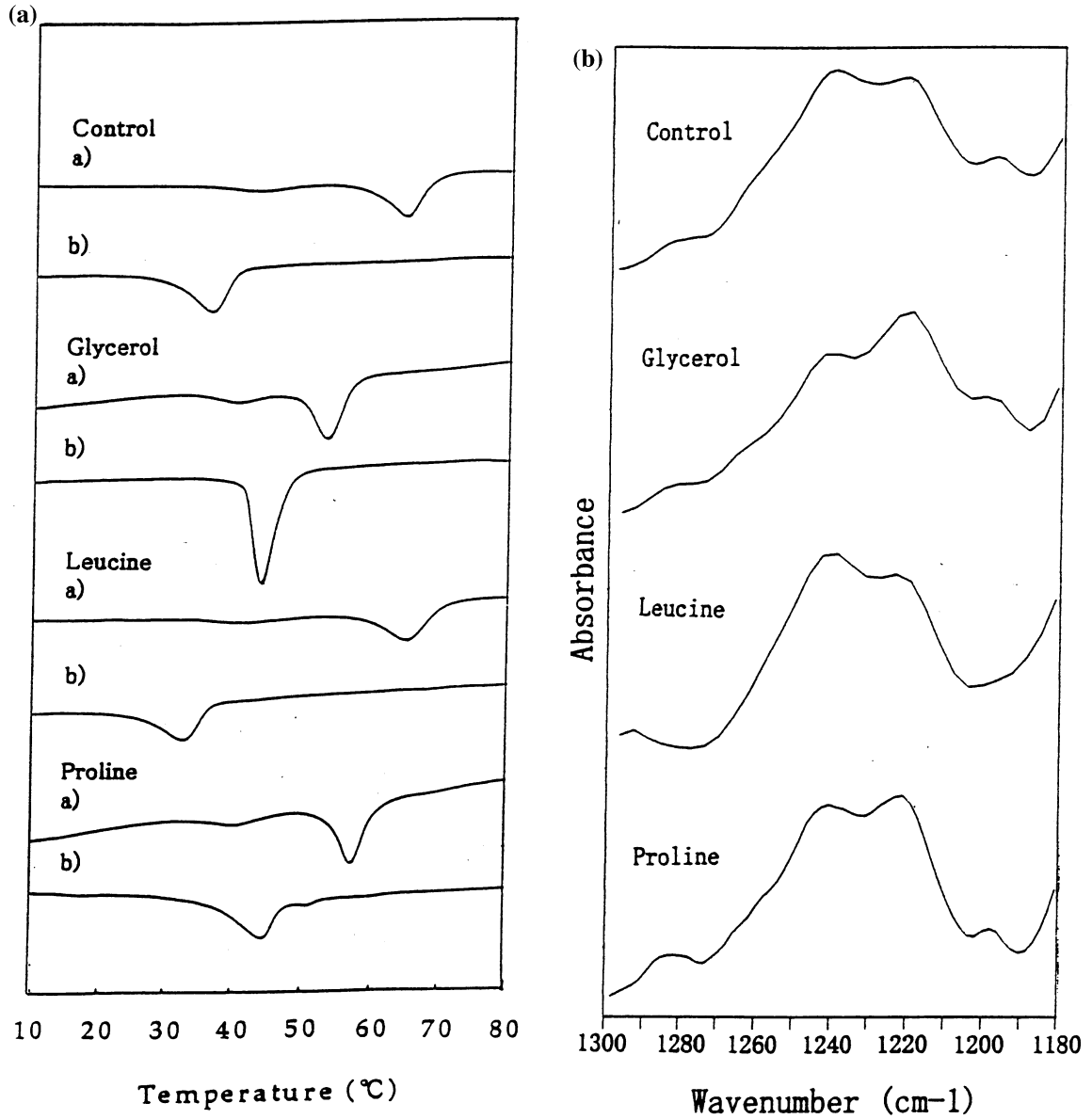

Fig. 3. a, First and second DSC scans of DPPC freeze-dried liposomes in the presence of additives with CF-6. a) before annealing; b) after annealing; control, CF-6 without any additives. b, FTIR spectra of the phosphate asymmetric stretching band region of DPPC freeze-dried liposomes in the presence of additives with CF-6.

with CF-6 was investigated using an FTIR spectrometer. As shown in Fig. 3b, the phosphate asymmetric stretching of freeze-dried liposomes was observed to be 1240 and 1224 cm^{-1}. For additives such as leucine, which did not improve the retention of calcein, the intensity of absorbence at 1224 and at 1240 cm^{-1} were almost the same as that of CF-6 only. In contrast, in the additives such as glycerol, which improved the retention of calcein, the intensity of absorbence at

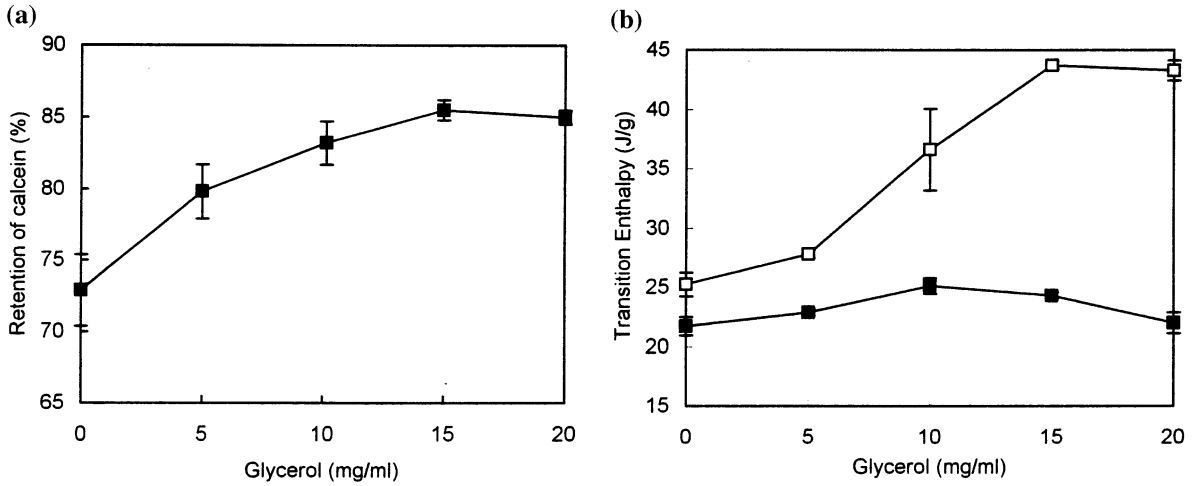

Fig. 4. a, Effect of glycerol content on the retention of calcein of DPPC freeze-dried liposomes with CF-6 (100 mg/ml) ($n = 3$, $\pm$ S.D.). b, Effect of glycerol content on the ΔH of DPPC freeze-dried liposomes with CF-6 (100 mg/ml) ($n = 3$, $\pm$ S.D.). ■, Before the annealing process; □, after the annealing process.

1224 cm^{-1} increased. Ethylene glycol and propylene glycol showed the same results as glycerol (data not shown).

3.4. The reinforcement effect of glycerol on the calcein retention and on ΔH of freeze-dried liposomes with CF-6

The effect of addition of varying amounts of glycerol to CF-6 (100 mg/ml) on the retention of calcein was investigated. As shown in Fig. 4a, the retention of calcein increased in accordance with the increase in the amount of glycerol from 0 to 15 mg/ml and reached a maximum at 15 mg/ml. As shown in Fig. 4b, the effect of glycerol on ΔH after the annealing process was investigated. The absolute value of ΔH increased as the glycerol amount increased from 0 to 15 mg/ml and reached a maximum at 15 mg/ml.

With the ratio of glycerol:CF-6 fixed at 0.15, the effect of the ratio of CF-6:lipid on the retention of calcein was investigated. Before lyophilization the lipid concentration of all samples was adjusted to 20 mg/ml. As shown in Fig. 5, the retention of calcein increased in accordance with the increase in the ratio of the CF-6:lipid from 0 to 2 and reached a maximum at 2. This result was almost the same as that in our previous study (Ozaki and Hayashi, 1996), where only CF-6 was used as a cryoprotectant. Before annealing, as shown in Fig. 6a, at a CF-6:lipid ratio below 2, the T_m decreased with an increase in the ratio. But at a ratio above 2, the T_m was held constant at about 50°C. After annealing, as shown in Fig. 6b, at a CF-6:lipid ratio below 2, two different endothermic peaks were observed, and when using a

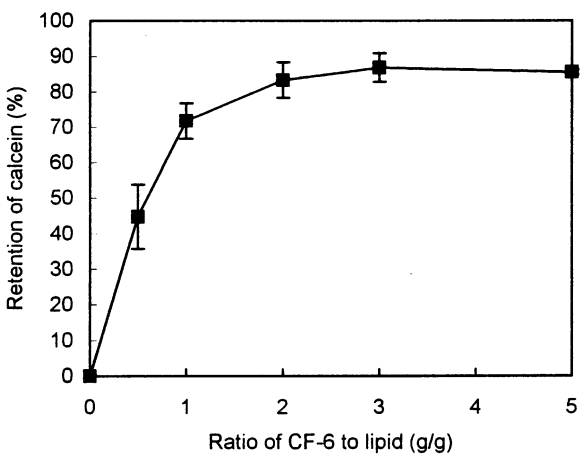

Fig. 5. Effect of the CF-6:lipid ratio (w/w) on the retention of calcein in DPPC freeze-dried liposomes with glycerol. The glycerol:CF-6 ratio was 0.15 ($n = 3$, $\pm$ S.D.).

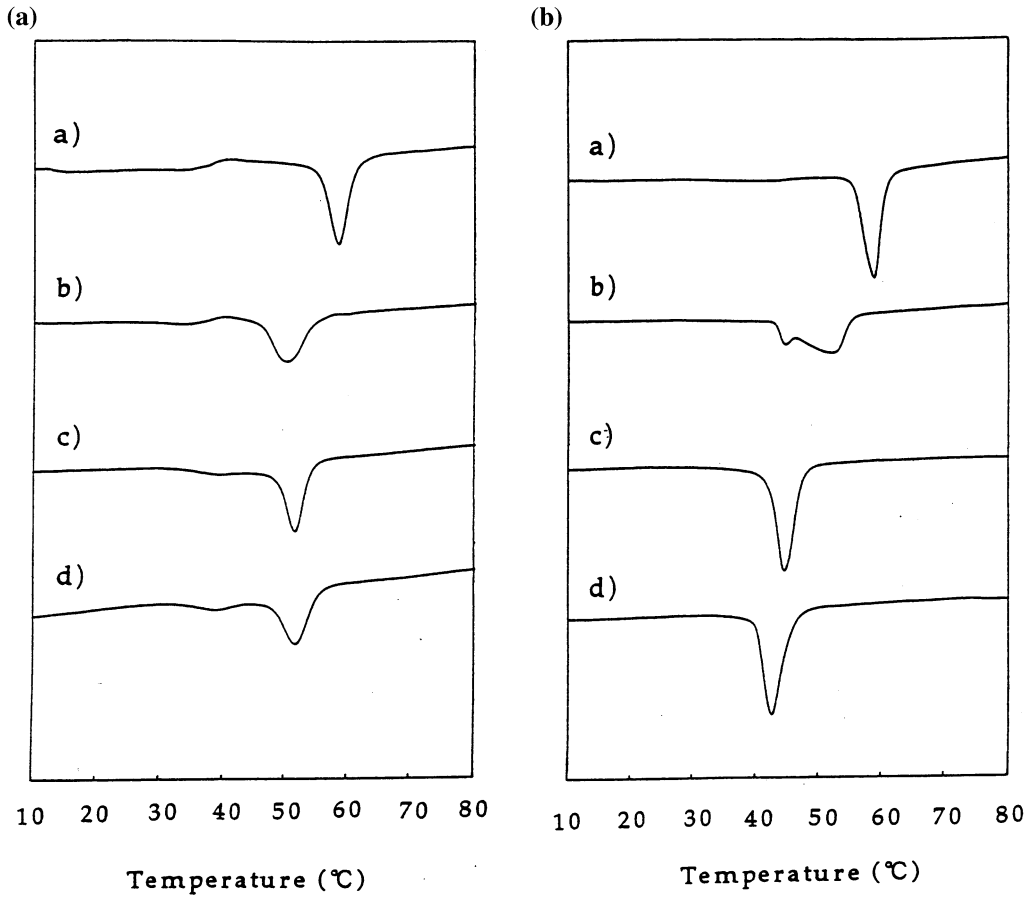

Fig. 6. a, Effect of the CF-6:lipid ratio (w/w) on DSC scans before the annealing of DPPC liposomes freeze-dried with glycerol. The glycerol:CF-6 ratio was 0.15. a) 0.5; b) 1; c) 2; d) 5. b, Effect of the CF-6:lipid ratio (w/w) on DSC scans after the annealing of DPPC liposomes freeze-dried with glycerol. The glycerol:CF-6 ratio was 0.15. a) 0.5, b) 1, c) 2, d) 5.

ratio above 2, only the low-temperature endotherm was observed. As shown in Fig. 7 the absolute value of ΔH of the low-temperature endotherm reached a maximum at a ratio of 2 and did not change above this ratio.

4. Discussion

In our results four additives (ethylene glycol, propylene glycol, glycerol and proline) increased the ability of CF-6 to improve the retention of calcein. As shown in Fig. 3b the phosphate stretching band was observed at 1220 cm^{-1}. Crowe et al. (1988) reported that in the case of trehalose, the band at 1220 cm^{-1} may be attributed to the hydrogen bonding between the phosphate group of the lipid and the hydroxyl group of the sugars. As shown in Fig. 3b the band at 1220 cm^{-1} for the four additives which improved the retention of calcein became stronger than that of the control (CF-6 alone). This suggested that the CF-6 directly interacted with the lipid by hydrogen bonding, and that the four additives which improved the retention of calcein also interacted with the lipid by hydrogen bonding.

As shown in Table 1, before the annealing process the T_m of the four additives which improved the retention of calcein decreased over 10°C compared to the control. This suggested that the opportunity for van der Waal's interaction among the acyl chains was decreased. In other words, it suggested that the distance between the lipids was increased compared with the control. This increase in the distance between the lipids may be attributed to the hydrogen bonding between phospholipid and sugar. The formation of hydrogen bonds between the lipids and sugars is thought to maintain a critical distance between the lipid molecules. This may weaken the intermolecular interaction of the lipids and keep them in a similar state to hydrated lipids.

The scans after annealing (second scan) of freeze-dried liposomes with sugars was already investigated by some researchers and the T_m of freeze-dried liposome with disaccharides decreased drastically once the samples annealed over their liquid–crystalline phase (Tanaka et al., 1992; Mobley and Schreier, 1994; Ozaki and Hayashi, 1996). In this paper, these results after annealing are discussed as described below in order to compare our data with those by Koster et al. (1994). As shown in Table 1 and Fig. 3a, in the case of the control and the additives which did not improve the retention of calcein, the T_m after annealing decreased drastically similarly to the case of disaccharides. But the T_m for the DPPC liposomes containing either ethylene glycol, propylene glycol or glycerol did not decrease as much. The T_g of these three samples was about 32–38°C measured by DSC and was lower than the T_m of hydrated DPPC. Koster et al. (1994) reported that vitrification of sugar was sufficient for cryoprotection of liposomes during freeze-drying and when the T_g of the lipids dried with sugar was lower than the T_m of the hydrated lipid, the T_m of the sample was approximately the same temperature as the T_m of the hydrated lipid. Their explanation seemed to agree approximately with our results, but if the T_g of the sample was the main reason for cryoprotection of the liposomes, the sugar with the higher T_g was the better cryoprotectant. On the other hand, the T_g of CF-6 was about 50°C measured by DSC and the T_g of the samples which contained the three additives were lower than the T_g of CF-6 only. Our results suggested that vitrification was not enough to explain the reason for reduction the T_m of the liposomes and supported the results of Crowe et al. (1996).

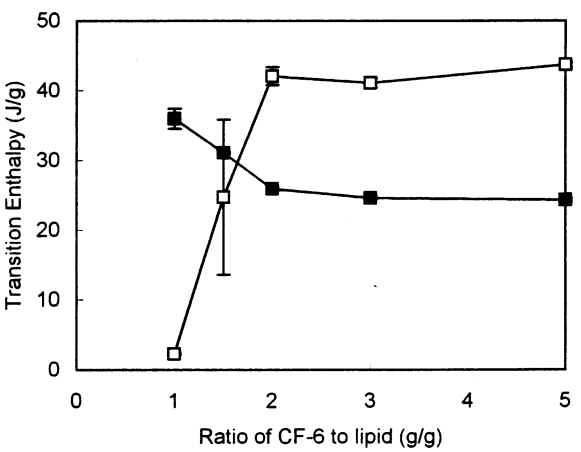

Fig. 7. Effect of the CF-6:lipid ratio (w/w) on the ΔH of DPPC liposomes freeze-dried with glycerol. The glycerol:CF-6 ratio was 0.15 ($n = 3$, $\pm$ S.D.). ■, Before the annealing process; □, after the annealing process.

Finally, the cryoprotection of liposomes was due mainly to the interaction between CF-6 and the lipids, and ethylene glycol, propylene glycol, glycerol, and proline also interacted with the lipids.

5. Conclusion

The presence of glycerol, ethylene glycol, propylene glycol and proline reinforced the retention of calcein in freeze-dried liposomes with CF-6, compared to CF-6 only. The main mechanism of this improvement was due to the interaction between CF-6 and the lipids and also the interaction between the additives and the lipids. For these interactions a critical distance between the lipid molecules was maintained. Because of this distance the intermolecular interaction of the lipids may be weakened.

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