Cryoprotective Mechanism of Saccharides on Freeze-Drying of Liposome

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The cryoprotective effects of various additives such as saccharides and polyalcohols on sonicated liposomes during freeze-drying were investigated. Fusion of liposomes was measured as energy transfer and size distribution. There was considerable difference among the additives in their cryoprotective ability. Polyalcohol systems showed considerable fusion. Although monosaccharides completely prevented the fusion of liposomes during freeze-drying similarly to disaccharides, they showed far less ability to retain the entrapped calcein of liposomes than disaccharides. The heating thermograms of differential scanning calorimetry of dipalmitoylphosphatidylcholine (DPPC) in the state of sonicated liposomes freeze-dried with various additives were measured. Disaccharides and monosaccharides again markedly differed in their effects on the thermal property of the DPPC. The reason for the variety in their cryoprotective ability was attributed to the difference in the strength of their interaction with phospholipid head group.

Keywords liposome; freeze-drying; retention; fusion; saccharide; polyalcohol; cryoprotective mechanism; differential scanning calorimetry (DSC)

Introduction

Freezing[1,2] and freeze-drying[3,4] of liposomes have recently been challenged with stabilizers such as saccharides, and the stabilization mechanism of saccharides has been investigated by several researchers.[2,4,5] Formation of hydrogen-bonding between saccharides and lipid head groups is confirmed to be indispensable for the cryoprotection.[5] But it is still not well understood how the hydrogen-bonding essentially protects the liposomes from damage during the freeze-drying process.

Crowe *et al.*[3,5,6] proposed the following mechanism: The damage of liposomes composed of phospholipid with low phase-transition temperature can be ascribed to both their fusion and the phase transition from gel to liquid-crystalline during freeze-drying. Saccharides inhibit fusion and depress the transition temperature in the dry lipids. In the case of liposomes composed of dipalmitoylphosphatidylcholine (DPPC), they concluded that the damage was due solely to fusion. Harrigan *et al.*[7] proposed that part of the protective effect of saccharides might result from their ability to work as a spacing matrix between liposomes, thus preventing fusion. But they did not find further leakage by passing freeze-dried DPPC liposomes through the phase transition temperature, and were suspicious of the contribution of phase transition. They found that the addition of saccharides only for the outside of liposomes completely prevented fusion but not the leakage, suggesting that saccharides play a role other than the prevention of fusion in maintaining the membrane permeability barrier.

Some worker pointed out that there is considerable variety among the saccharides in their ability to stabilize the dry liposomes[5,8]; but the reason for these differences has not be defined.

In this paper we pursued the reason for the difference in the cryoprotective ability of various additives such as saccharides and polyalcohols: Their effects on the thermal property of DPPC in the state of freeze-dried sonicated liposomes were investigated in detail after reexamination of their cryoprotective effects. It is concluded from the results that the cryoprotective ability is higher when the interaction between saccharides and phospholipid head groups is stronger. Further insight into the cryoprotective mechanism is provided: Keeping the phospholipid membrane in a more fluid state during the drying process is suggested to be important to obtain high retention.

Experimental

Materials Egg yolk-L-α-phosphatidylcholine (EggPC) was supplied from Asahi Kasei Co., Ltd. DPPC was purchased from Nihon Seika Co., Ltd. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) was obtained from Molecular Probes Co., Ltd. *N*-(Lissamine-rhodamine-B-sulfonyl)dioleoyl-L-α-phosphatidylethanolamine (Rh-PE) was supplied from Avanti Co., Ltd. Saccharides and polyalcohols were obtained from Wako Chemical Co., Ltd. and they were used after drying *in vacuo* at room temperature on P_2O_5 for 3 d. Calcein was purchased from Dojin. Water was purified by deionization and distillation.

Measurements Calcein-entrapped sonicated liposomes were prepared as previously described.[9] The inside solution of liposomes was a 10 mM Tris–HCl buffer solution containing 70 mM calcein and 50 mM trehalose (pH = 7.2), and the outside buffer solution contained 365 mM of either saccharide or polyalcohol to keep it isotonic with the inside solution. EggPC concentration was 4 mM. The freeze-drying procedure was as follows. Normally 100 μl-aliquots of liposome dispersion were freeze-dried in a chamber freeze-dryer (RL-10NA by Kyowa-Shinku Co., Ltd.). The sample was frozen to a terminal temperature of −45 °C at a cooling rate of about 5 °C/min, and dried at a pressure of 0.01 Torr for 18 h, the temperature was kept at −45 °C. Thereafter, shelf temperature was set at 25 °C and the drying was continued at about 0.003 Torr for 12 h. Calculation of percent of retention of entrapped calcein (R%) and measurement of liposome size, *i.e.*, size distribution and weight average diameter ($\bar{D}_w$), were presented in a previous paper.[9]

Fusion Fusion of liposomes by freeze-drying was estimated by the probe dilution assay of resonance energy transfer technique as described by Struck *et al.*[10] Fluorescence-labeled liposomes containing 1.0 and 0.3 mol% of NBD-PE and Rh-PE in the membrane were prepared in each 365 mM saccharide buffer solution. The labeled liposomes were mixed with the unlabeled liposomes at a ratio of 1 : 1 and a total EggPC concentration of 4 mM. The efficiency of energy transfer (E) was obtained before and after the freeze-drying from the fluorescence emission intensity of NBD-PE at 530 nm with excitation at 460 nm by Eq. 1,

$$E = 1 - F/F_0 \quad (1)$$

F_0 and F are fluorescence intensities in the presence and absence of triton X-100, respectively. Under this experimental condition, decrease in measured energy transfer efficiency was proportionally related to the decrease in the surface density of the energy acceptor (Rh-PE), indicating fusion of the liposomes.[10]

Differential Scanning Calorimetry (DSC) Sonicated liposomes composed on DPPC were prepared in each saccharide aqueous solution to give a weight ratio of saccharide to DPPC equal to unity. At this ratio,

the cryoprotective effect of each additive is known to be maximal,[3,9] and further addition of saccharides does not alter it. Therefore, the results of DSC measured at the low weight ratio are comparable with the data on cryoprotective effects, *i.e.*, retention and fusion measured at the high weight ratio. After freeze-drying, samples were immediately weighed in an aluminum sample holder in an atmosphere of dry nitrogen, sample holders were hermetrically sealed. DSC thermograms were obtained using a high sensitivity Seiko calorimeter (TC-100). The samples were scanned from 0 °C to 120 °C at a heating rate of 2 °C/min. The heat absorbed was determined from the area under the peaks using the heat of pure palmitic acid melting as a standard.

Residual Water Content Residual water content of freeze-dried samples was determined gravimetrically. Samples in a freeze-dry holder were brought to atmospheric pressure with dried nitrogen and immediately weighed. Weight loss upon drying at 105 °C and at 0.05 Torr on P_2O_5 for 1 d was also measured. In all systems the water content was in a range from 0.5% to 1.0%.

Results

Retention and Fusion of Sonicated Liposomes during Freeze-Drying Values of retention percentage of entrapped calcein (R%) and weight average diameter of liposomes ($\bar{D}_w$) before and after the freeze-drying in the presence of various additives are determined as shown in Table I. Size distribution before and after the freeze-drying in the systems with trehalose, glucose and mannitol are represented in Fig. 1. The addition of disaccharides, *i.e.*, trehalose, sucrose and maltose, results in a high retention percentage (89%), and liposome size does not change. Monosaccharides provide much lower R% (25%), but no increase in liposome size is found, indicating that fusion of liposomes is not the cause of the decrease of R% in these systems. Polyalcohols gave far lower R%, especially in mannitol (0%). Great increase in liposome size is found in the systems with inositol and mannitol. This result shows that disaccharides and monosaccharides completely prevent the fusion of liposomes during freeze-drying, while polyalcohols do not. In order to obtain information on the fusion of liposomes directly, values of the energy transfer efficiency (E) before and after the freeze-drying in the system with trehalose, glucose and mannitol are measured as represented in Table II. With trehalose and glucose, the values of E before and after the freeze-drying are the same. On the contrary, with mannitol, it decreases about 50%, which indicates that all of the input liposomes participate in fusion events. These findings are consistent with results presented in Table I and Fig. 1.

Crowe *et al.*[3] also investigated R% and the fusion of liposomes in the presence of various additives. They reported that disaccharides were, in general, superior to other sugars, and this well agrees with our results. But they found considerable fusion in the monosaccharide systems, contradicting the present results which show no fusion of liposomes in the monosaccharides systems. The discrepancy may arise from differences in experimental conditions and the method of monitoring the fusion; they used the probe mixing method, while we used the probe dilution method. Disagreement has often been reported between the results obtained by these two methods.[11] The probe mixing technique is likely to monitor not only the fusion but also

TABLE I. R% and $\bar{D}_w$ before and after the Freeze-Drying in the Presence of Various Saccharides and Polyalcohols

Additive	R%	$\bar{D}_w$ (nm)	
		Before	After
Disaccharide			
Trehalose	89±1	28±7	30± 4
Sucrose	88±1	26±5	27± 4
Maltose	89±1	28±4	32± 4
Monosaccharide			
Glucose	26±5	28±6	29± 4
Mannose	25±3	28±4	27± 6
Galactose	26±3	20±4	21± 4
Polyalcohol			
Sorbitol	26±3	27±5	37± 15
Inositol	17±2	26±7	1760±350
Mannitol	0	28±4	1900±441

TABLE II. Value of Transfer Efficiency (E) of Various Liposome Systems before and after the Freeze-Drying

Additive	E	
	Before	After
Trehalose	0.44	0.42
Glucose	0.40	0.41
Mannitol	0.43	0.23

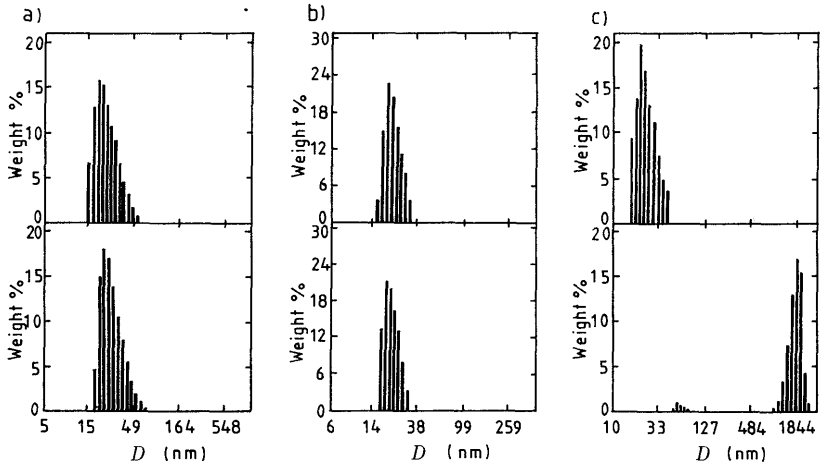

Fig. 1. Size Distribution of Sonicated Liposomes before (Above) and after (Blow) the Freeze-Drying in the Presence of Various Additives
a) trehalose; b) glucose; c) mannitol.

the aggregation and adhesion, while the probe dilution method may be more reliable for detection of the fusion. Moreover, fusion is not found in our results of size-distribution.

Inositol and mannitol show very poor cryoprotective ability compared to other additives. This is probably attributable to the formation of the eutectic mixture in the frozen state.[2,12] The fusion observed in these systems is probably caused by the mechanical breaking during the crystal formation of ice and these compounds.[2]

DSC Studies The effect of various additives on the thermal property of DPPC in the state of freeze-dried sonicated-liposomes is investigated. The DSC heating thermogram of each system shows metastability as follows: The first scan thermogram from 0 °C to 120 °C of the system with trehalose shows a large endothermic transition peak at around 75 °C (Fig. 2a). On the second scan, the peak shifts to lower transition temperature at around 26 °C as shown in Fig. 2b. The thermogram on the third scan (Fig. 2c) is almost the same as that of the second. The behavior of the thermograms well agrees with that reported by Crowe and Crowe[13] in the same system. From their results, they concluded that for maximal effect, it was necessary for the trehalose to interact with DPPC which was in liquid-crystalline phase. As they mentioned, the thermogram on the second scan most probably reflects the state of DPPC interacting with additives on a maximal level.

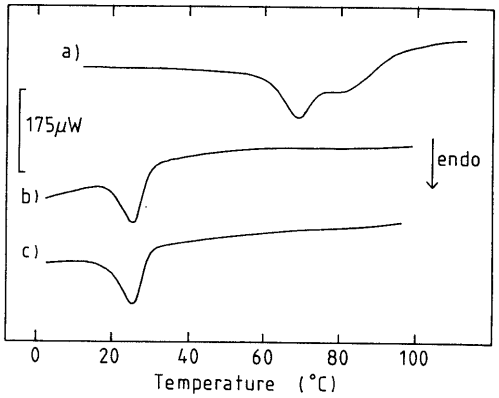

Fig. 2. DSC Heating Thermograms of Sonicated DPPC Liposomes Freeze-Dried with Trehalose

a) first scan from 0 °C to 120 °C; b) the second scan; c) the third scan.

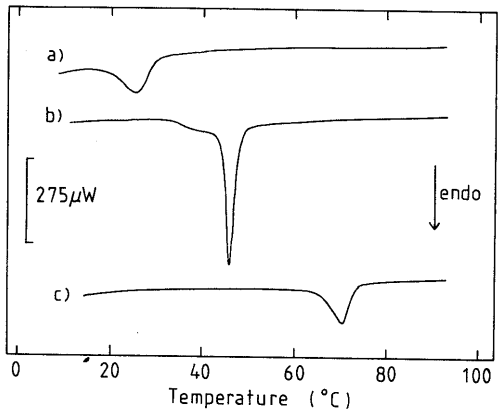

Fig. 3. DSC Heating Thermograms on the Second Scan of Sonicated DPPC Liposomes Freeze-Dried with Various Additives

a) trehalose; b) glucose; c) inositol.

In Fig. 3a, b and c, the thermograms on the second scan of the system with trehalose, glucose and inositol are shown. The systems with maltose and sucrose give similar thermograms as that of trehalose, and those of galactose and sorbitol resemble that of glucose. The thermogram of the monosaccharide system has higher transition temperature, larger ΔH, and narrower peak width than that of disaccharide. The systems with inositol and mannitol show the highest transition temperature, but the peak temperature varies rather widely from test to test in the range of 70—80 °C. The thermodynamic parameters of these thermograms, *i.e.*, transition temperature (T_m), ΔH, the width of the peak at half height ($\Delta t_{1/2}$) and cooperative unit (n) of the transition are summarized in Table III.

These freeze-dried systems show metastability when they are stored at 4 °C for a prolonged time after the second scan. The way of change during storage in the system with disaccharide (Fig. 4) is quite different from the system with monosaccharide (Fig. 5). Figure 4b and c represents the thermograms of the system with trehalose stored at 4 °C for 2 weeks and 6 weeks, respectively. The systems with maltose and sucrose give results similar to trehalose. The storage at 4 °C causes the peak to shift to higher temperature, accompanied by an increase in ΔH and a decrease in the peak width. After 6 weeks of storage, a small and broad endothermic peak at around 80 °C is observed; the origin of this new peak is unknown at the moment. Rescanning of each stored sample results in the same thermogram as

TABLE III. Thermodynamic Parameters of the Transition on Second Scan of Freeze-Dried Sonicated DPPC Liposomes in the Presence of Various Additives

Additives	ΔH (kcal/mol)	T_m (°C)	$\Delta t_{1/2}$ (°C)	$n^{a)}$
Trehalose	5.6	25.4	6.7	15
Maltose	5.8	26.0	6.6	16
Sucrose	5.1	28.7	10.5	12
Glucose	9.7	44.9	2.3	28
Galactose	10.1	44.1	2.2	36
Sorbitol	10.3	42.6	2.7	26

a) The cooperative unit (n) is obtained from $n \fallingdotseq 7(273+T_m)^2/1000 \cdot \Delta t_{1/2} \cdot \Delta H$ as described by Tasaka and Mio.[16]

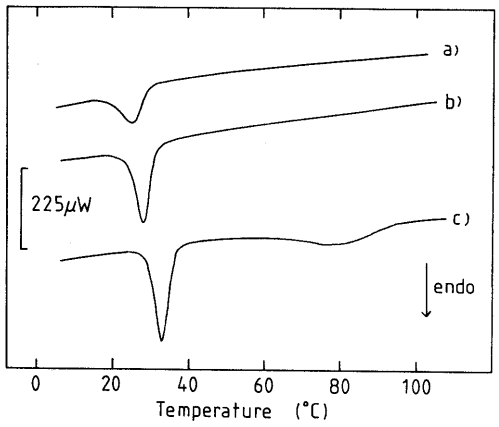

Fig. 4. DSC Heating Thermograms of Sonicated DPPC Liposomes Freeze-Dried with Trehalose

a) the second scan (T_m=25.4 °C, ΔH=6.0 kcal/mol); b) after storage at 4 °C for 2 weeks (T_m=28.6 °C, ΔH=8.3 kcal/mol); c) after storage at 4 °C for 6 weeks. There are two peaks at 33.7 °C (8.9 kcal/mol) and around 80 °C.

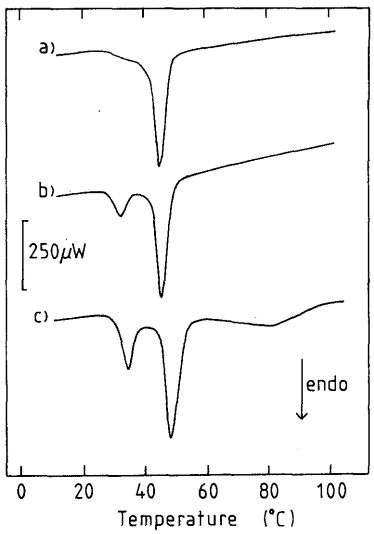

Fig. 5. DSC Heating Thermograms of Sonicated DPPC Liposomes Freeze-Dried with Glucose

a) the second scan (T_m=44.9 °C, ΔH=9.7 kcal/mol); b) after storage at 4 °C for 1 week. There are two apparent peaks at T_m=31.7 °C (1.8 kcal/mol) and 45.1 °C (8.9 kcal/mol); c) after storage at 4 °C for 5 weeks. There are three transitions at T_m=34.6 °C (3.2 kcal/mol), 48.3 °C (9.3 kcal/mol) and around 80 °C.

that shown in Fig. 4a. Some researchers[13,14] also found this kind of metastability in the dry mixture system of DPPC and trehalose. Lee *et al.*[14] prepared samples by freeze-drying of the mixture of trehalose and DPPC with organic solvent in contrast to the present sample preparation. Though the appearance of thermograms differs from ours, they found similar metastability to that in the present study and attributed it to the trapping of the lipid acyl chains in a disordered state.

Figure 5 shows the change with storage at 4 °C in the thermograms of the system with glucose. The endothermic peak at 45 °C changes little except for a slight shift of T_m to higher temperature. There is a clear, new endothermic peak at 32 °C in the thermogram after 1 week storage (Fig. 5c), and its T_m and ΔH increase slightly with length of storage. This peak is observed as a shoulder in the second scan thermogram (Fig. 5a). A broad endothermic peak at around 80 °C is also shown in the thermogram after 5 weeks (Fig. 5c). Results of the system with galactose are almost the same as those of glucose.

Discussion

The formation of hydrogen-bonding between the saccharides and phospholipid molecules is indispensable for the stabilization of liposomes during freeze-drying.[5] It significantly reduces the transition temperature of anhydrous DPPC from the gel to liquid-crystalline in a similar fashion to water.[5] In order to have the maximal effect of DPPC, saccharides must interact with DPPC at above phase transition temperature.[13] As a matter of fact, the transition temperature decreases drastically once the system has passed through the liquid-crystalline phase, as shown in Fig. 2. That is to say, the thermograms on the second scan (Figs. 2b and 3) can be regarded as representing the thermal property of DPPC when it interacts with saccharides on a maximal level. Based on this interpretation, DSC heating thermogram on the second scan is investigated to elucidate the effect of each saccharide on DPPC.

In the dry mixture system of DPPC and trehalose, Lee *et al.*[14] proposed designating the phase at above phase transition temperature as the λ-phase. The fatty acyl chains of DPPC are thought to be highly disordered and the head groups are immobilized due to binding to trehalose in the λ phase according to their deuterium nuclear magnetic resonance (NMR) study.[14] They also reported that a prolonged time is necessary for the λ-phase to convert to the stable gel phase due to the trapping of acyl chains in disordered state.[14] This slow reversibility is represented as the metastability in the second scan thermogram during the long term storage at 4 °C as shown in Fig. 4. T_m and ΔH increase and the peak width decreases during storage at 4 °C (Fig. 4). Such changes in the thermodynamic parameters mean that the gel phase of this system becomes more stable, *i.e.*, van der Waal's interaction between acyl chains becomes stronger during storage at 4 °C. In other words, the acyl chains in the gel phase in Fig. 4a are in a significantly disordered state. This is probably caused by the strong interaction between trehalose and the head group of DPPC. At the maximal level of the interaction, deep intercalation of trehalose into the head groups may be accompanied by the formation of hydrogen-bonding. The intercalation may induce the expansion of the phospholipid head group spacing; this was suggested by the molecular modeling studies by Gaber *et al.*[15] The fact that other disaccharides such as maltose and sucrose gave similar results as trehalose shows that such strong interaction is general in disaccharide molecules.

The thermograms on the second scan of the monosaccharide systems differ markedly from those of disaccharides as shown in Fig. 3 and Table III. The former systems have higher T_m, larger ΔH and narrower peak width. Smaller $\Delta t_{1/2}$ and hence larger n value indicate higher cooperativity of the phase transition in the monosaccharide systems,[16] suggesting that they have less effect on the interaction between DPPC molecules than disaccharide. The thermogram changes during 4 °C storage are also quite different from those of disaccharides (Figs. 4 and 5). The peak for the main transition at 44 °C is found almost unchanged during storage. This means that the conversion to stable gel phase in the monosaccharide system is faster than that in disaccharide system. A new peak at around 32 °C disappears when the stored samples immediately scanned again without further storage. This new peak may be ascribed to the transition to more thermodynamically favorable crystalline phases, which is also observed in the hydrated DPPC system during prolonged storage at low temperature.[17] This mode of metastability strongly suggests that the interaction between acyl chains is stronger in the monosaccharide system. This means that the interaction between monosaccharide and DPPC head group is weaker than that of disaccharide, though the hydrogen-bonding is formed in both cases.

These differences between monosaccharide and disaccharide in the interaction with phospholipid head group is probably the reason for the difference in their cryoprotective effects: despite monosaccharide completely preventing the fusion of liposomes during freeze-drying, it retains far less entrapped calcein of liposomes than disaccharides. In order to obtain a high percentage of retention, maintenance of the liposome membrane in a

more flexible state during the drying process is considered important. This is well satisfied by the addition of disaccharide to a liposome system, but is not by monosaccharide.

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