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Effects of glucose and its oligomers on the stability of freeze-dried liposomes

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Abstract

The effects of glucose and its oligomers (maltodextrins) on the stability of sonicated liposomes during freeze-drying were studied by monitoring the retention of the fluorescent dye, Calcein, entrapped in the liposomal inner aqueous phase and by the use of differential scanning calorimetry (DSC). Glucose showed weak cryoprotective effects on dioleoylphosphatidylcholine (DOPC) or egg yolk phosphatidylcholine (eggPC) liposomes, while it had a relatively high cryoprotective effect on dipalmitoylphosphatidylcholine (DPPC) liposomes. Maltose and maltotriose showed high cryoprotective effects on eggPC liposomes, while other maltodextorin, longer oligomers, showed low cryoprotective effects. No saccharide was effective to protect DOPC liposomes. The fluidity and/or packing of lipid membranes had considerable influences on the stability of liposomes during the lyophilization. Maltodextrins showed relatively high cryoprotective effects on DPPC liposomes at low saccharide/lipid molar ratios, although the cryoprotective effects decreased with the increase in the molar ratios. Size measurements suggested that glucose and maltose completely prevented the aggregation and/or fusion of liposomes during lyophilization, and that other maltodextrins induced them due to their weak hydrophobic properties.

Keywords: Liposome; Freeze-dry; Glucose; Maltodextrin; Saccharide; DSC; Lyophilization

1. Introduction

Recently in the pharmaceutical fields freezing [1,2] and freeze-drying of liposomes [3–17] have been attempted using stabilizers such as saccharides, and some of them are commercially available. The stabilization mechanism of saccharides has been studied by numerous workers [1–17]. Crowe et al. [10] proposed that the leakage of markers entrapped in the inner aqueous phase of liposomes can be ascribed to the liposomal fusion and the phase separation of liposomal membranes during drying and rehydration. The addition of certain saccharides to the liposomal solutions before drying can inhibit the fusion and phase separation, and depress the transition temperature in the dry lipids [10]. In the case of dipalmitoylphosphatidylcholine (DPPC) liposomes, they concluded that the damage of liposomes was due solely to fusion [10]. Harrigan et al. [12] proposed that part of the cryoprotective effect of saccharides might result from their ability to work as a spacing matrix between liposomes, thus preventing fusion. They also found that when saccharides were added only to the outside of liposomes, fusion of liposomes was completely prevented but leakage of an aqueous marker was not prevented, suggesting that saccharides play a role in maintaining the membrane permeability barrier other than prevention of fusion [12].

Many works suggested that there is a direct interaction between the sugar and the polar head group of the phospholipid [4,7,18–21]. Recently, a hydrogen-bonding formation between saccharides and lipid head group has been proposed to be indispensable for cryoprotection [7]. More recently, we found that there is no direct interaction between the saccharide molecules and DPPC molecules in the frozen state, suggesting that the hydrogen-bonding can form during the drying process [14].

Abbreviations: PC, lecithin; eggPC, egg yolk L-α-phosphatidylcholine; DOPC, dioleoyl-L-α-phosphatidylcholine; DPPC, dipalmitoyl-L-α-phosphatidylcholine; DSC, differential scanning calorimetry; T_c, phase transition temperature of hydrated liposomes; T_m, phase transition temperature of dehydrated liposomes.

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Thus, some workers have pointed out the presence of a variety of cryoprotective effects among the saccharides but the reason for these differences has not been elucidated [13]. Furthermore, the effect of mono- and di-saccharides as cryoprotectants has been mainly studied [1–17]. However, few data on oligo-saccharides have been reported.

Here, the effects of glucose and its oligomers (maltodextrins) with different numbers of glucose residues on the stability of sonicated liposomes during freeze-drying were studied by monitoring the retention of fluorescent dye, Calcein, entrapped into the liposomal inner aqueous phase and the apparent size changes, and by the use of differential scanning calorimetry (DSC). Also, the effects of the fatty acyl composition of lipids on the cryoprotective ability of a variety of saccharides were studied by using egg yolk, dioleoyl or dipalmitoyl lecithin as a liposomal component.

2. Materials and methods

2.1. Chemicals

Egg yolk L-α-phosphatidylcholine (eggPC) (iodine value of 65) was a gifted from Asahi Kasei (Tokyo, Japan). Dioleoyl-L-α-phosphatidylcholine (DOPC) (99%, P-1013) and dipalmitoyl-L-α-phosphatidylcholine (DPPC) (99%, P-0763) were purchased from Sigma (St. Louis, MO, USA). The stock solutions of lecithins (PCs) were prepared in chloroform solution, and they were kept in the freezer under a nitrogen gas atmosphere in the dark until use.

Glucose, maltose, maltotriose, maltotetraose, maltohexaose and maltoheptaose were purchased from Wako Pure Chemical Industries (Tokyo, Japan), without further purification. A fluorescent dye, Calcein, 3,3′-bis(*N*,*N*-bis(carboxymethyl)aminomethyl)-fluorescein, was obtained from Dojindo Laboratories (Kumamoto, Japan).

Water was double distilled with a quart still. All other agents were of analytical grade.

2.2. Preparation of liposomes

The PC stock solution was dried in a rotary evaporator under reduced pressure to form a lipid film on the wall of a round-bottomed flask. This film was left in vacuo for at least 12 h to ensure complete removal of the solvent. The Calcein-containing buffer solution, composed of 70 mM Calcein, 1 mM EDTA and 50 mM saccharides, adjusted at pH 7.0 using NaOH solution, was added to the thin film containing lipids. Nitrogen gas was bubbled to remove any dissolved oxygen, and the lipid was hydrated at about 55°C for DPPC or at room temperature, ca. 20°C in the case of eggPC or DOPC. During these incubations, the sample was vortexed periodically.

Sonicated and Calcein-trapped liposomes were obtained as follows [2,15,22]: The lipid suspensions were sonicated for 15 min under a nitrogen gas atmosphere at the same temperature as the hydration procedure. To eliminate Calcein un-trapped into the inner aqueous phase of the liposomes, the liposomal solution was gel-filtered through a Sephadex G-50 column (15 mm I.D. × 30 cm) using a 10 mM Tris-HCl buffer solution (pH 7.0) as an eluent at about 20°C, and orange-colored fractions in the eluted solution were collected. Then, various amounts of each saccharide were added to the liposomal solutions to provide the desired mole ratios of saccharides/lipids. In the gel-filtration step, the isotonicity was not maintained during the gel-permeation under our experimental conditions. In our previous study, however, little leakage in 50 mosM of osmotic gradient was found in our systems (< 1%) [15].

The concentrations of lipids were determined as those of P_i according to Bartlett's method [23], which was slightly modified in our laboratory.

2.3. Freeze-drying

In a chamber freeze-dryer Model RL-10 NA (Kyowa-Shinku, Tokyo, Japan) 100 μl of liposomal dispersions were freeze-dried. The sample was frozen to a terminal temperature of −45°C, and dried at −45°C of the shelf temperature at a pressure of 1.3 Pa for 18 h. Then, the shelf temperature was set at 25°C, and drying was continued at about 0.4 Pa for 6 h. The samples were rehydrated to their original volume with distilled water. The details were described previously [15].

Residual water content of freeze-dried samples was in a range from 0.2% to 1.0%, determined by Karl-Fisher method.

2.4. Calculation of percent retention of calcein

The percentage of Calcein retained after the freeze-drying was determined by measuring the fluorescent intensity of Calcein at 520 nm with excitation at 490 nm using a FB-550 fluorometer (Jasco, Tokyo, Japan) at 25°C. The percent retentions were calculated according to the method proposed by Crowe and Crowe [8].

2.5. Estimation of liposomal size

Liposomal size was estimated by dynamic light scattering and sedimentation methods on a Photal laser particle analyzer Model LPA-3100 (Ohtsuka Electronics, Osaka, Japan), which can estimate the particle diameter in a range from 3 nm to 100 μm. The weight average diameter and distribution of particle size were determined by the histogram method [24]. These technique was validated by using latex beads, of which average diameter and polydispersity were authorized. To determine the viscosity and refractive index, the values for each saccharide solution were used [25].

2.6. Differential scanning calorimetry (DSC)

Sonicated liposomes were prepared in each saccharide aqueous solution to give a molar ratio of 5:1 of saccharides to PCs except for glucose (10:1). After lyophilization, the sample was immediately weighed in an aluminum sample holder in an atmosphere of nitrogen gas, and the holder was hermetically sealed. DSC thermograms were obtained at a heating rate of 2°C/min on a differential scanning calorimeter Model DSC-3200S (MAC Science, Tokyo, Japan). The temperature of the DSC apparatus was calibrated using the melting point of gallium as a reference: 29.78°C. DSC measurements were performed at least three times, and the reproducibility of DSC curves was good.

The tradition temperature should not be evaluated as the peak temperature of the DSC-curve but as onset of the transition because the former depends on the scan rate. In some cases, however, it was difficult to estimate exact onset-values because of broad tradition peaks. Therefore, in this study the transition temperature was described as peak temperature of the transition curve.

3. Results and discussion

3.1. Retention of entrapped calcein

Fig. 1 shows the retentions of Calcein entrapped in the rehydrated liposomes after freeze-drying as a function of saccharide/PC molar ratios in a diversity of PC liposomes. The retentions were not dependent upon only the kind of glucose oligomers or PCs but also saccharide/PC molar ratios.

In all cases the retention increased with increasing molar ratios in small molar ratios. The addition of maltose or maltotriose to the eggPC liposomal solutions led to a high stabilization of them during dehydration and rehydration but glucose was not an adequate cryoprotectant for eggPC liposomes. On the other hand, in the cases of DPPC liposomes, glucose was slightly less effective than maltose.

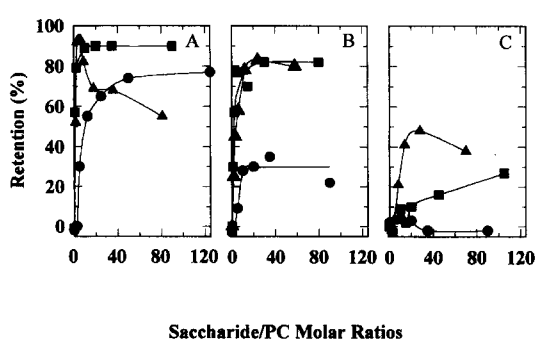

Fig. 1. Retentions of Calcein entrapped in the inner aqueous phase of liposomes after freeze-drying as a function of saccharide/PC molar ratios in various PC liposomes. PCs used are A, DPPC; B, eggPC; and C, DOPC. Saccharides are glucose (●), maltose (■) and maltotriose (▲).

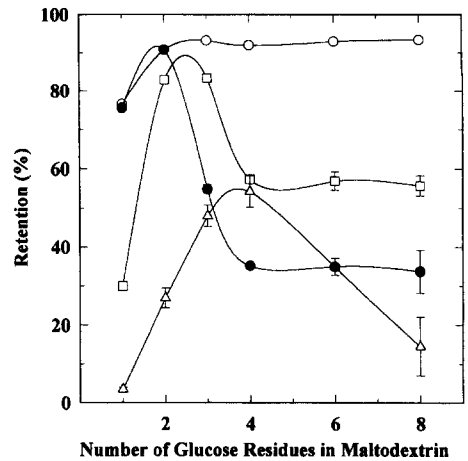

Fig. 2. Effects of the residual number of glucose on the maximum and plateau retention values of various liposomes after freeze-drying and rehydration in the presence of glucose and maltodextrins. Maximum retentions in PCs: ○, DPPC; □, eggPC; and △, DOPC. ●, The plateau values, referred to the retention at the highest saccharide/PC molar ratio observed in the DPPC liposome (in Fig. 1A).

For DOPC liposomes, no saccharide was a good cryoprotectant in the lyophilization.

In the presence of maltotriose, the retention reached its maximum at very small saccharide/PC molar ratios, and then, decreased with the increase in the molar ratios. This phenomenon was also observed when maltodextrins with more than two residues (maltodextrin) were used.

Fig. 2 shows the effects of glucose residual number on the maximum retentions of various liposomes after freeze-drying and rehydration in the presence of glucose and a variety of maltodextrins. Fig. 2 also contains plots of values, referred to as the values of retention at the highest saccharide/PC molar ratios observed in the DPPC liposome. The plateau values have a maximum at two of glucose residues (corresponding to maltose), following the decrease in the retention with increasing the residual number. This maximum retention is quite similar to the finding that the retentions were decreased with the increase in the maltotriose/PC molar ratios after the maximum, as shown in Fig. 1A–C. These findings indicate that there is specific interaction between longer glucose oligomers and PCs or liposomes. These phenomena have already been found in the freeze-thawing process [2]. Based on the fact that the hydrophobicity increases with the number of glucose residue, it could be ascribed to aggregation and/or fusion of liposomes through the hydrophobic interactions of saccharide molecule with PCs or liposomal membranes [2]. The details will be discussed again in Section 3.3.

For DPPC, all saccharides showed high cryoprotective effects at or over a certain saccharide/PC ratio. The plateau value in DPPC was 55% in maltotriose and 35% in maltodextrin having more than four glucose residues. In the case of eggPC, maltose and maltotriose showed high cryoprotective effects. The maximum value of retention for

eggPC liposomes was approximately 55% with maltodextrin in the case of more than three glucose residues. The maximum value of DOPC was about 50% with maltotriose and maltotetraose, and other saccharides gave a retention value of less than 50%. These findings indicate that acyl chain structures of PCs as well as numbers of glucose residues affect the retention during lyophilization, and that the presence of the *cis*-double bond in the PC acyl chains decreases the retention.

3.2. Differential scanning calorimetry

Fig. 3 depicts DSC thermograms of sonicated DPPC liposomes in the presence or absence of various saccharides. The phase transition temperature of the hydrated liposomes (T_c) at 39°C was changed to 104.2°C (the phase transition temperature of the dehydrated liposomes, T_m) by the dehydration in the absence of saccharides, as shown by curve A in Fig. 3. This was in good agreement with the reported value [9,10]. In the presence of saccharides the lyophilization led to lower T_m. The lower shift of T_m with saccharides is very similar to the phenomena, reported by Crowe and coworkers although they used trehalose as a saccharide [9,10].

The DPPC liposome, lyophilized with glucose, showed a peak of T_m at 40.5°C (a curve B in Fig. 3), which was not significantly different in the second scan of the same sample from the first scan (not shown). On the other hand, the DPPC liposome, freeze-dried in the presence of maltose, showed higher T_m (66.5°C) than in the presence of glucose, and in the second scan it shifted to a lower temperature (26.8°C) than that in the first scan (a curve C in Fig. 3). After the second scan, the peak did not change. Fig. 4 shows the effects of the number of glucose residues on the T_m of DPPC liposomes in the first and second scans after freeze-drying in the presence of glucose and various

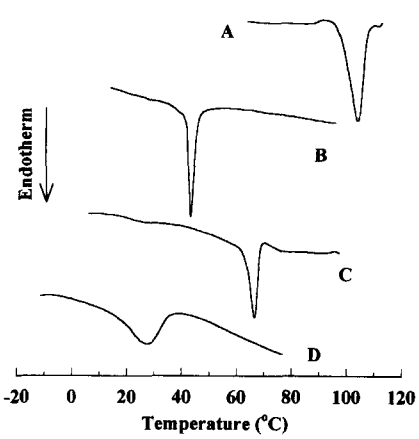

Fig. 3. DSC curves of dry liposomes after freeze-drying. DPPC liposomes lyophilized without saccharides at 1st heating scan (A), with glucose at 1st heating scan (B) and with maltose at 1st (C) and 2nd (D) heating scan. Saccharide/PC molar ratio was 5:1, except for glucose (10:1). The results are the means ± S.E. of three independent measurements.

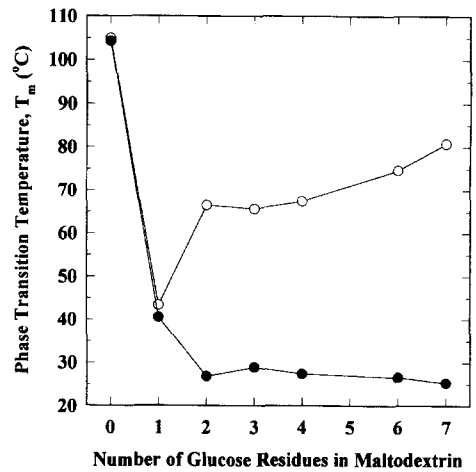

Fig. 4. Phase transition temperatures (T_m) of dry DPPC liposomes freeze-dried with glucose and maltodextrins. T_m values are at 1st (○) and 2nd (●) heating scans.

maltodextrins. The same phenomena as maltose were also observed in the presence of other maltodextrins. Crowe and Crowe [9], and Crowe et al. [10] reported the same effect of trehalose on the T_m as that of maltodextrins. These transitions have been ascribed to be gel to liquid-crystal phase transition of dry liposomal membranes [20,26,27].

Ausborn et al. suggested that lipid head groups and saccharides can rearrange during the fluid phase, resulting in an observed shift [28]. The agreement of T_m between the first scan and the second scan in the presence of glucose and the lower shift of T_m at the second scan in the presence of maltodextrins suggests that the interaction of glucose with the head group of lipids is weaker than that of maltodextrins because of its weaker hydrophobicity [29,30]. In other words, glucose can not provide sufficient lipid-separation to lower the phase transition temperature as much as maltodextrins. This interpretation is consistent with the model, proposed by Lee et al. [20]. They suggested that sugar molecules can occupy some space between the head groups of lipid molecules [20].

Fig. 5 presents the DSC thermograms of eggPC liposomes with and without various saccharides. The eggPC liposomes lyophilized with glucose were at a gel state at room temperature, as shown by curve A in Fig. 5. As hydrated eggPC liposomes are in a liquid-crystalline phase above about −15°C [31], the gel to liquid-crystalline phase change in the rehydration process was surely responsible for Calcein leakage, as shown by circle symbols in Fig. 1B. However, eggPC liposomes freeze-dried in the presence of maltose is in a fluid-like state at room temperature, as presented by curve C in Fig. 5, and the phase transition during the rehydration does not take place. In the case of eggPC and DOPC, it is necessary to maintain liposomal membranes in a fluid-like state because they are in a liquid-crystalline phase at the hydrated state. However, in

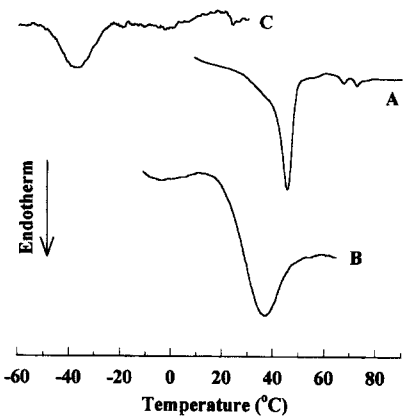

Fig. 5. DSC curves of dry liposomes after freeze-drying at the first heating scans. The eggPC liposomes lyophilized without saccharides (A), with glucose (B,) and with maltose (C).

the case of DPPC it is not necessary because the DPPC membrane is in a rigid state (gel phase) at the hydrated state as well as dry state, and the phase change during the rehydration does not occur [10]. One of the reasons for the leakage of aqueous marker from liposomes at the rehydration has been said to be a phase change [10]. These findings well support the mechanism for stabilization of dry liposomes by sugars, proposed by Crowe et al. [10].

As mentioned above, the cryoprotective roles of saccharides for two kinds of liposomes, eggPC and DPPC liposomes during the rehydration process are different; the former takes place from liquid-crystalline to liquid-crystalline transition, the latter from gel-like state to gel state. The DOPC liposome is also in a liquid-crystalline state at room temperature [33]. We can not find any saccharide to protect the DOPC liposome from freeze-drying damage. The detailed reasons for the results are still an open question.

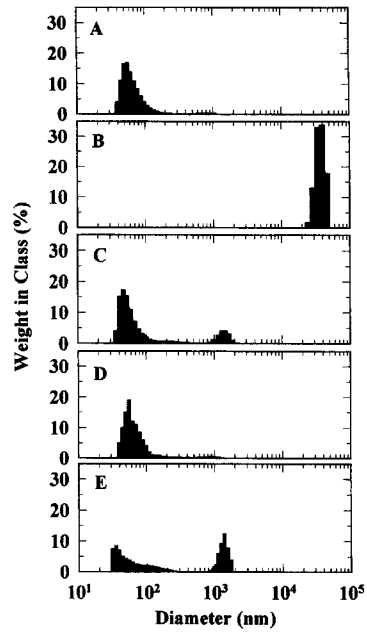

Fig. 7. Apparent size distributions of DPPC liposomes before freeze-drying and after rehydration at various maltotriose concentrations. A, before freeze-drying. B, C, D and E, after rehydration in the presence of maltotriose of various concentrations: B, 0 mM; C, 7.0 mM; D, 10.5 mM; and E, 175.0 mM. The concentration of DPPC is 1.75 mM.

3.3. Size distribution

Fig. 6 shows the size distributions of eggPC liposomes before dehydration and after rehydration in the presence of glucose or maltodextrins. In the case of glucose, no increase in liposome size was found (not shown), indicating that fusion and/or aggregation of eggPC liposomes is not the reason for the low cryoprotectant efficiency of glucose, in agreement with the previous results [16]. Maltose and maltotriose also prevented the aggregation and/or fusion of eggPC (shown in Fig. 6A–D), DPPC or DOPC (not

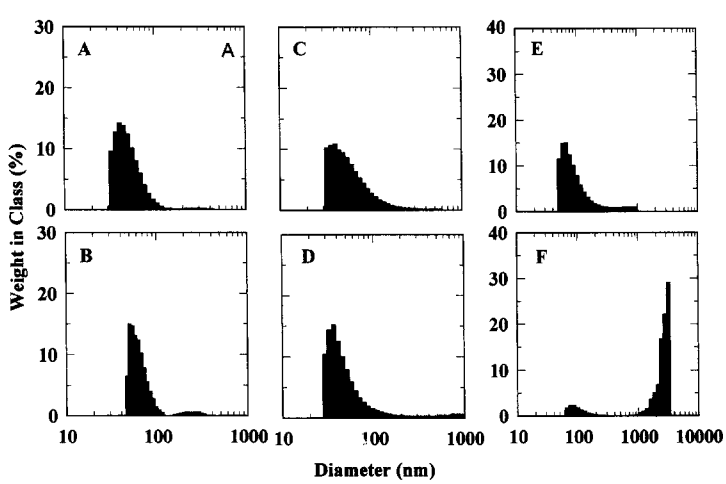

Fig. 6. Apparent size distributions of eggPC liposomes before freeze-drying and after rehydration in the presence of various maltodextrins. The saccharides are maltose (A and B), maltotriose (C and D) and maltotetraose (E and F). A, C and E, before freeze-drying; and B, D and F, after rehydration.

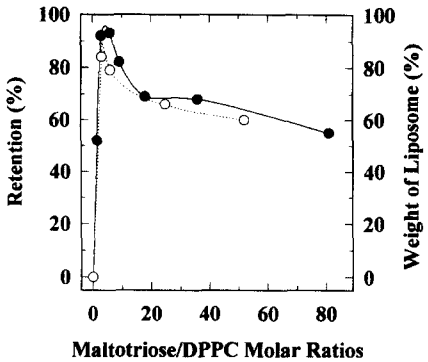

Fig. 8. Correlation between retentions of Calcein entrapped in the inner aqueous phase of DPPC liposomes after freeze-drying and weight of DPPC liposomes as a function of the maltotriose/DPPC molar ratios. •, Retentions of Calcein; ◦, weight of DPPC liposomes.

shown) liposome during lyophilization. However, the eggPC liposome lyophilized with maltotetraose was aggregated and/or fused notably (in Fig. 6F), resulting in a large leakage of Calcein, as shown in Fig. 2. These phenomena were also observed for DOPC liposomes (not shown). For eggPC and DOPC, maltodextrins with a large number of glucose residues caused aggregation and/or fusion of liposomes, resulting in low cryoprotectant ability. These phenomena were also observed in the freeze-thawing process [2]. In general the saccharide molecule has a weak hydrophobic character, which originates from the hydrophobic surface formed by the CH- and CH_2-groups of the saccharide molecules [32]. Therefore, the hydrophobicity of maltodextrins increases with the number of glucose residues [33], suggesting that these oligomers can interact with the membrane phospholipid molecules and/or the surface through hydrophobic bonding, resulting in aggregation and/or fusion of the liposomes [2].

Fig. 7 shows the size distributions of DPPC liposomes before and after the lyophilization in the presence of various concentrations of maltotriose. The aggregation and/or fusion of DPPC liposomes were prevented by 10.5 mM maltotriose (Fig. 7D), but the presence of 7 and 15 mM maltotriose (Fig. 7C and E) resulted in a small suppression.

Fig. 8 shows the correlations between Calcein retention and weight of DPPC liposomes, the size distribution of which was estimated, as a function of the maltotriose/DPPC molar ratios. The two curves coincide well with each other, clearly indicating that the leakage can be intimately related with the aggregation and/or fusion of liposomes.

4. Concluding remarks

There are a number of factors concerning the cryoprotective activities of glucose or maltodextrins. One is the difference in the tendency to induce aggregation and/or fusion of liposomes. Another is the lowering of the phase transition temperature of dry lipid, in other words, to prevent phase changes during the rehydration process. The latter factor was the same as that reported by Crowe [10] although they found the factor using saccharides different from ours [10]. We found that the fluidity and/or packing of lipid membranes also had considerable influences on the stability of liposomes during the lyophilization. Oligo-saccharides such as maltodextrins are not good stabilizers for liposomes except DPPC liposomes during freeze-drying because they induce aggregation and/or fusion due to the increase in hydrophobicity with the number of sugar residues.

Acknowledgements

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