The Effects of Glucose Oligomers (Maltodextrins) on Freeze-Drying Liposomes

Kazuhiko OZAKI*,[a] and Masahiro HAYASHI[b]

Pharmaceuticals Development Laboratory, Mitsubishi Chemical Corporation,[a] 14 Sunayama, Hasaki-machi, Kashima-gun, Ibaraki 314-02, Japan and Faculty of Pharmaceutical Sciences, Science University of Tokyo,[b] 12 Ichigaya, Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan.

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Liposomes were freeze-dried with glucose oligomers (maltodextrins) consisting of 2 (maltose) to 7 (maltoheptaose) glucoside units, and the effects of the glucoside unit number of the maltodextrin on the lyophilization of the liposomes were investigated. When the molar ratio of the glucoside units of maltodextrins to lipids was reduced below 6, two distinct endotherms were observed after annealing the freeze-dried L-α-dipalmitoylphosphatidylcholine (DPPC) liposome by differential scanning calorimetry (DSC). When the molar ratio was raised above 6, only the lower of the two endotherms was observed in all maltodextrins tested. At a molar ratio of 6, the gel to liquid crystalline transition temperature (T_m) of the first scan of these samples was measured. The T_m with maltose was observed to be *ca.* 65 °C, whereas the T_m with the other maltodextrins was observed to increase as the number of glucoside units was increased. Using Fourier transform IR, the phosphate asymmetric stretching band of DPPC liposomes lyophilized with these maltodextrins was observed to shift to lower frequencies. In all cases, the phosphate asymmetric stretching was observed to be roughly 1240 and 1224 cm^{-1} in the presence of these saccharides. The ratio of the absorbance at 1224 to that at 1240 cm^{-1} of DPPC liposomes freeze-dried with maltose was greater than the ratio of those stabilized with any of the other maltodextrins tested. These results suggest that the rate of hydrogen bonding between the phosphate of the lipid and maltodextrins was highest when maltose was used as the cryoprotectant. Because of this interaction, the space between the lipid molecules may become wider, causing an increase in the flexibility of the liposomal membrane.

Key words liposome; freeze-dry; maltose; maltodextrins

The lyophilization of liposomes in the presence of cryoprotectants is one of the most promising ways to keep liposomes stable during long-term storage.[1,2] The most important aspect of freeze-drying liposomes is to protect the membrane structures, and therefore, the presence of cryoprotectants are necessary during freeze-drying. Cryoprotectants for freeze-drying liposomes have been investigated in detail, and it is well-known that sugars, particularly disaccharides such as trehalose or maltose, are some of the most effective cryoprotectants for freeze-drying liposomes.[3-8] Investigations of the mechanism by which saccharides protect the membrane structure during freeze-drying have revealed two different effects. The first is the prevention of the mechanical rupture of membranes caused by ice crystal formation during the freezing process. The concentrated saccharide solution or the glass state mixture of saccharide and water surrounding the liposomes prevent the penetration of ice crystals. The second is the prevention of membrane disruption during drying and rehydration by maintaining the membrane of the liposome in a flexible state. The formation of hydrogen bonds between the lipid and saccharides maintains a critical distance between the lipid molecules, which allows the membrane to retain flexibility. One of the disadvantages of di-saccharides is their low collapse temperature. Although the collapse temperature increases with an increase in the number of glucoside units of oligosaccharides,[9] the effects of glucoside unit number on the freeze-drying of liposomes is currently not well understood.

In this paper, we investigated the effects of the glucoside unit number of maltodextrins on the freeze-drying of liposomes and confirmed the mechanism of protection of the membrane structure during freeze-drying. The glucoside unit examined was from 2 (maltose) to 7 (maltoheptaose), as shown in Fig. 1.

Experimental

Materials L-α-Dipalmitoylphosphatidylcholine (DPPC) was purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan); maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose from Hayashibara Co., Ltd. (Okayama, Japan); and calcein from Dojindo (Kumamoto, Japan). Water was purified by deionization and distillation. Polycarbonate membrane filters were purchased from Costar (Cambridge, MA, U.S.A.). The other chemicals used in this study were of reagent grade or better.

Preparation of Liposomes Multilamellar vesicles (MLV) were prepared by Bangham's method.[10] The lipids were dissolved in chloroform in a round bottomed flask and were dried *via* rotary evaporation to make a thin film. The residual solvent was removed in a desiccator. Calcein solution (95 mM, pH 7.0) was added to the round bottomed flask, and the flask was shaken by a vortex mixer for about 10 min above 60 °C to make the MLV liposomes. The osmotic pressure of the calcein solution was adjusted to the same value as the solution outside the liposomes, in which the cryoprotectants were added. To prepare liposomes of uniform size, the liposome solution (>60 °C) was extruded through two polycarbonate membrane filters with a pore

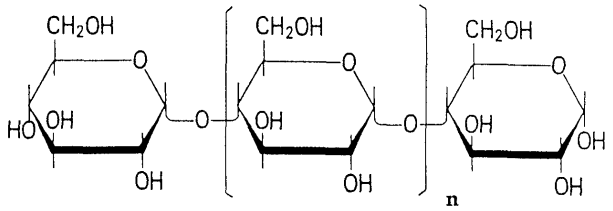

Fig. 1. The Structures of Maltodextrins (n=0—5)

n=0: maltose, n=1: maltotriose, n=2: maltotetraose, n=3: maltopentaose, n=4: maltohexaose, n=5: maltoheptaose.

* To whom correspondence should be addressed.

diameter of 100 nm . The extrusion was repeated 10 times. Untrapped calcein was removed on a Sepharose-6FF column using distilled water as the eluent. Subsequently, the cryoprotectants were added to the liposomal solution to make it isotonic with respect to the solution inside of the liposomes, and finally the concentration of lipids was adjusted to 20 mg/ml.

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 (Coulter Electronics, Inc., U.S.A.).

Freeze-Dried Liposomes Two ml of the liposomal solution was placed into 10 ml glass vials. The vials were partially capped with rubber stoppers. The vials were then lyophilized using an RL-100BSW lyophilizer (Kyowa Vacuum Engineering, Ltd., Japan). The liposomes were frozen to 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 its temperature was set to 20 °C for 10 h. Dried nitrogen gas was emitted into the drying chamber and the vials were sealed inside the drying chamber. The liposomal size and calcein leakage of each sample was measured after rehydration with 2 ml of distilled water.

Retention of Calcein (%) Any free calcein, due to leakage from 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 at 60 °C. Both the amount of free calcein and the amount of calcein retained inside the liposomes were determined by fluorescence measurement using an RF-5000 fluorescence spectrophotometer (Shimadzu, Japan) set to 490 nm for excitation and 520 nm emission wavelengths.

Differential Scanning Calorimetry (DSC) Measurement Fifty mg of DPPC was dissolved in chloroform and dried *via* rotary evaporation. Then, 100 mg of maltodextrin in 1 ml of distilled water was added. MLV and sized liposomes were made by the method described above. Aluminum DSC pans filled with 20 mg of liposomal solution were placed in 10 ml vials. The samples were lyophilized by the freeze-dry procedure described above. After that, the aluminum pans were hermetically sealed. The thermograms were analyzed using a DSC 8230C (Rigaku, 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 endotherm peaks, and the enthalpy was measured as endotherms corrected for phospholipid masses.

FTIR Analysis All liposome samples were prepared and freeze-dried by the same methods as for the DSC measurement described above. Then, each sample was applied to the surface of the diamond of a Golden Gate Single Reflection Diamond Attenuated Total Reflectance (ATR) apparatus (Graseby Specac, Belgium), followed by compression between the sapphire and diamond anvil. Next, the samples were analyzed with an FIS-165 Fourier transform IR (FTIR) spectrophotometer (Bio-Rad Laboratories, U.S.A.), running 64 scans with a resolution of 4 cm^{-1}.

Results

The Effect of the Glucoside Unit Number of Maltodextrins on the T_m and Enthalpy (ΔH) of Freeze-Dried Liposomes The effects of the glucoside unit number of maltodextrins used as cryoprotectants for the freeze-drying of liposomes on the T_m and ΔH of DPPC were investigated by DSC. As shown in Figs. 2 and 3, when the molar ratios of maltose or maltoheptaose to lipid were adjusted below 3 and 0.9, respectively, two distinct endotherms were observed after heating the freeze-dried liposomes above their transition temperatures. Below those molar ratios, a high temperature endotherm appeared at about 50—70 °C. As the maltodextrin content was increased, the high temperature endotherm diminished and the low temperature endotherm (25—35 °C) increased, until finally the enthalpies of the low temperature endotherms became saturated in cryoprotectant. When the molar ratios of maltose and maltoheptaose to lipid were above those ratios (3 and 0.9 respectively), only the low endotherm was observed in both cases. Converting the molar number of these saccharides into the molar number of glucoside units, the ratios for each of the saccharides was determined to be about 6, as shown in Fig. 4. Furthermore the same result was obtained for all the six maltodextrins that we tested (data not shown). The T_m and ΔH were determined for the freeze-dried liposomes with the six maltodextrins used at this ratio, and the results are shown in Table 1. After the annealing process, the T_m of all six samples was *ca.* 26 °C, and the ΔH was *ca.* −7 cal/g. In contrast, the T_m of the first scan (no annealing before measurement) showed very different results. In this case, the T_m of

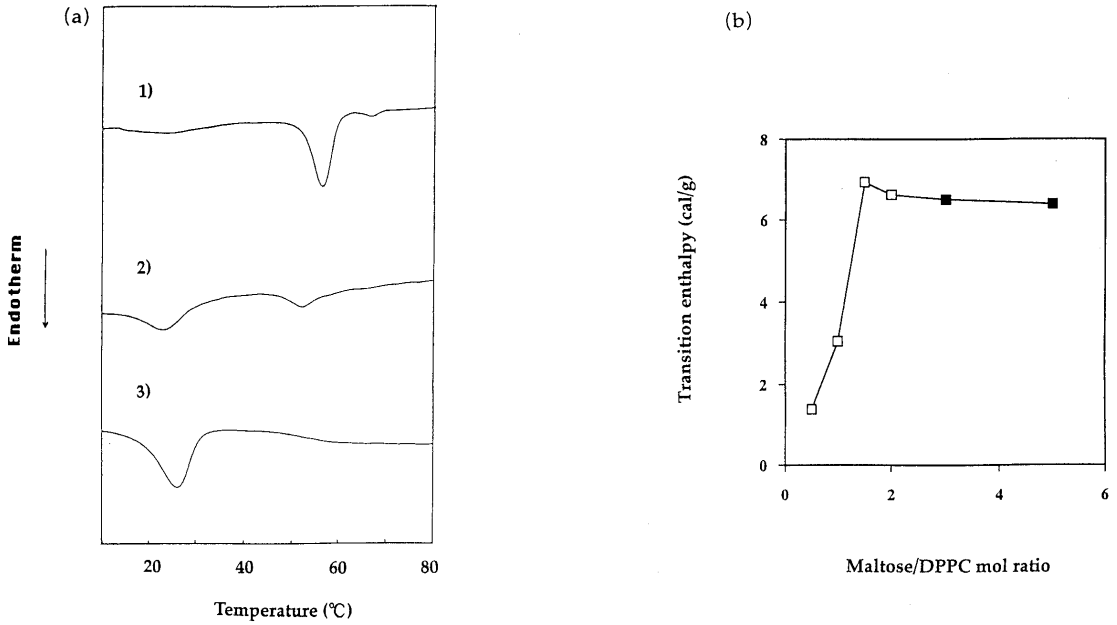

Fig. 2a. DSC Thermograms after the Annealing Process of DPPC Liposomes Freeze-Dried with Maltose

The molar ratios of maltose to DPPC were 1) 0.5, 2) 1, 3) 3.

Fig. 2b. Effect of the Maltose to DPPC Molar Ratio on the Low Temperature Transition Enthalpy

□, observed two peaks; ■, observed low temperature peak only.

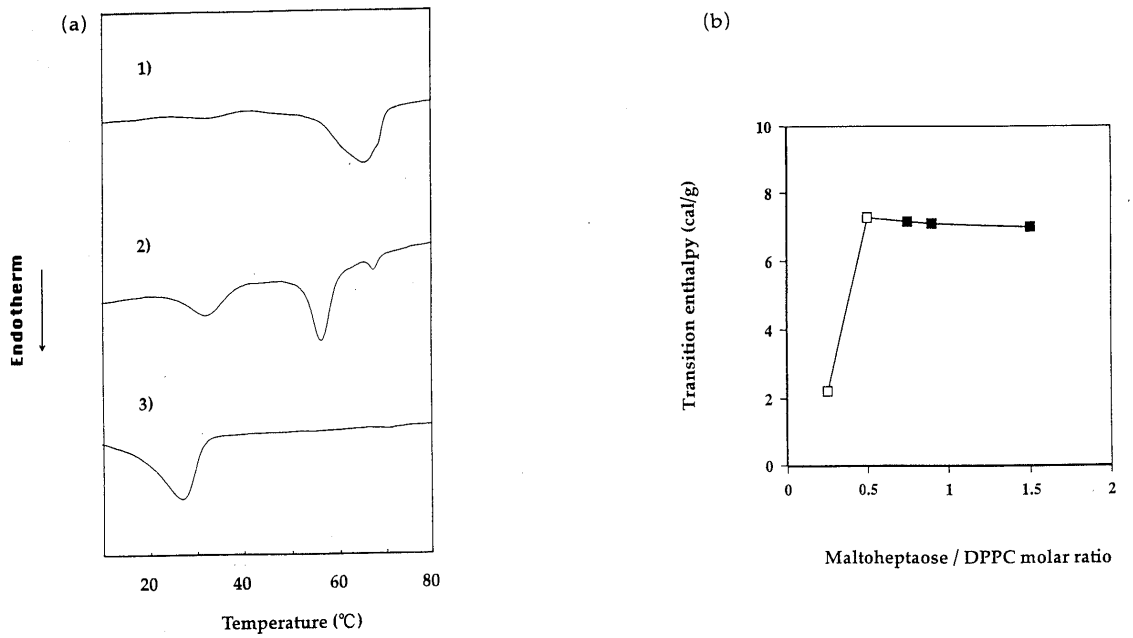

Fig. 3a. DSC Thermograms after the Annealing Process of DPPC Liposomes Freeze-Dried with Maltoheptaose
The molar ratios of maltoheptaose to DPPC were 1) 0.15, 2) 0.25, 3) 0.8.
Fig. 3b. Effect of the Maltoheptaose to DPPC Molar Ratio on the Low Temperature Transition Enthalpy
□, observed two peaks; ■, observed low temperature peak only.

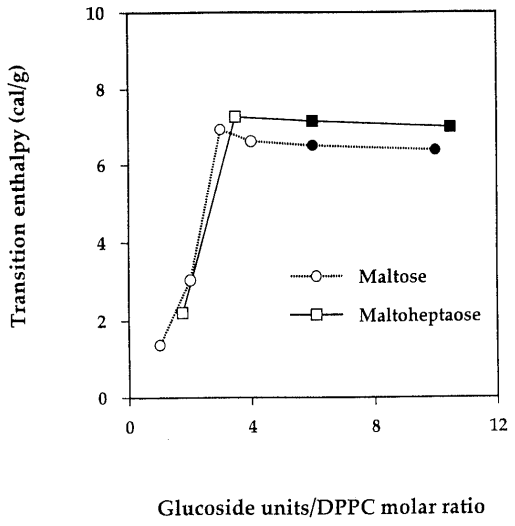

Fig. 4. Effect of the Glucoside Units of Maltodextrin to DPPC Molar Ratio on the Low Temperature Enthalpy
□, ○, observed two peaks; ■, ●, observed low temperature peak only.

maltose was observed to be about 65 °C, whereas the T_m of the other maltodextrins was observed to be steadily higher as their glucoside unit number increased.

The Effect of Glucoside Unit Number of Maltodextrins on the Calcein Retention of Freeze-Dried Liposomes The effect of the molar ratio of maltose and maltoheptaose to lipids on the retention of calcein was investigated. As shown in Fig. 5a, in the case of maltose, as the ratio increased from 0.5 to 3, the calcein retention increased from 50 to 95% and reached a maximum at a ratio above 3. In the case of maltoheptaose, as the ratio increased from 0.25 to 0.9, the calcein retention increased from 22.6 to 80% and reached a maximum at a ratio above 0.9. As shown in Fig. 5b, the molar ratio of glucoside unit to lipid, at which the retention of calcein reached a maximum, was about 6 in both cases. The effect of the glucoside unit number of the six maltodextrins at a molar ratio of 6 on the retention of calcein was also measured. As shown in Fig. 6, the retention of calcein with maltose and maltotriose was 95 and 90%, respectively. The retention of calcein with maltodextrins with a high number of glucoside units (4 to 7) was *ca.* 80—85%.

The Effect of Glucoside Unit Number of Maltodextrins on the Phosphate Asymmetric Stretching Band The effect of the glucoside unit number on the phosphate asymmetric stretching band was investigated by FTIR. As shown in Fig. 7, various conditions of freeze-dried liposomes using maltose were measured by FTIR. The phosphate asymmetric stretching of dried DPPC was centered at *ca.* 1260 cm^{-1}. The same band of freeze-dried liposomes using maltose was shifted down to 1240 and 1224 cm^{-1}. Further, if the sample was annealed, the ratio of absorbance at 1224 to that at 1240 cm^{-1} increased. The same results were observed in all the maltodextrins that we tested. As shown in Fig. 8a and 8b, the absorbance of the phosphate asymmetric stretching of freeze-dried liposomes using maltodextrins before and after annealing was measured. The ratio of the absorbances at 1224 to those at 1240 cm^{-1} was the highest using maltose irrespective of whether annealing had taken place. In addition, the absorbance ratio decreased as the number of glucoside units increased from 2 (maltose) to 4 (maltotetraose), but was unchanged from 4 to 7 (maltoheptaose) glucoside units.

Discussion

As shown in Fig. 5b, the maximum retention of calcein of freeze-dried liposomes with maltose was almost the

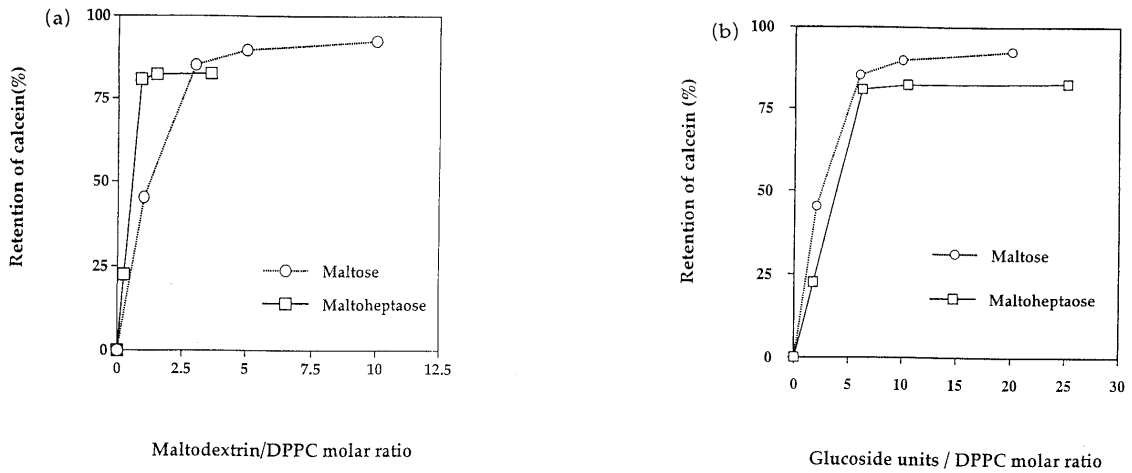

Fig. 5. Effect of the Molar Ratio of Maltodextrin (a) and Glucoside Units of Maltodextrin (b) to DPPC on the Retention of Calcein

Table 1. Transition Temperature and Transition Enthalpy of Freeze-Dried Liposomes with Maltodextrin

		Maltose	Maltotriose	Maltotetraose	Maltopentaose	Maltohexaose	Maltoheptaose
First scan	Transition temperature (°C)	64.5	68.1	69.2	69.9	70.3	72.9
	Transition enthalpy (cal/g)	−7.2	−6.0	−6.7	−6.3	−6.5	−6.6
Second scan	Transition temperature (°C)	26.1	25.6	26.5	26.8	26.9	26.7
	Transition enthalpy (cal/g)	−6.9	−6.9	−6.5	−7.0	−7.2	−7.1

The molar ratio of glucoside units of maltodextrin to lipid was 6.

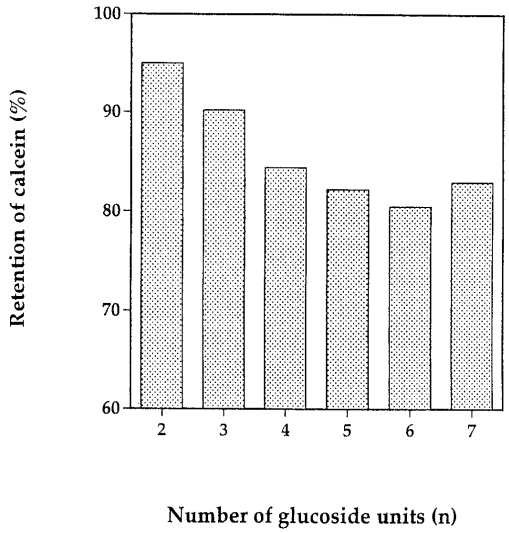

Fig. 6. Effect of the Glucoside Units Number of Maltodextrin on the Retention of Calcein

The molar ratio of glucoside unit of maltodextrin to lipid was 6. $n=2$: maltose, $n=3$: maltotriose, $n=4$: maltotetraose, $n=5$: maltopentaose, $n=6$: maltohexaose, $n=7$: maltoheptaose.

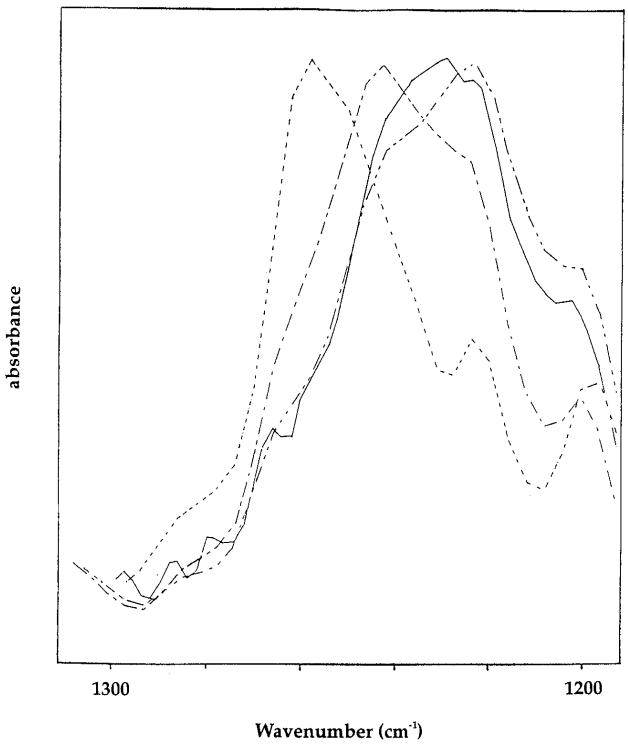

Fig. 7. FTIR Spectra of the Phosphate Asymmetric Stretching Band Region of DPPC Liposomes

– – –, dry DPPC; — — —, freeze-dried liposomes with maltose; — · — · —, freeze-dried liposomes with maltose after annealing; ——, liposome in solution.

same as the results reported by Crowe *et al.*[6] and Miyajima *et al.*[4] Further, the retention of calcein reached a maximum when the molar ratio of glucoside units to lipid was above 6. This ratio was virtually the same as the ratio at which only the low temperature endotherm was observed after annealing. These two results indicate that above this ratio, the cryoprotective effects of maltodextrins reached a maximum and the calcein retention and endotherm appearance did not change if the maltodextrins were added above this ratio. These results also suggest that the ratio at which the cryoprotective effects reached a maximum did not depend on the type of maltodextrins nor on the molar number of maltodextrins, but rather on the molar number of glucoside units. Recently, Suzuki *et al.*[7] reported that when using the maltodextrins which had more than three glycoside units for cryoprotection during the freeze-drying of liposomes, the retention of calcein entrapped in the inner aqueous phase reached its maximum

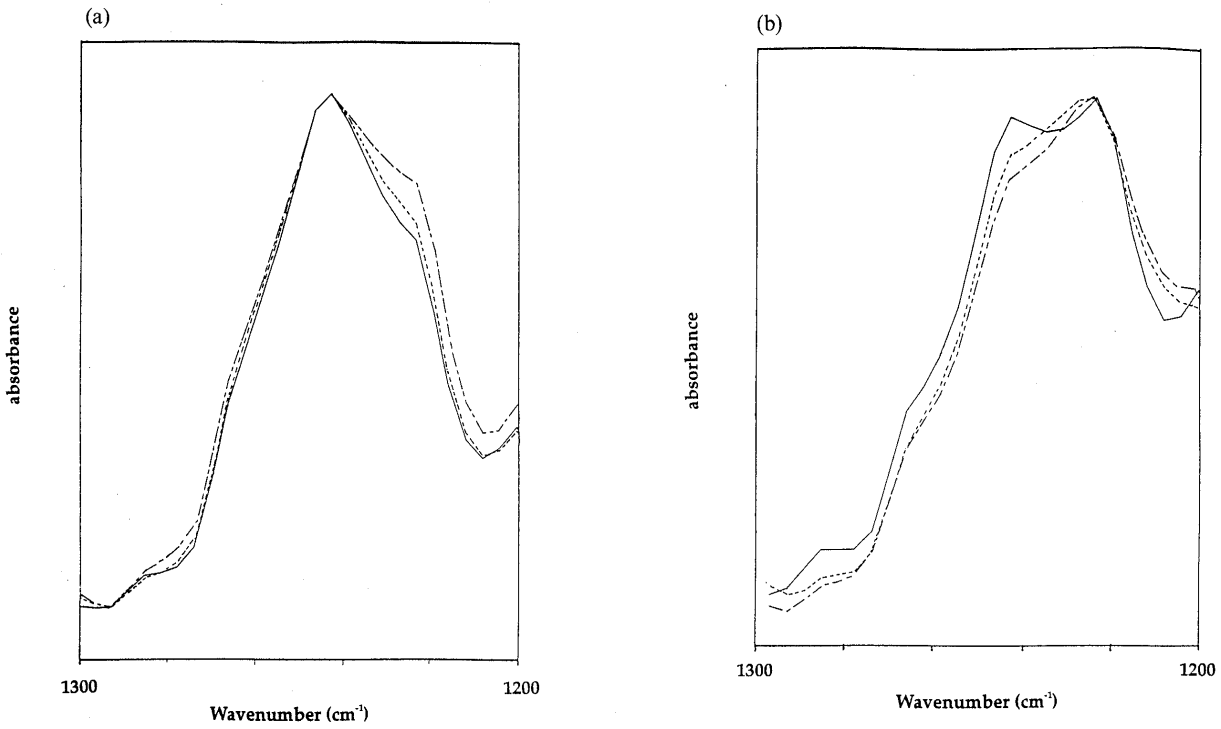

Fig. 8. FTIR Spectra of the Phosphate Asymmetric Stretching Band Region of DPPC Liposomes Freeze-Dried with Maltodextrins before (a) and after (b) Annealing

———, maltose; – – –, maltotriose; ——, maltoheptaose.

at a very small saccharide to lipid ratio, and decreased with an increase in the molar ratio. Their results apparently differed from our results (Fig. 5a and 5b). However, we obtained the retention of calcein at a smaller saccharide to lipid molar ratio, considering its practical use as a pharmaceutical formulation. As far as these smaller ratio, both results are considered almost equal.

As shown in Fig. 7, a phosphate asymmetric stretching band was observed at 1224 cm^{-1} when freeze-dried liposomes with maltodextrins were analyzed. Crowe *et al.*[8] reported that in the case of trehalose, the shift may be attributed to the hydrogen bonding between the phosphate group of the lipid and the hydroxyl of the sugars. This suggests to us that the interaction between DPPC and maltose was due to hydrogen bonding. To test this, asymmetric stretching of the phosphate group was examined for various preparations before and after annealing. As shown in Fig. 8a, using maltose as a cryoprotectant, the hydrogen bonding, as indicated by the largest shift in the phosphate asymmetric stretching, was greatest. The order of hydrogen bonding correlated with the order of retention of calcein, as shown in Fig. 6. This suggests that the formation of hydrogen bonding between lipids and sugars maintains a critical distance between lipid molecules. Because of this distance, the intermolecular interaction of lipids may be weakened and the fluidity of the acyl chain increased. The result was also supported by a decrease in T_m during the first DSC scans, as shown in Table 1. Miyajima *et al.*[11] suggested that if the membrane maintains its flexibility during freeze-drying and rehydration, the ability to resist and recover from membrane structural change increases. Therefore, as the glucoside units of maltodextrins increased, the hydrogen bonding between lipid and maltodextrins decreased. Consequently, maltose exhibited the strongest cryoprotectant effects, and increasing the number of glucoside units in the maltodextrins decreased the cryoprotectant effects. It is suggested that the reasons for these differences in the hydrogen bonding rate between lipids and sugars involved an increase in hydrophobicity[12] and steric hindrance as the glucoside unit number of maltodextrins was increased.

As shown in Table 1, the T_m after the annealing process was about 26 °C, which was much lower than the value before annealing. This indicates that the interaction between lipid and maltodextrins became stronger and the membrane became more flexible. This result was also supported by the shift observed in the phosphate asymmetric stretching using FTIR, as shown in Fig. 7.

Conclusion

The effects of the glucoside unit number of maltodextrins on cryoprotection were investigated. The larger the glucoside unit number, the less hydrogen bonding between lipid and saccharides was observed. Similarly, the space between lipid molecules was also decreased, the fluidity of the acyl chains of lipid decreased, and the membrane flexibility decreased. Because of these molecular level changes, the structure of the liposome membrane could not be perfectly maintained during drying and rehydration. The retention of calcein in liposomes using these six maltodextrins was also largely influenced by these effects. In conclusion, short chain maltodextrins such as maltose ($n=0$) and maltotriose ($n=1$) are considered very useful as cryoprotectants of liposomes during freeze-drying, whereas maltodextrins with large glucoside unit numbers are poor cryoprotectants due to their poor hydrogen

bonding with lipids.

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