Interactions of Cyclodextrins with DPPC Liposomes. Differential Scanning Calorimetry Studies

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The interaction of cyclodextrins (CDs) with dipalmitoylphosphatidylcholine (DPPC) liposomes has been studied using differential scanning calorimetry (DSC). The phase transition temperature and the enthalpy change due to the gel-to-liquid crystalline phase transition of the liposomes were measured in the presence of α-CD, β-CD, γ-CD, heptakis (2,6-di-*O*-methyl)-β-CD (DOM-β-CD), heptakis (2,3,6-tri-*O*-methyl)-β-CD (TOM-β-CD) and 2-hydroxylpropyl β-CD, respectively. The effects on the change of enthalpy of the transition and transition temperature were remarkable in the order of DOM-β-CD > α-CD > TOM-β-CD. The residual CDs caused scarcely detectable changes in the enthalpy changes and the transition temperatures.

In order to clarify the DSC curves in the presence of the CDs mentioned above, the type of interactions which occurred between CDs and DPPC liposomes were studied. Consequently, it was found that DOM-β-CD forms a soluble complex and α-CD forms an insoluble complex with DPPC liposomes, whereas only a slight amount of TOM-β-CD was suggested to penetrate the matrix of the liposomes.

Key words cyclodextrin; dipalmitoylphosphatidylcholine; liposome; differential scanning calorimetry; inclusion complex

Cyclodextrins (CDs) have been known to contain a hydrophobic cavity and to form inclusion complexes with many substances using the cavity.[1] In pharmaceutics, CD has been used for the purpose of increasing the stabilization and the solubility of drugs.[1] Also, it has been reported that the drugs given by oral administration in the style of the inclusion complexes are better absorbed in the living body than those free from such complexes,[1] if the drugs are slightly soluble. It has also been reported that CDs induce hemolysis by reacting with the erythrocyte membrane.[2] Therefore, the interaction of lipids, being the main constituent of the biomembrane, with CDs was investigated as follows.

Multilamellar liposomes consisting of L-α-dipalmitoylphosphatidylcholine (DPPC) were prepared, and the effects of CDs on the gel-to-liquid crystalline transition of DPPC were studied by differential scanning calorimetry (DSC). Then, in order to clarify the calorimetric traces observed in the presence of CDs, the types of interactions which occurred between CDs and DPPC were studied. No studies concerning the interaction of CDs with DPPC liposomes by DSC have been reported except for a few reports that CDs interact scarcely with phospholipids.[3,4]

Experimental

Materials α-CD, β-CD, γ-CD, heptakis (2,6-di-*O*-methyl)-β-CD (DOM-β-CD), heptakis (2,3,6-tri-*O*-methyl)-β-CD (TOM-β-CD), purchased from Nacalai Tesque Co., were used after being purified by recrystallization from water and 2-hydroxylpropyl β-CD (HP-β-CD), which was purchased from Aldrich Chemical Company, Inc. The average substitution degree of 2-hydroxyl group per β-CD was 5.6. The CDs were dried for 12 h at 110 °C in a vacuum before use. DPPC (purity >99.0%) purchased from Sigma was used without further purification. The water used was obtained by twice distilling water purified with ion-exchange resin. Reagent grade chloroform purchased from Nacalai Tesque Co. was distilled before use. All other chemicals used were of reagent grade.

DSC Measurements For preparation of the samples used for DSC, a given amount of DPPC was dissolved in chloroform; the solvent was evaporated to dryness first under nitrogen gas by rotary evaporation to give the deposition of a thin DPPC film on the inside wall of the flask. The latter was dried in a vacuum overnight. Tris–HCl buffer (10 mM Tris/0.1 mM EDTA/100 mM NaCl, pH 7.4) was added to the lipid film. The above mixture was subsequently vigorously vortexed for approx. 40 min, during which time the mixture was warmed at 50 °C above the transition temperature of the DPPC liposomes. Then, aliquots of the lipid suspension (4.0 μl) and each CD buffer solution (11.0 μl) having various concentrations were added to the aluminum sample cell and sealed. The concentration of DPPC was kept constant at 8.0×10^{-3} M. Then, the sealed mixture was incubated for 1—5 h at 50 °C above the transition temperature of DPPC liposomes. Calorimetric scans were performed at a rate of 2.0 °C/min, using the buffer as reference, with a differential scanning calorimeter, Rigaku DSC 8280, controlled by thermal analysis station TAS 100. The enthalpy measurements were calibrated using indium as a standard.

Measurement of Binding of CD to DPPC Liposomes and Solubilization of DPPC Liposomes by CD To investigate the interaction of CD and DPPC liposomes, multilameller liposomes (3.0×10^{-2} M) were prepared in the same way as those for the DSC measurements. Various concentrations of CDs were added to the aliquot of the liposomes, and suspensions with the same concentrations of DPPC liposomes (8.0×10^{-3} M) and CDs as those used for DSC measurements were prepared. These mixtures, obtained in this manner, were vortexed for 10 min at 50 °C, followed by incubation for 1 h at the same temperature. Then, after the obtained milky suspension was centrifuged at $20000 \times g$ and at 43 °C, using the centrifugal separator Hitachi SCR 20 B, the concentrations of CDs and DPPC in the supernatant solution were determined as follows, to investigate whether CDs bind to DPPC liposomes or DPPC liposomes are solubilized by CDs.

(i) Determination of CD: The determination of CD was carried out using a high-performance liquid chromatograph (HPLC) after the supernatant solution was filtered with Sample Prep-LCR (Millipore). HPLC analyses were conducted with a Shimadzu RID-R6A refractive index detector and Shimadzu Chromatopac CR-6A. The column temperature was maintained at 40 °C. The interactions of α-CD, β-CD, γ-CD, and HP-β-CD were performed with a Shin Kakoh column ULTRON VX-ODS (4.61 i.d. × 250 mm) using methanol : water = 5 : 95 (v/v) at 0.5 ml/min as a mobile phase. Also, the determinations of DOM-β-CD and TOM-β-CD were performed with a Nova-Pak C18 cartridge (3.9 i.d. × 150 mm) employed in a Waters-z-Module radial compression separation system (Waters Assoc.) using 2 propanol : water = 19 : 81 (v/v) and 22 : 78 (v/v), respectively, at 0.6 ml/min as a mobile phase. Each CD was determined by the calibration curves made from each peak area according to Chromatopac CR-8A.

(ii) Determination of DPPC: After the supernatant solution was filtered with a new Steradisc 13 (Kurabo), the concentration of DPPC in the filtrate was determined by Phospholipid C-Test Wako supplied by Wako Pure Chemical Industries, Ltd. Color-producing reagents, 3.0 ml of Phospholipid C-Test Wako, were added to the filtrate, 0.2 ml, then the

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reaction mixture was heated for 5 min at 37 °C and the absorbance at 600 nm was measured using the calibration curve made in advance.

Results and Discussion

DSC Measurements The interaction of DPPC liposomes with CD was studied by DSC. The effects of incubation times above the phase transition temperature mentioned in the experimental section on the DCS traces were initially examined. As a series of preliminary investigations, the incubation times of the mixtures of CD and DPPC liposomes were examined using α-CD and DOM-β-CD, which have strong interactions with DPPC liposomes. As shown in Fig. 1, it was found that the enthalpy change, ΔH_t, due to the gel-to-liquid crystalline phase transition of the DPPC liposomes in the presence of α-CD decreased with the incubation time for up to 5 h. ΔH_t, was evaluated from the area of the endothermic peak. Also, in the presence of DOM-β-CD, ΔH_t became constant with the incubation time for up to 5 h. Therefore, the subsequent experiments were carried out by being incubated for 5 h.

Figure 2 shows representative DSC scans of DPPC liposomes in the presence of α-CD, with increasing α-CD/DPPC molar ratios. Identical DSC curves were obtained after repeated scans of the samples, indicating that the structures formed by CD and DPPC do not vary with time and represent an equilibrium distribution of the two components. In Fig. 2 a, the endothermic peak due to the main phase transition of DPPC liposomes is observed in the neighborhood of 42 °C. Also, a pretransition was observed in the neighborhood of 36 °C. In the presence of α-CD, the area of the endothermic peak decreased with increasing α-CD/DPPC molar ratios (Fig. 2 b—d). Finally, the endothermic peak disappeared for α-CD/DPPC molar ratios = 6.3 (Fig. 2 e). However, it was found that the peak temperature is scarcely influenced by the presence of α-CD. From the calorimetric results presented here, the interaction between α-CD and DPPC liposomes was assumed to be as follows: In the presence of α-CD, the transition temperature was not changed, and only ΔH_t decreased. This suggests that α-CD substracts DPPC molecules from the liposomes and forms an inclusion complex with DPPC out of a membrane matrix; therefore, the number of phospholipid molecules constituting the membrane decreases, resulting in a decrease in ΔH_t.

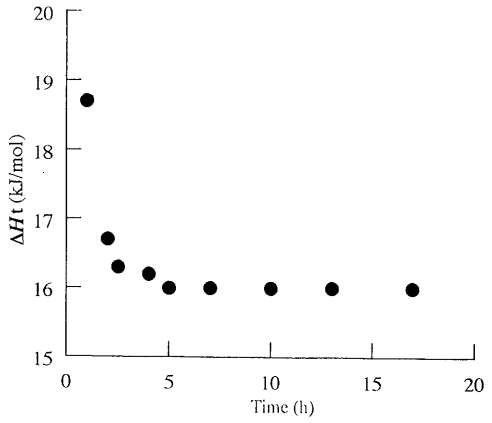

Fig. 1. The Effect of Incubation Time on ΔH_t of DPPC Liposomes in the Presence of α-CD (2.7×10^{-2} M)

Figure 3 shows representative DSC scans of DPPC liposomes with and without DOM-β-CD. In the presence of DOM-β-CD, the area of the endothermic peak decreased with increasing DOM-β-CD/DPPC molar ratios (Fig. 3 b—e) in a manner similar to α-CD. The endothermic peak disappeared for DOM-β-CD/DPPC molar ratios = 5.9. Also, the peak temperature was scarcely influenced by the presence of DOM-β-CD. Therefore, it is estimated that DOM-β-CD substracts DPPC molecules from the liposomes to form an inclusion complex with DPPC out of the membrane matrix in the same way as α-CD. Further, the interaction of DOM-β-CD with DPPC liposomes was a little stronger than that of α-CD judging from the molar ratio at which the endothermic peak disappeared.

Figure 4 shows representative DSC scans of DPPC liposomes with and without TOM-β-CD. In the presence of TOM-β-CD, the broadening of the endothermic peak was observed without any appreciable change in the area of the peak and with a slight lowering of the peak temperature. In the case of TOM-β-CD, as TOM-β-CD has a hydrophobic character,[5] it is estimated that it

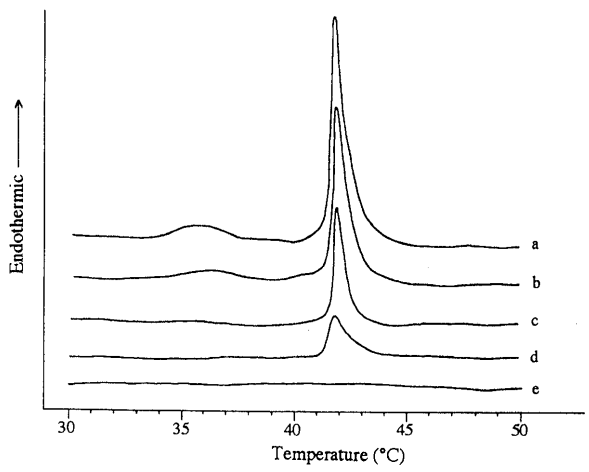

Fig. 2. DSC Scans of DPPC Liposomes in the Presence of α-CD

(a) DPPC liposomes alone 8.0×10^{-3} M, (b) α-CD 1.7×10^{-2} M, (c) α-CD 2.7×10^{-2} M, (d) α-CD 3.7×10^{-2} M, (e) α-CD 5.0×10^{-2} M.

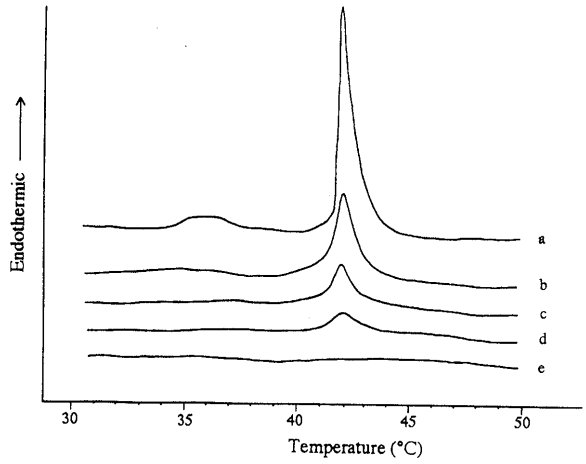

Fig. 3. DSC Scans of DPPC Liposomes in the Presence of DOM-β-CD

(a) DPPC liposomes alone 8.0×10^{-3} M, (b) DOM-β-CD 1.7×10^{-2} M, (c) DOM-β-CD 2.7×10^{-2} M, (d) DOM-β-CD 3.7×10^{-2} M, (e) DOM-β-CD 4.7×10^{-2} M.

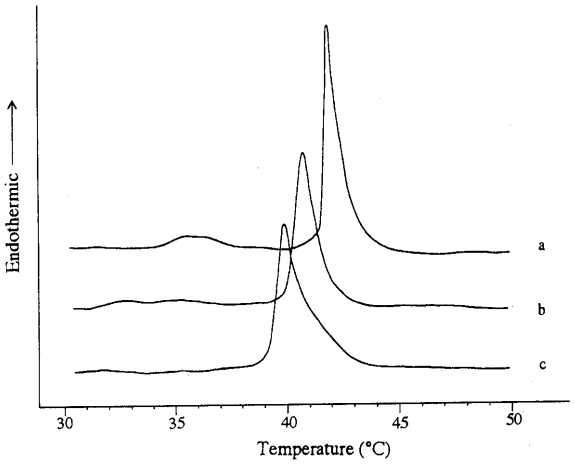

Fig. 4. DSC Scans of DPPC Liposomes in the Presence of TOM-β-CD

(a) DPPC liposomes alone 8.0×10^{-3} M, (b) TOM-β-CD 2.7×10^{-2} M, (c) TOM-β-CD 3.7×10^{-2} M.

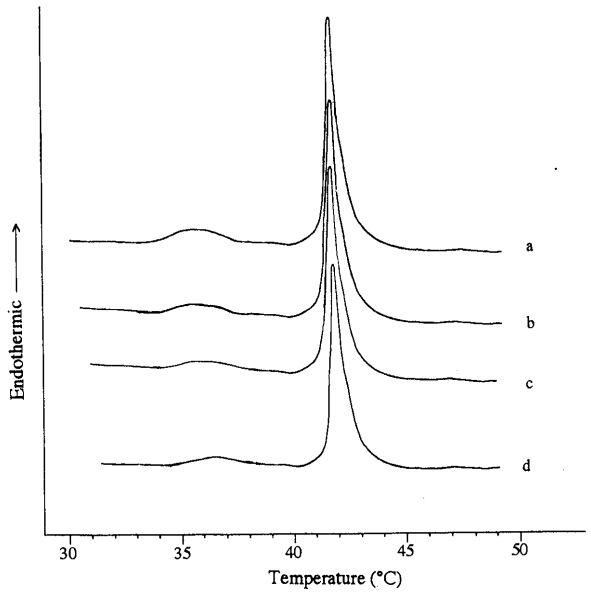

Fig. 5. DSC Scans of DPPC Liposomes in the Presence of β-CD, γ-CD, and HP-β-CD

(a) DPPC liposomes alone 8.0×10^{-3} M, (b) β-CD 2.2×10^{-2} M, (c) γ-CD 3.2×10^{-2} M, (d) HP-β-CD 3.2×10^{-2} M.

penetrates the membrane, as has been shown in many hydrophobic drugs,[6] and includes the acyl chain of DPPC in the matrix of the membrane. However, judging from the facts that seven glycosidic oxygen atoms of TOM-β-CD form an elliptically distorted heptagon,[7] the inclusion ability for acyl chains might be weak. In addition, TOM-β-CD is bulky.[7] Therefore, it is presumed that the stabilization of the gel state of liposomes decreases in the presence of TOM-β-CD within the membrane matrix. Respresentative DSC scans of DPPC liposomes with and without β-CD, γ-CD and HP-β-CD are shown in Fig. 5. In Fig. 5b, the concentration of β-CD added was 0.022 M (β-CD/DPPC molar ratio = 2 : 75), which is the solubility limit of β-CD. Also, in Fig. 5c and d, the concentrations of γ-CD and HP-β-CD added were both 0.032 M (CD/DPPC molar ratio = 4.0). As is apparent from Fig. 5, the peak areas and peak temperatures of the phase transition were scarcely influenced by the presence of β-CD, γ-CD and HP-β-CD, respectively. The dependence of the enthalpy change, ΔH_t, due to phase transition on the concentrations of CDs added, is shown in Fig. 6. From the calorimetric results, it has become apparent that α-CD and DOM-β-CD strongly interact with DPPC liposomes, followed by TOM-β-CD, though the interaction of TOM-β-CD is considerably weaker than that of the former, and the interactions of other CDs with DPPC liposomes are slight.

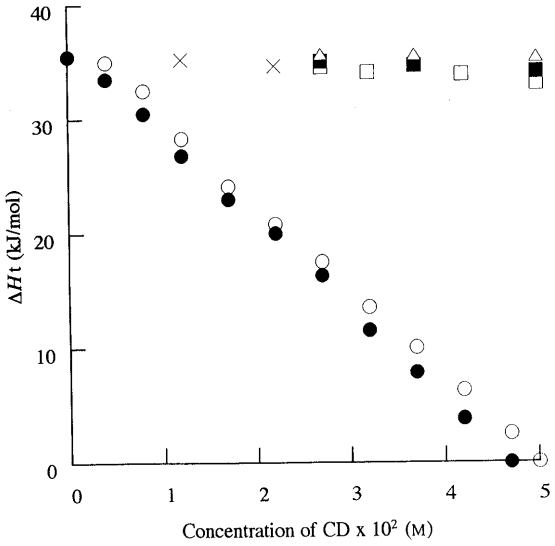

Fig. 6. The Effect of CD on the Enthalpy Change Due to the Phase Transition of DPPC Liposomes

○, α-CD; ×, β-CD; □, γ-CD; ●, DOM-β-CD; △, TOM-β-CD; ■, HP-β-CD.

Mode of Interaction The mode of the interaction of CD and DPPC liposomes, namely, whether CD permeates to the membrane matrix, or CD forms a soluble or insoluble complex in an aqueous solution out of the membrane matrix, was investigated to clarify the above considerations. α-CD of various concentrations and DPPC liposomes, with a concentration of 8.0×10^{-3} M were reacted. After centrifuging the suspension mixtures, the amount of α-CD in the supernatant was determined by HPLC, as described fully in the experimental section. In Fig. 7, the concentration of α-CD in the supernatant was plotted *versus* that of added α-CD; the concentration of added α-CD is presented with solid line. It was found that the concentration of α-CD in the supernatant was much smaller than that of the added α-CD. The concentration of α-CD in the supernatant increased only slightly until the added α-CD concentration reached about 4.9×10^{-2} M. However, the concentration of α-CD in the supernatant increased abruptly when that of added α-CD was increased beyond 4.9×10^{-2} M. This phenomenon is explained as follows: Added α-CD reacted with DPPC liposomes to form an insoluble complex with DPPC molecules constituting the liposomes. At the concentration of 4.9×10^{-2} M α-CD, all the DPPC molecules in the liposomes were expended to form an insoluble complex with α-CD. Therefore, the amount of α-CD added beyond the concentration remains in the supernatant as it is. The insoluble complex formation was confirmed because the production of a white precipitation was observed when α-CD and DPPC liposomes were mixed. Also, it is ap-

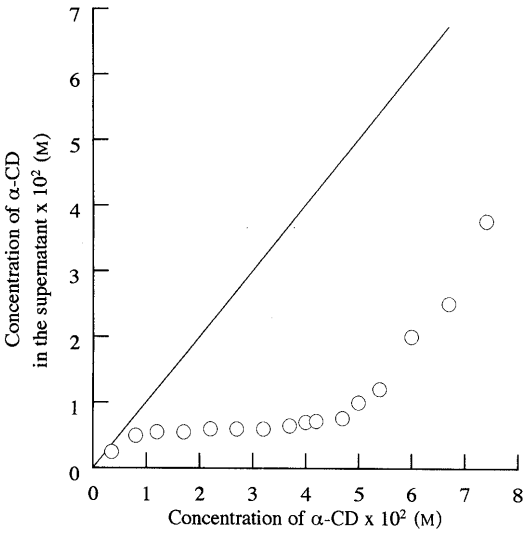

Fig. 7. The Concentration of α-CD Dissolved in the Presence of DPPC Liposomes

Table 1. Concentrations of TOM-β-CD in the Supernatants and Liposomes

Concentration of total added TOM-β-CD (M)	Concentration in the supernatant (M)	Concentration in the DPPC liposomes (M)
1.70×10^{-2}	1.67×10^{-2}	3×10^{-4} (1.8%)
2.70×10^{-2}	2.64×10^{-2}	6×10^{-4} (2.2%)
3.70×10^{-2}	3.61×10^{-2}	9×10^{-4} (2.4%)
5.00×10^{-2}	4.88×10^{-2}	1.2×10^{-3} (2.4%)

parent that the difference of α-CD concentration, 4.1×10^{-2} M between 4.9×10^{-2} M and 0.8×10^{-2} M in the supernatant, namely the concentration difference of α-CD between the α-CD concentration added beyond 4.9×10^{-2} M and that in the supernatant was expended to form an insoluble complex with DPPC liposomes, 8.0×10^{-3} M. Thus, the composition of the insoluble complex between α-CD and DPPC, with a molar ratio of α-CD to DPPC, is about 5.1, in which approximately 5.1 mol α-CD bind to 1 mol DPPC on average. This value agreed with the composition of precipitate formed between small unilamellar liposomes of DPPC with calcein in the inner aqueous phase and α-CD.[8] Also, it was found that the composition of the insoluble complex is constant, independent from unilamellar or multilamellar liposomes.

Then, the concentration of TOM-β-CD in the supernatant was investigated after the TOM-β-CD was added to the liposomes, and the suspension mixture was treated according to the method described above. The results are presented in Table 1. The concentrations of TOM-β-CD in the supernatant are smaller than the added ones. The differences in both concentrations can be attributed to the fact that TOM-β-CD precipitates with the DPPC liposomes, though the differences are only slight. It was suggested that TOM-β-CD penetrates DPPC liposomes, though only slightly, and the penetrating amount of TOM-β-CD increases with an increase in the added concentration as shown in the third line in Table 1. On the other hand, in the presence of β-CD, γ-CD, DOM-β-CD and HP-β-CD, each CD concentration in the supernatant agreed with each added CD concentration. Together with the consideration of the results of DSC measurements, it is suggested that DOM-β-CD forms a soluble complex which exists as both complex and free forms in the supernatant. It seems reasonable to consider that other CDs scarcely interact with DPPC liposomes, since they exist as a free rather than complex form in the supernatant.

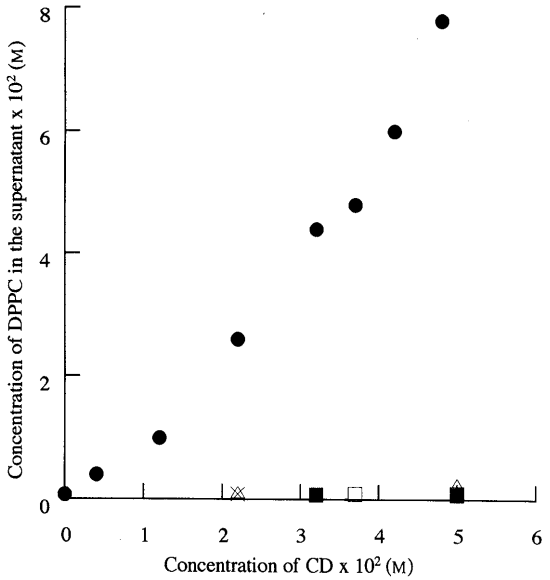

Fig. 8. The Concentration of DPPC Dissolved from Liposomes in the Presence of CD

×, β-CD; □, γ-CD; ●, DOM-β-CD; △, TOM-β-CD; ■, HP-β-CD.

To better clarify the interactions of CDs with DPPC liposomes, after the CDs were added to the liposomes and the suspension mixtures were treated in the method described above, another component, the DPPC concentration in the supernatant, was investigated. The DPPC concentration dissolved by the presence of CD was plotted *versus* the added concentration of CD. The results are shown in Fig. 8. In the addition of DOM-β-CD to DPPC liposomes, the concentration of DPPC in the supernatant increased significantly with the increase in added DOM-β-CD concentration. On the other hand, the concentration of DOM-β-CD in the supernatant coincided with that of added DOM-β-CD, as described already. Therefore, it is reasonable to conclude that DOM-β-CD includes a DPPC molecule in its cavity and forms a soluble complex with the DPPC molecule, subtracted from DPPC liposomes. In the case of DOM-β-CD, the endothermic peak due to the phase transition of DPPC liposomes (8.0×10^{-2} M) disappeared in the presence of DOM-β-CD, of which concentration is 4.75×10^{-2} M. Also, in the presence of 4.75×10^{-2} M DOM-β-CD, 7.9×10^{-2} M DPPC was solubilized, as shown in Fig. 8. Therefore, it was further confirmed that DOM-β-CD forms a soluble complex with DPPC being subtracted from the liposomes, out of the membrane matrix, and then, the DPPC molecules constituting liposomes decrease, resulting in a decrease in the endothermic peak area due to phase transition.

In the addition of the other CDs to the DPPC liposomes, the concentrations of DPPC in the supernatant barely

increased with the increase in added CD concentrations (Fig. 8). In the addition of α-CD, the concentration of DPPC in the supernatant did not increase because the DPPC subtracted from the liposomes by α-CD forms insoluble inclusion complex. Also, in the addition of TOM-β-CD, the concentration of DPPC in the supernatant did not increase. This result is consistent with the consideration that TOM-β-CD penetrates the matrix of the liposomes rather than subtracting a DPPC molecule out of the membrane as mentioned above.

Further, with the addition of β-CD, γ-CD, and HP-β-CD, the concentrations of DPPC in the supernatant barely increased. It is apparent that these CDs barely interact with DPPC liposomes, in addition to the considerations of experimental results mentioned above. However, the DPPC liposome suspension containing these CDs tended to rise to the surface with partial sedimentation in spite of centrifugal separation treatment. The tendencies of the liposomes containing γ-CD and HP-β-CD to rise to the surface increased with an increase in the concentration of both added CDs. In the case of β-CD, no significant tendency to rise to the surface was observed because the solubility of β-CD is low and a high concentration of added β-CD is not obtained. From the experimental results mentioned above, it is likely that these three CDs do not form an inclusion complex with DPPC. It has also been shown that water-structure making reagents such as sugars may perturb a hydrated layer surrounding phospholipid liposomes, indirectly causing structural changes of the bilayer surface.[9] A recent X-ray study has indicated that the addition of trehalose causes an increase in the interbilayer spacing of hydrated multilamellar DPPC liposomes, but exerts no notable effect on the lipid chain packing.[10] Also, it has been reported that water-structure breaking reagents such as urea may disrupt the water structure near the polar head group of DPPC in such a way as to alter the pattern of intermolecular hydrogen bonding among DPPC, thus leading to an altered packing of the polar head groups. CDs such as γ-CD and α-CD have been reported to be water-structure making reagents,[11] whereas β-CD is considered to be a water-strucure breaking reagent.[12] Also, HP-β-CD can be presumed to be a water-structure making or breaking reagent in view of the structure. Therefore, these CDs may interact with hydrated water molecules on the DPPC liposome surface and then slightly weaken the condensation forces of DPPC molecules constituting the liposomes. As the results, only a slight decrease in ΔH_t, due to phase transition is observed in the presence of these three CDs, as shown in Fig. 6. In addition, the tendency to rise to the surface of the suspension was observed and is considered attributable to the slight decrease in the density of DPPC packing.

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