Alteration in the Temperature-Dependent Content Release Property of Thermosensitive Liposomes in Plasma

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The effect of plasma components on the temperature-dependent content release property of thermosensitive liposomes has been described. Temperature-sensitive liposomes containing mitomycin C (MMC) were prepared from dipalmitoylphosphatidylcholine (DPPC liposomes) and a 7 : 3 mixture of DPPC and dipalmitoylphosphatidylglycerol (DPPC/DPPG liposomes). We defined in this study the difference in the content release between 38 °C and 44 °C as an index of the temperature-dependent content release efficiency (Δ% release). In the absence of rat plasma, the Δ% release of the DPPC liposomes and the DPPC/DPPG liposomes was 83% and 71%, respectively. However, when the release study was conducted with rat plasma, the Δ% release increased to about 96% for both liposomes. In addition, while the DPPC liposomes were destabilized by rat plasma below the gel-to-liquid crystalline phase transition temperature (T_m), MMC leakage from the DPPC/DPPG liposomes below T_m was suppressed by rat plasma. Moreover, the plasma protein binding onto lipid bilayer was concomitant with the gel-to-liquid crystalline phase transition and then enhanced the temperature-dependent release from the DPPC/DPPG liposomes. The possible mechanism of interaction between liposomes and plasma proteins, especially serum albumin, was discussed based on differential scanning calorimetry and protein binding experiments.

Key words temperature-sensitive; phase transition release; mitomycin C; phosphatidylglycerol

Liposomes have been proposed as carriers for the delivery of molecules to cells. They have been shown to enhance the efficacy of encapsulated drugs, prolonging the circulation time and reducing side effects. Moreover, the targeting of liposomes to desirable sites has been attempted.[1,2] In addition, a number of attempts have been made to develop functional liposomes, which can regulate the release of drugs responding to various stimuli, such as pH,[3—6] light[7,8] and temperature.[9—15]

The liposomes injected into blood would be opsonized by serum components, and rapidly taken up by the reticuloendothelial system (RES) including the liver and spleen.[16—20] This is a major problem for researchers, because it is a significant disadvantage for the delivery of drugs to non-RES tissues. Moreover, some serum components have a destabilizing effect upon lipid vesicles and thereby cause leakage of liposomal contents. For example, high-density lipoprotein (HDL) is known to cause the disintegration of liposomes.[21,22]

Yatvin *et al.* and Weinstein *et al.* reported an unique approach to controlling the release of drugs using temperature-sensitive liposomes in conjunction with local hyperthermia.[9—11] The barrier efficiency of the membrane abruptly decreases near the gel-to-liquid crystalline phase transition temperature (T_m) of the phospholipid membrane. The temperature-sensitive liposomes have been designed to release a drug in response to local hyperthermia, during which a tumor was heated at temperatures of 41 to 45 °C. Other strategies have been used for the production of temperature-sensitive liposomes. One example is the use of polymers, which are attached to the liposome to exhibit temperature-sensitivity and cause release of the internal content above a certain temperature.[14,15]

In the development of the liposome, pharmaceutical scientists have been confronted with two difficulties. The first is how to prolong circulation time. Recently, the liposome surface has been modified with GM1 ganglioside[23] and polyoxyethylene derivatives,[24,25] which have some ability to escape the RES. The second is the problem regarding the stability of liposomes in systemic circulation after injection. In general, it is favorable for a drug delivery system (DDS) to have liposomes that are stable in the blood circulation, especially for chemotherapy and gene therapy. In addition, when trying to develop stimulus-sensitive liposomes, one should consider the influence of the serum components on the function of liposomes. Liu and Huang have observed that DOPE-based pH-sensitive liposomes lost their pH-sensitivity in the presence of serum.[26]

Anionic lipids are frequently used in preparing liposomes for drug delivery. In the fluid state, negatively charged liposomes composed of phosphatidylglycerol (PG) interacted with serum components and caused an increase in the leakage of the encapsulated drug.[27] Therefore, it is necessary to investigate the effects of serum components on the temperature-dependent release property of thermosensitive liposomes. For this purpose, we prepared a temperature-sensitive liposome by adding dipalmitoylphosphatidylglycerol (DPPG) to the lipid composition of dipalmitoylphosphatidylcholine (DPPC). In this study, we investigate the temperature-dependent content release from thermosensitive liposomes in the presence of rat plasma, because we expected that certain plasma proteins interact with the negatively charged lipid bilayer and cause the enhancement of temperature-dependent release. The effect of the plasma proteins on the temperature-dependent release property of thermosensitive liposomes has been described using mitomycin C (MMC, see Fig. 1) as a

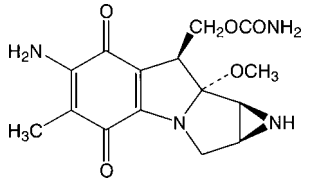

Fig. 1. Chemical Structures of Mitomycin C

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model drug.

Experimental

Materials MMC was produced industrially by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). DPPC and DPPG were purchased from Nippon Fine Chemical Co., Ltd. (Osaka, Japan). Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DOWEX 50W-X2 cation exchange resin (proton form, 100—200 mesh) was purchased from Dow Chemical Co. (Midland, MI, U.S.A.) and used after washing with distilled water. All other chemicals were of reagent grade and obtained from Kanto Chemical Co. (Tokyo, Japan). Rat plasma was obtained from male Wister rats and frozen at −80 °C before use.

Preparation of Liposomes Large unilamellar vesicles (LUVs) were prepared by the reverse-phase evaporation method (REV).[28] Briefly, 100 mg of DPPC or a 7 : 3 mixture by weight of DPPC and DPPG was dissolved in an organic phase consisting of 5 ml of diethyl ether and 1 ml of chloroform. One and a half milliliters of the aqueous phase containing 6 mM MMC and 280 mM mannitol was added to the lipid/organic solvent mixture and then mixed by a vortex mixer. The obtained w/o emulsion was homogenized with a bath-type sonicator for 5 min. The organic phase was slowly removed *in vacuo* until liposomes were formed. Free MMC in the LUV suspensions was removed with cation exchange resin. One milliliter of the liposomal suspensions was mixed with 80 mg of DOWEX 50W-X2 cation exchange resin for about 1 min. The mixture was centrifuged at 2000 rpm for 1 min. Then the pH of the liposomal suspensions was adjusted to 6—8 by mixing the supernatant with the same volume of 10 mM Na_2HPO_4 containing 280 mM mannitol.

Vesicle size distributions were determined by quasielastic light scattering employing a DLS-700 Dynamic Light Scattering Spectrophotometer (Otsuka Electronics Ltd., Osaka, Japan).

Measurement of MMC Release from Liposomes The lipid concentration of the MMC-loaded liposomal suspensions was diluted to 2.8 mM with 10 mM phosphate buffer (pH 7) containing 280 mM mannitol. When the influence of plasma proteins was investigated, 30% (v/v) rat plasma was included. One milliliter of each sample was incubated at a specified temperature for 4 min. The released MMC was removed from the liposomal suspensions with cation exchange resin. The concentration of MMC in the liposomal suspensions was measured by HPLC after the disruption of liposomes with 5% Triton X-100.

HPLC Analysis MMC was determined by HPLC on a μ-Bondapak C18 ODS column (10 μm, 3.9×300 mm, Waters, Milford, MA, U.S.A.). The mobile phase consisted of a mixture of 1/300 M phosphate buffer (pH 7.0) and methanol at 8 : 3 (v/v) and was delivered at a flow rate of 1.0 ml/min. A UV detector operated at 254 nm was used to detect and quantitate MMC.

Differential Scanning Calorimetry Differential scanning calorimetry (DSC) of liposomes was performed with a DSC10 (Seiko E & I, Tokyo, Japan) equipped with a SSC/580II Thermal Controller (Seiko E & I, Tokyo, Japan). Aliquots (10 μl) of liposomal suspensions which contained 0.14 μmol of phospholipid were analyzed at a heating rate of 1 °C per minute using water as a reference. When the cycle heating was conducted, samples were heated to 45 °C and cooled to about 15 °C, and then heated again at a rate of 1 °C per minute.

Protein Binding Removal of lipids from rat plasma was performed with ether as described by Scanu and Edelstein.,[29] and then powdered plasma proteins were obtained by drying with nitrogen gas *in vacuo*. The powdered proteins were reconstituted with distilled water. Insoluble particulate matter was removed by centrifugation (3000 rpm for 10 min at 5 °C) and then the supernatant was filtered through a polysulfone membrane (0.2 μm). The protein concentration in solution was 30 mg/ml, determined using the Bio-Rad Protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

For binding studies, a mixture of 0.2 ml of liposomal suspension and 0.2 ml of protein solution was incubated at 45 °C for 5 min. In the case of BSA binding studies, phosphate buffered saline containing 30 mg/ml of BSA was incubated with liposomal suspensions at the indicated temperature for 4 min. The liposomal suspensions were then eluted with phosphate-buffered saline on a 1.5×20 cm column of Sepharose CL-6B (Pharmacia, Piscataway, NJ, U.S.A.) to remove free BSA. The protein concentration in the liposomal fractions was determined using protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Polyacrylamide Gel Electrophoresis Protein molecular mass was determined by SDS-PAGE. Electrophoresis was performed under reducing conditions by using precast ATTO PAGEL (5—20% acrylamide gradients; ATTO Corporation, Tokyo, Japan) with ATTO electrophoresis AE-3131 (ATTO Corporation). To visualize the protein, the gel was stained with coomassie brilliant blue (CBB R-250). Prestained molecular weight markers were used: Myosin, 200 kD; β-galactosidase, 116.3 kD; Phosphorylase b, 97.4 kD; Serum albumin, 66.2 kD; Ovalbumin, 45 kD; Carbonic anhydrase, 31 kD; Trypsin inhibitor, 21.5 kD; Lysozyme, 14.4 kD; Aprotinin, 6.5 kD.

ζ Potential Measurements ζ potentials of liposomes were determined in 10 mM phosphate buffer (pH 7.4) by electrophoretic measurements using a dynamic laser scattering detection electrophoretic apparatus (ELS-800, Otsuka Electronics Ltd., Osaka, Japan).

Statistical Analysis Data were expressed as the means±S.D. of three experiments. Statistical difference was analyzed by two-way analysis of variance or Student's *t*-test. Statistical significance was considered when $p<0.05$.

Results

MMC (Fig. 1) is an effective antitumor antibiotic used extensively in cancer chemotherapy. MMC has also been reported to have a mutual effect in combination with hyperthermia.[30,31] In general, to achieve an ideal temperature-dependent release from liposomes, the drug chosen should be hydrophilic and have a low affinity for the lipid membrane. MMC has an *n*-octanol/buffer partition coefficient (log *P*) of −0.5 at pH 6.5 and thus is considered suitable as a model compound for developing temperature-sensitive liposomes.

Preparation of MMC-Loaded Temperature-Sensitive Liposomes We prepared MMC-loaded liposomes with a lipid composition of DPPC for the neutral form (DPPC liposomes) and a 7 : 3 mixture, by weight, of DPPC and DPPG for the negatively charged form (DPPC/DPPG liposomes) by means of the reverse phase evaporation technique. DPPG was selected as an anionic lipid component because it has been reported that PG can interact with serum components in its fluid phase.[27] By utilizing DOWEX 50W-X2, a cation exchange resin, we could remove completely the free MMC from the liposomal suspensions. This procedure enabled us to prepare the MMC-loaded liposomes quickly and easily, because conventional techniques to remove unentrapped materials from liposomes such as gel filtration, dialysis and centrifugation are troublesome and time-consuming. The MMC encapsulation efficacy for these liposomes was 30—35%, regardless of the lipid composition. It should be noted that the liposomes obtained with this method contain 0.30—0.35 mg/ml of MMC, which is a sufficient concentration for clinical application. The mean particle size of the liposomes was determined as 200—300 nm by dynamic light scattering (Fig. 2).

Temperature-Dependent Content Release Figure 3 represents % release of MMC from liposomes as a function of temperature after incubation in phosphate buffer for 4 min throughout the temperature range used in hyperthermia. To evaluate the influence of the serum components on the temperature-dependent content release, the liposomes were incubated with 30% (v/v) rat plasma. As shown in Fig. 3, the DPPC liposomes without rat plasma showed minimal release below the experimental temperature of 38 °C with a gradual increase to 87% at 44 °C. The DPPC/DPPG liposomes without rat plasma exhibited a release of about 9% below 38 °C, which increased to 63% at 42 °C and further to 81% at 44 °C. However, when the study was conducted with rat plasma, a complete release of MMC was achieved at 44 °C, regardless of the lipid composition.

Influence of Rat Plasma on MMC Release from Liposomes When developing liposomes as drug carriers, it is necessary to consider the stability in the circulation as well

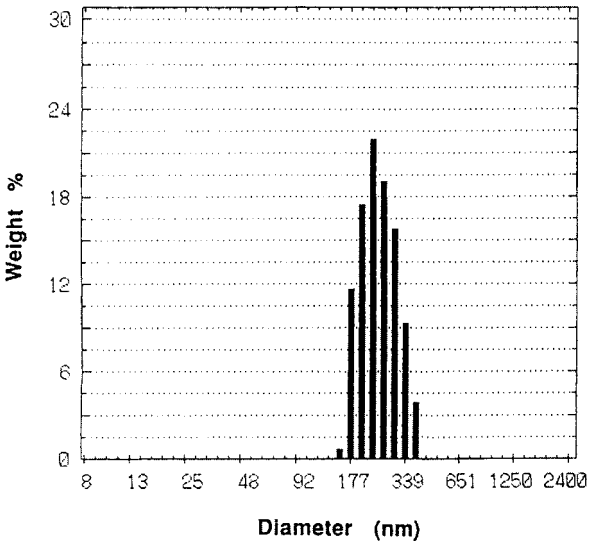

Fig. 2. Representative Particle Size Distribution of the DPPC/DPPG Liposomes

Weight average particle size of this distribution is 250.9 nm.

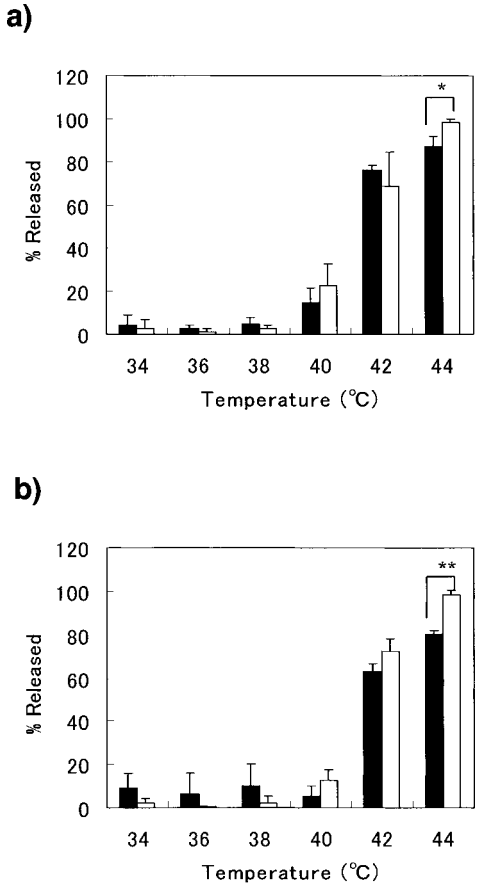

Fig. 3. Temperature-Dependent Release of Mitomycin C from the DPPC Liposomes (a) and the DPPC/DPPG Liposomes (b) in the Absence (■) and Presence (□) of Rat Plasma

* Indicates $p<0.05$, and ** indicates $p<0.01$ as compared with the respective control group. Data represent the means±S.D. of three experiments.

as to assess the functional drug release. Figure 4 represents % release of MMC as a function of incubation time. The liposomes were incubated at 37 °C and 42 °C for 60 min with or without rat plasma and the release profiles were evaluated.

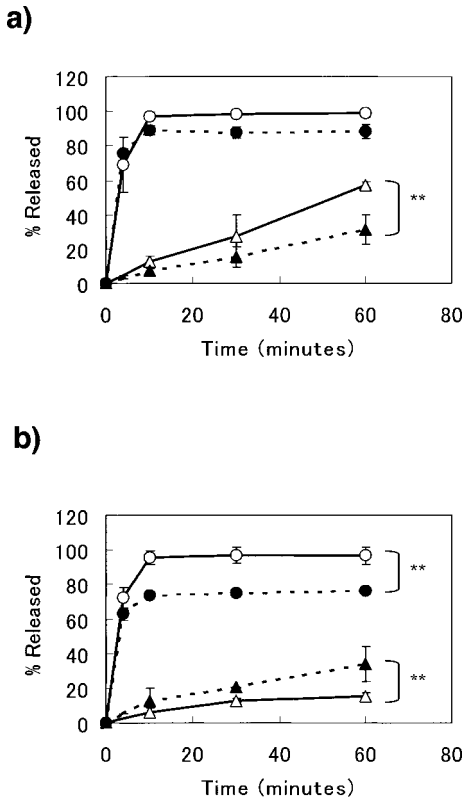

Fig. 4. Mitomycin C Release from the DPPC Liposomes (a) and the DPPC/DPPG Liposomes (b) at 37 °C (▲, △) and at 42 °C (●, ○) in the Absence (Filled Symbol) and Presence (Open Symbol) of Rat Plasma

** Indicates $p<0.01$ by ANOVA between the groups. Data represent the means ±S.D. of three experiments.

For the DPPC liposomes, the content release reached a plateau of 88% at 42 °C without rat plasma, while it increased to 99% even at 42 °C in the presence of rat plasma. The DPPC/DPPG liposomes showed the same tendency and the % release increased from 76% without rat plasma to 97% with rat plasma at 42 °C. Both of the liposomes exhibited an excellent phase transition release at 42 °C in the presence of rat plasma.

However these liposomes showed a remarkable feature, retaining the incorporated drug at temperatures below the T_m. The leakage of MMC from the liposomes at 37 °C without plasma was quite extensive and increased gradually to about 30% after 60 min, regardless of the lipid composition. The release profiles of these liposomes at 37 °C without rat plasma was little different. When the release study was conducted with rat plasma, the MMC leakage from the DPPC liposomes was significant and gradually increased to 57% after 60 min. In contrast, the MMC leakage from the DPPC/DPPG liposomes was suppressed in the presence of rat plasma and reached a plateau of 15%. This means that the DPPC liposomes are destabilized by rat plasma below the T_m, while the plasma components enhanced the barrier efficiency of the DPPC/DPPG liposomes.

Plasma Protein Binding to Lipid Membrane Table 1 summarizes the ζ potential of the liposomes at pH 7.4. The ζ potential of the DPPC liposomes was +1 mV. That of the DPPC/DPPG liposomes was −46 mV. These values reflected the lipid composition of each kind of liposomes. After the liposomal suspensions were mixed with the rat plasma, the ζ

Table 1. ζ Potential of DPPC and DPPC/DPPG (7 : 3, w/w) Liposomes

	ζ Potential (mV)	
	DPPC liposomes	DPPC/DPPG liposomes
Phosphate buffer (pH 7.4)	+1±0	−46±1
+Rat plasma	+2±1	−31±0
+Rat plasma +42 °C-4 min	±0±0	−31±1

Values represent the means±S.D. of three experiments.

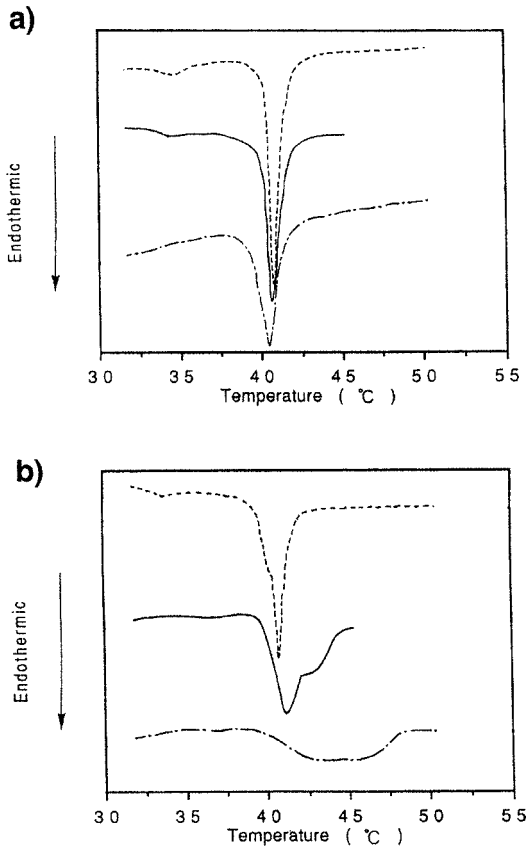

Fig. 5. Differential Scanning Calorimetry of the DPPC Liposomes (a) and the DPPC/DPPG Liposomes (b) in the Absence of Rat Plasma (-----) and the First (——) and Third (–·–·–) DSC Scan in the Presence of Rat Plasma

The scan rate was maintained at 1 °C/min.

potential of the DPPC/DPPG liposomes increased to −31 mV but that of the DPPC liposomes did not change. After heating to 42 °C and cooling to 25 °C, there was no change in the value of ζ potential. These findings suggest that the DPPC/DPPG liposomes interact with the plasma components, probably plasma proteins, through electrostatic attraction.

Thermal analysis provides a valuable tool with which to evaluate the lipid/protein interaction in terms of excess free energy.[32] Figure 5 shows scanning calorimetric curves of the DPPC and the DPPC/DPPG liposomes. Without rat plasma, both of the liposomes exhibited a sharp thermal transition with a midpoint at 41 °C and a pronounced pretransition at about 33 °C. Next we conducted calorimetric experiments on

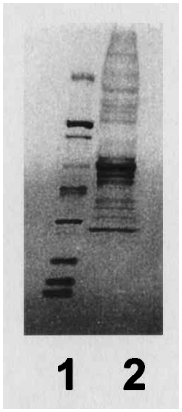

Fig. 6. Gradient Gel Electrophoresis of Proteins Binding to the DPPC/DPPG Liposomes

Lane 1, molecular weight markers (Myosin, 200 kD; β-galactosidase, 116.3 kD; Phosphorylase b, 97.4 kD; Serum albumin, 66.2 kD; Ovalbumin, 45 kD; Carbonic anhydrase, 31 kD; Trypsin inhibitor, 21.5 kD; Lysozyme, 14.4 kD; Aprotinin, 6.5 kD); lane 2, proteins binding under reducing conditions.

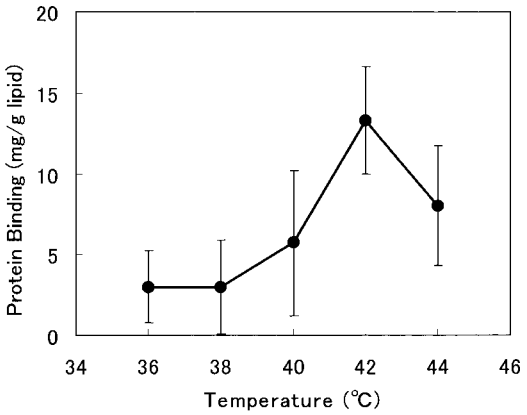

Fig. 7. Temperature-Dependent Binding of Albumin to the DPPC/DPPG Liposomes after Incubation for 4 min

Data represent the means ±S.D. of three experiments.

lipid membranes in the presence of rat plasma. A cycling scan was made. In the case of the DPPC liposomes, the calorimetric curve broadened slightly and the midpoint transition temperature lowered accompanying a decrease in the phase-transition enthalpy (ΔH), though the thermal transition was rather sharp (Fig. 5a). In contrast, the calorimetric curve of the DPPC/DPPG liposomes broadened markedly and shifted to a higher temperature after cycling scanning, and then the midpoint transition peak disappeared in scan 3 (Fig. 5b).

The DPPC/DPPG liposomes were isolated after incubation with the plasma proteins by gel filtration. The proteins associated with the liposomes were analyzed by SDS-PAGE under reducing conditions (Fig. 6). There are several major polypeptides associated with the DPPC/DPPG liposomes, nine of them having molecular weights of 123, 70, 66, 57, 52, 39, 35, 31, and 25 kD. The molecular weights of the others could not be accurately determined because of their broadened pattern. In these peptides, the peak with the molecular weight of 66 kD was the major component fraction, according for more than 50% of associated proteins. Serum albumin, the molecular weight of which is 66.2 kD, normally comprises 60% of the plasma protein. Therefore, one of the

proteins associated with the DPPC/DPPG liposomes was considered to be a serum albumin. Serum albumin is known to interact with numerous materials in the blood, such as fatty acids, organic anions, inorganic anions, and hydrophobic materials. The DPPC liposomes did not show any protein association. We conducted the protein binding study using the serum albumin. Figure 7 shows the binding of BSA to the DPPC/DPPG liposomes as a function of temperature. Below the T_m, BSA exhibited minimal binding to the lipid membrane (3 mg/g lipid). However, once the temperature reached the T_m, BSA showed temperature-dependent binding and the amount bound increased to a maximum of 13 mg/g lipid.

Discussion

The aim of the present study was to examine the effects of serum components on the temperature-dependent release property and influence on the stability of thermosensitive liposomes. We also investigated the interaction of plasma proteins with the liposomes dependent upon the membrane morphology. The liposomes used in this study had a mean particle size of 200—300 nm. When the diameter of the liposomes is below 70 nm, the surface curvature affects the properties of the lipid bilayers such as the T_m, enthalpy, and entropy.[33] However, at these sizes, there is no effect of curvature on these properties.

The clearance and bio-distribution of liposomes after injection have been considered related to the opsonization of plasma proteins.[34] There are many reports regarding the binding of plasma proteins onto the surface of liposomes or emulsions and influence on their metabolic pathway.[35—38] Furthermore, the interaction of liposomes with serum components can cause the leakage of materials entrapped in the liposomes.[21,22] A number of studies have indicated that serum components increase the release efficiency of liposomes.[39] It is also reported that the apoprotein in HDL interacts strongly with liposomes near the T_m and alter the liposomal structure.[40,41] Thus, it is considered that a certain kind of plasma protein is weakly interacting with the bilayers even below T_m, thus causing the leakage of drug from the DPPC liposomes (Fig. 4a). In contrast, the rat plasma suppressed the leakage from the DPPC/DPPG liposomes at temperatures below the T_m in this experiment (Fig. 4b). This is probably due to the presence of plasma proteins adsorbed onto the DPPC/DPPG lipid bilayer through electrostatic interaction rendering the bilayer more stable and less prone to a casual release of internal content. This argument is supported by the fact that PG can interact with the positively charged polymers, proteins and divalent cations *via* electrostatic forces.[32,42—47] On the other hand, at temperatures higher than the T_m of lipids, rat plasma only slightly enhanced the temperature-dependent content release from liposomes regardless of the lipid composition (Fig. 4). The bilayer disruption in this case could be accompanied by an interaction of plasma proteins with the lipid bilayer that is more severe than the gel-to-liquid crystalline phase transition. For both liposomes, the MMC release was enhanced greatly at temperatures between 38 and 44 °C (Figs. 3a, b). Therefore, we defined in this study the difference in the content release between these temperatures as an index of the temperature-dependent content release efficiency (Δ% release). In the absence of rat plasma, the Δ% release of the DPPC liposomes and the DPPC/DPPG liposomes was 83% and 71%, respectively. For both liposomes, the Δ% release increased to about 96% with the rat plasma. The effect of the rat plasma on the temperature-dependent content release of the DPPC/DPPG liposomes was greater than that of the DPPC liposomes; the difference in Δ% release with and without rat plasma was 25% for the DPPC/DPPG liposomes and 13% for the DPPC liposomes.

Based upon the results of ζ potential and DSC, it could be said that below the T_m, plasma protein bound to the DPPC/DPPG lipid bilayer simply through electrostatic interaction. However, when the temperature reached the T_m, we found that the interaction with the plasma proteins suppressed the ΔH and the main phase transition broadened until finally the gel-to-liquid crystalline phase transition peak disappeared. This effect is indicative of hydrophobic insertion. It can be explained by the penetration of the plasma proteins into the lipid bilayer core, which expands, destabilizes, and undergoes a rearrangement of the phospholipid structure. The strong binding of BSA to the DPPC/DPPG membrane, which could not be separated by gel filtration, supports this notion. As a result, it is possible to argue that serum albumin is one of the plasma proteins participating in the induction of the phase transition release of MMC from the DPPC/DPPG liposomes. Serum albumin is known to interact with organic anions. Therefore, it was suggested that electrostatic interaction between the acidic phosphate of DPPG and cationic residues in serum albumin was responsible for the initial electrostatic binding, which is nonspecific. It could be said that the hydrophobic interaction of proteins with lipid bilayers is induced by the phase separation at T_m. The finding that the amount of BSA bound to the DPPC/DPPG membranes reaches a maximum at T_m would support this idea (Fig. 7). However, other plasma proteins, such as lipoproteins or apolipoproteins, should also participate in this reaction, because SDS-PAGE shows that more than ten proteins bound to the DPPC/DPPG membranes (Fig. 6). This result indicates that relatively nonspecific reactions may be important for the interaction of peripheral proteins with membranes. I hope to investigate this in future studies.

Weinstein *et al.*[48] studied the protein-mediated phase transition release from temperature-sensitive liposomes based on the interaction of proteins with lipid bilayers using a neutral lipid, such as DPPC and distearoylphosphatidylcholine (DSPC). In their reports, lipoproteins and apolipoproteins, especially HDL, interacted with the lipid membrane and induced a protein-mediated phase transition release of entrapped materials. However, they also reported that most of the non-lipoprotein plasma constituents, such as albumin and immunoglobulin, had no significant effect on the release. Their findings are inconsistent with ours. These discrepancies could be attributed to the difference of lipid compositions used. We used a negatively charged lipid, DPPG, and the findings suggested the possibility of a serum albumin-mediated phase transition release. In truth, serum albumin exhibited no evidence of binding to the DPPC liposomal membrane in our study (data not shown); these findings are consistent with those obtained by Weinstein *et al.* Scherphof *et al.*[21,22] have reported that HDL remove the phospholipids from the bilayer membrane and this results in a destabilization of the liposomes in blood. This also explains our results

of the MMC release from the DPPC liposomes in the presence of rat plasma. It was revealed that serum albumin is one of the plasma proteins participating in the protein-mediated phase transition release from the DPPC/DPPG liposomes. Moreover, below the T_m, plasma components suppressed the MMC leakage from the DPPC/DPPG liposomes. Consequently, it is of interest to understand the interaction of plasma proteins with negatively charged lipid membranes. Not only is this relevant to the application of temperature-sensitive liposomes for clinical use and related studies, but it also serves as a model for the interaction of peripheral proteins with membranes.

In this study, we used conventional lipids to prepare the temperature-sensitive liposomes with the aid of gel-to-liquid crystalline phase transition. Other strategies have been used for the production of sterically stabilized temperature-sensitive liposomes.[14,15] One of the most effective methods of modification has been the use of polymers, which exhibit temperature-sensitivity and cause release of the internal content above a certain temperature. Kim *et al.*[14] have covalently attached a copolymer to liposomes for this purpose, but the spontaneous interaction of plasma proteins with anionic vesicles discussed here is a simpler means to achieve this end. Considering the application of temperature-sensitive liposomes to site-specific drug delivery, it is important to know their temperature-dependent content release property in the presence of plasma components.

In summary, we prepared temperature-sensitive MMC-loaded liposomes and examined the influence of plasma proteins on release efficiency and leakage. Moreover, we investigated the interaction of plasma proteins with the liposomes dependent upon the membrane morphology. Plasma components destabilized the conventional DPPC liposomes. However in the case of negatively charged liposomes composed of a 7 : 3 mixture of DPPC and DPPG, plasma components suppressed the leakage at the lower temperature and enhanced the temperature-dependent release at the T_m. In this study, we used rat plasma and BSA to investigate the protein binding to the DPPC/DPPG membranes. Our results indicate that these reactions are nonspecific and we expect the DPPC/DPPG liposomes to show the same performance in human plasma when applied in a clinical study. The findings from the present study should help in the development of liposomes for clinical applications in the future.

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