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# Effect of soybean-derived sterols on the in vitro stability and the blood circulation of liposomes in mice

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**Abstract**

The stability and long lifetime in blood circulation of liposomes are important when using them as a reservoir for drugs. A soybean-derived sterol mixture (SS) was used to stabilize and extend the blood circulation lifetime of liposomes. In the present study, the effects of SS on the stability and lifetime in blood of liposomes entrapping calcein were investigated by measuring the leakage of calcein and the concentration of calcein in blood. The results indicate that SS has a greater ability to stabilize the DPPC liposomes than cholesterol which is usually used as a stabilizer. The stabilizing effect was greatest at a molar ratio of DPPC and SS of 7:4.

*Keywords:* Liposome; Dipalmitoylphosphatidylcholine; Soybean-derived sterol; Stability; Blood residence time

## 1. Introduction

Liposomes have demonstrated considerable promise as a carrier for the delivery of drugs in vivo. Enhancement of therapeutic efficacy and reduction of toxicity of a variety of drugs have been demonstrated with liposome-encapsulated dosage forms (Ostro, 1987; Gregoriadias et al., 1988). However, the stability of liposomes is still a problem. Factors affecting the clearance from the blood of liposomes have been discussed regarding particle size and lipid composition, etc. (Senior, 1987; Juliano, 1988). For example, small unilamellar vesicles (SUV) tend to remain in blood circulation rather than larger ones, and incorporation of cholesterol (Ch) into the lipid bilayer membrane generally enhances the stability of liposomes in serum and reduces the clearance rate from the blood (Senior and Gregoriaids, 1982).

We intended to make the liposomes stable with the addition of a soybean-derived sterol mixture (SS). The SS used in this study was a mixture of $\beta$-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%). Many researchers have routinely used Ch to stabilize liposomes. Animal cells mainly contain Ch and plants contain sterols. Sterols have been reported to show a function similar to that of Ch in membranes (Demel and De Kruijff, 1976).

We have reported that SS stabilizes liposomes entrapping calcein by measuring the leakage of calcein in vitro by differential scanning calorimetry and fluorescence anisotropy (Muramatsu et

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al., 1994a). In this study, we investigated the influence of the composition and size on the stability and the blood circulation of liposomes in mice using SS as a stabilizer for the liposomes.

## 2. Materials and methods

### 2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) was purchased from Nippon Oil & Fats Co., Ltd (Tokyo, Japan). SS was kindly provided by Ryukakusan Co., Ltd (Tokyo, Japan). Ch was purchased from Sigma Chemical Co. (St. Louis, MO). Calcein was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals used were of reagent grade.

Male ddY mice weighing about 30 g (7 weeks old) purchased from Saitama Experimental Animal Supply (Saitama, Japan) were used in all experiments.

### 2.2. Preparation of liposomes

Liposomes were prepared with 35 $\mu$mol DPPC and various molar ratios of SS (DPPC/SS liposomes, DPPC:SS = 7:0–7) or Ch (DPPC/Ch liposomes, DPPC:Ch = 7:2) according to a reverse-phase evaporation method (Szoka and Papahadjopoulos, 1978). As a marker, calcein was entrapped in the DPPC liposomes. The lipid mixture was dissolved in 3.0 ml of organic solvent (isopropyl ether and chloroform (1:1, v/v)) and mixed with 1.0 ml of phosphate-buffered saline (1:10 dilution of phosphate-buffered saline in distilled water: 137 mM NaCl/2.6 mM KCl/6.4 mM $Na_2HPO_4 \cdot 12H_2O$/1.4 mM $KH_2PO_4$; pH 7.31; PBS). The PBS contained 20 mM calcein. The mixture was sonicated to give a w/o emulsion using a bath-type sonicator, and the organic solvent in the emulsion was evaporated under reduced pressure using a water aspirator at 50–55°C to form a reverse-phase evaporation vesicle (REV) suspension. Liposomes were successively extruded through polycarbonate membranes (Nuclepore, U.S.A.) of 1000, 400 and/or 200 nm pore size at about 60°C. Nonencapsulated calcein was removed by gel filtration of the liposome suspension through a Sephadex G-50 column (1.8 × 35 cm; Pharmacia, Sweden) with the PBS in all fractions. Each fraction contained 4.5 ml.

The concentration of DPPC in the liposomes was determined by enzymatic assay using a Phospholipid B-test Wako (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

The size of the liposomes was measured using a Nicomp 370 Submicron Particle Analyzer (Pacific Scientific, CA, U.S.A.).

### 2.3. Measurement of the amount of calcein entrapped in liposomes

The amount of calcein entrapped in the liposomes was determined using a fluorescence spectrometer (excitation at 490 nm and emission at 520 nm; Hitachi F-4010, Tokyo, Japan) as follows: firstly, the liposomes were diluted 1000-fold with PBS, and then the fluorescence intensity ($F_b$) was measured. Secondly, the liposomes were completely disrupted using 30 $\mu$l of 10% Triton X-100 solution in a 1 ml sample, and then the fluorescence intensity ($F_a$) was measured. The amount of calcein entrapped in the liposomes ($F_{lipo}$) was calculated according to ($F_{lipo} = F_a - F_b$).

### 2.4. Turbidity of liposome suspension

The variation in turbidity at 550 nm was expressed as ($T_t/T_0 \times 100$), where $T_0$ and $T_t$ denote the turbidity of the liposome suspension initially and at time $t$, respectively. The dynamic sedimentation of the liposome suspension could be measured by comparison of the turbidity of the liposome suspension.

### 2.5. Leakage of calcein from liposomes in plasma in vitro

The in vitro leakage of calcein from DPPC liposomes was assessed by incubating the liposome suspension in 30% (v/v) of rat plasma at $37 \pm 0.5$°C, at a shaker speed of 100 $min^{-1}$. The calcein leakage was assayed by fluorescence spectrometry as previously described (Muramatsu et

al., 1994b). The percentage of calcein leakage was determined from the following equation:

$$\% \text{ of calcein leakage} = 100(F_t - F_0)/(F_\infty - F_0) \quad (1)$$

where $F_0$ and $F_t$ are the fluorescence intensities of the suspension of liposomes entrapping calcein initially and at time $t$, respectively, and $F_\infty$ represents the fluorescence intensity of calcein when the liposomes entrapping calcein were completely disrupted by Triton X-100.

### 2.6. Animal experiments

Calcein solution and the calcein entrapped in DPPC/SS (7:0–7) liposomes and DPPC/Ch (7:2) liposomes were injected via the tail vein at a dose of 2.5 $\mu$mol/kg weight of calcein. At 5, 15, 30, 60, 120, 240 and 360 min after injection, a 10 $\mu$l blood sample was taken from the tail vein. The concentration of calcein in the blood was then measured according to the method of Sawahara et al. (1991) with a few modifications. The sample was immediately poured into 1.25 ml of PBS and thoroughly agitated, then centrifuged at $1000 \times g$ for 10 min, and 1 ml of supernatant was obtained and stored at 4°C until measurement. On the other hand, blank blood samples with the addition of a known concentration of calcein were assayed, and the standard curve was constructed using the same method and conditions. The fluorescence of calcein is influenced by divalent metallic ions (Wallach and Steck, 1963) such as $Ca^{2+}$ or $Mg^{2+}$ in blood and, therefore, $Na_2EDTA$ was used to eliminate them. The total blood volume was assumed to be 7.3% of the body weight of mice, and the concentration of calcein in the blood was expressed as a percentage of the injected dose.

### 2.7. Pharmacokinetic analysis

The total area under the curve (AUC) from time 0 to infinity was estimated from the sum of successive trapezoids between each data point, plus an estimation of the tail area from the last concentration-time point to infinity. The elimination half-life ($t_{1/2}$) was estimated by a linear regression analysis of the blood concentration-time curve according to a one-compartment model (Yamaoka, 1986). Total body clearance (Cl) was calculated from the AUC values divided by the dose. The volume of distribution ($V_d$) was calculated from the dose divided by the initial concentration of calcein in the blood.

### 2.8. Statistical analysis

Data from the animal experiments using mice were compared using analysis of variance and Student's $t$-test. A $p$ value of 0.05 was considered significant.

## 3. Results

### 3.1. Characterization of DPPC / SS liposomes

The calcein was entrapped in DPPC liposomes by reverse-phase evaporation to produce REV liposomes. They are composed of DPPC and SS or Ch and have a high capture efficiency for calcein of about 23–52% (data not shown), the capture efficiency being found to be dependent on the liposomal composition. For comparison, the characterization of DPPC/SS (7:2) and DPPC/Ch (7:2) liposomes is summarized in Table 1; both the DPPC/SS and DPPC/Ch (7:2) liposomes were quite homogeneous in size distribution.

The mean diameters of liposome suspensions were 162 or 134 nm for DPPC/SS (7:2) liposomes, after extrusion through the 400 or 200 nm pore size polycarbonate membranes, respectively.

Table 1
Characterization of DPPC/SS (7:2) and DPPC/Ch (7:2) liposomes

| Composition (molar ratio) | Polycarbonate membrane (nm) | Encapsulated volume (l/mol lipid) | Mean diameter (nm) |
|---|---|---|---|
| DPPC/SS (7:2) | 0.4 | 6.66 | 162.1 |
| DPPC/SS (7:2) | 0.2 | 6.56 | 134.2 |
| DPPC/Ch (7:2) | 0.2 | 4.93 | 138.6 |

### 3.2. Turbidity of liposome suspension

Because the liposomes of a suspension have a density different from that of the medium, they will be subjected to a gravitational force which will tend to cause sedimentation of the liposomes during storage. As shown in Fig. 1, the relative turbidity ($T_t/T_0 \times 100$) value of a DPPC liposome suspension displays dramatic variation due to vesicle sedimentation during a period of 2.5 h at $25 \pm 0.5$°C. On addition of SS or Ch in the liposomes, the ($T_t/T_0 \times 100$) value at 2.5 h was almost the same as that at 0 h under the same conditions. It is clear that physical stability such as during sedimentation depends on the composition of the liposomes. The sedimentation rate decreased with the addition of SS and Ch.

### 3.3. Leakage of calcein from liposomes in plasma in vitro

As shown in Fig. 2, 23 and 43% of entrapped calcein leaked from the DPPC liposomes on incubation at 37°C for 1 and 17 h, respectively; however, only 1–3 and 4–11% of entrapped calcein leaked from the DPPC/SS liposomes on incubation at 37°C for 1 and 17 h, respectively. On the other hand, 4 and 19% of entrapped calcein leaked from the DPPC/Ch (7:2) liposomes at 1 and 17 h, respectively, which is greater than that of the DPPC/SS liposomes.

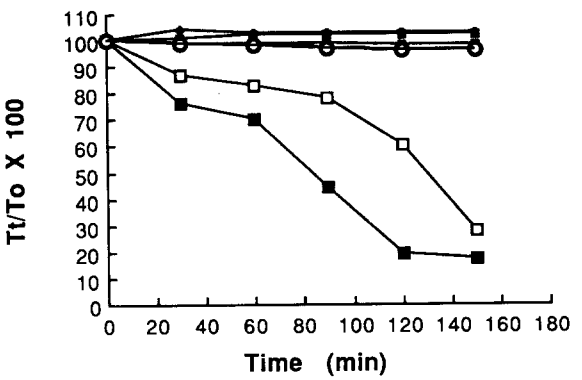


Fig. 1. Relative turbidity of liposome suspension. (■) DPPC liposome suspension extruded through 400 nm polycarbonate membrane; (□) DPPC liposome suspension extruded through 200 nm polycarbonate membrane. Other lines indicate the relative turbidity of DPPC/SS (7:2–7) and DPPC/Ch (7:2) liposome suspensions extruded through 400 or 200 nm polycarbonate membrane, respectively.

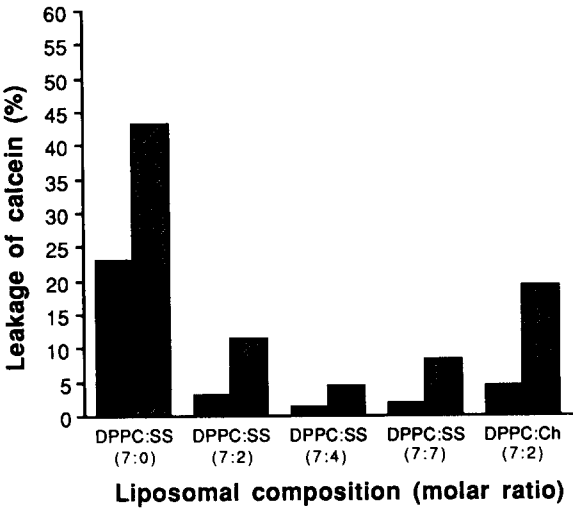


Fig. 2. Leakage of calcein from DPPC/SS (7:0–7) and DPPC/Ch (7:2) liposomes in 30% (v/v) rat plasma in vitro on incubation at $37 \pm 0.5$°C. (Filled bars) 1 h incubation; (empty bars) 17 h incubation. The liposome suspensions were extruded through 200 nm polycarbonate membrane. Value represents mean ($n = 2$).

It is clear that the leakage of calcein from DPPC/SS and DPPC/Ch liposomes decreased due to the addition of SS or Ch into the DPPC liposomes, and that calcein leakage from the liposomes was dependent on the amount of SS included. Calcein leakage decreased on addition of SS up to DPPC/SS (7:4) but reached a plateau thereafter. The collapse of liposomes by the proteins in plasma decreased correspondingly with increasing SS concentration.

### 3.4. Effect of SS content on the blood residence time of calcein entrapped DPPC / SS liposomes

The effect of increasing content of SS on the blood residence time of calcein entrapped DPPC-/SS (7:0–7) liposomes extruded through 200 nm polycarbonate membranes was examined in mice via tail vein injection. The blood clearance behavior for calcein solution and liposomal calcein is shown in Fig. 3.

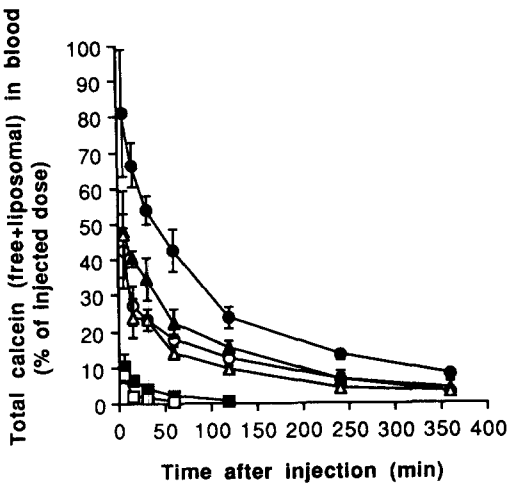


Fig. 3. Time course of blood residence of calcein entrapped in DPPC liposomes after injection in mice via tail vein. (■) Calcein solution, (□) DPPC, (○) DPPC/SS (7:2), (●) DPPC/SS (7:4), (▲) DPPC/SS (7:7) and (△) DPPC/Ch (7:2) liposomes. The liposome suspensions were extruded through 200 nm polycarbonate membrane. Bar represents S.D. ($n$ = 3–4).

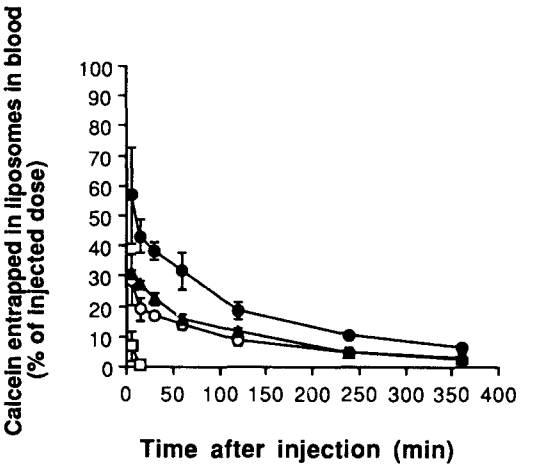


Fig. 5. Time course of blood residence of calcein entrapped in stable liposomes after injection via tail vein. (□) DPPC, (○) DPPC/SS (7:2), (●) DPPC/SS (7:4) and (▲) DPPC/SS (7:7) liposomes. The liposome suspensions were extruded through 200 nm polycarbonate membrane. Bar represents S.D. ($n$ = 3–4).

DPPC liposomes without SS led to a dramatic increase in the rate of removal of calcein from the blood, which was even faster than that observed for calcein solution. The blood calcein level immediately decreased to 0.69% of the injected dose within 60 min.

DPPC liposomes with SS resulted in a dramatic decrease in the rate of removal of calcein from the blood. Among them, DPPC/SS (7:4) liposomes presented the highest level of calcein in blood. The blood calcein levels conformed to the following order: DPPC/SS (7:4) > DPPC/SS (7:7) > DPPC/SS (7:2) > DPPC/Ch (7:2) > calcein solution > DPPC liposomes.

### *3.5. Effect of vesicle size on the blood residence time of calcein entrapped DPPC / SS liposomes*

Vesicle size is one of the most important factors affecting the clearance from the blood. When

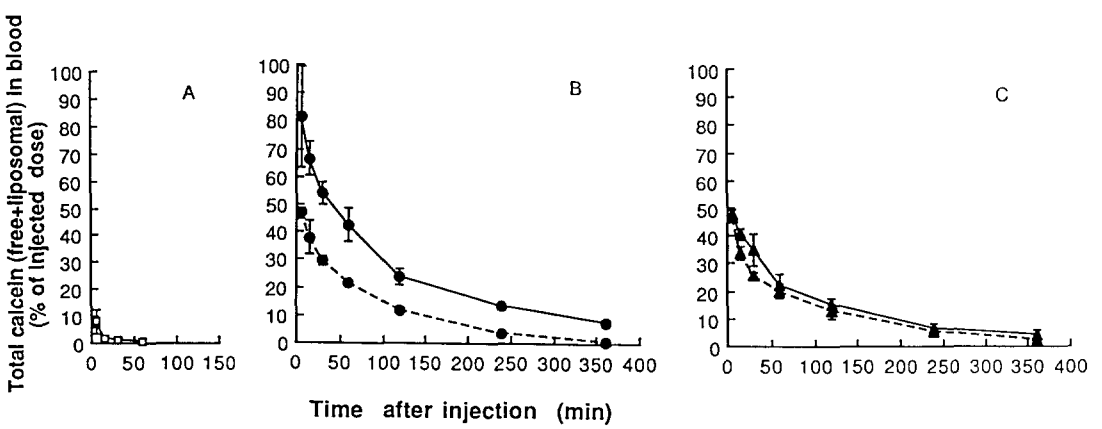


Fig. 4. Time course of blood residence of calcein entrapped in DPPC (A), DPPC/SS (7:4) (B) and DPPC/SS (7:7) (C) liposomes after injection in mice via tail vein. The dotted line represents 162 nm liposomes; the full line represents 134 nm liposomes. Bar represents S.D. ($n$ = 3).

liposomes are forced through filters with defined pore sizes, a liposomal population is obtained with a mean diameter that reflects the diameter of the filter pores (Gregoriadis, 1993). The effect of vesicle size on the blood residence time and blood levels of calcein entrapped DPPC/SS (7:0, 7:4, 7:7) liposomes was examined in mice via tail vein injection. As shown in Fig. 4, decreasing the mean vesicle diameter from 162 to 134 nm increases the blood level for DPPC/SS (7:4) liposomes. No significant effect was observed for the DPPC/SS (7:7) liposome preparation. In DPPC liposomes without SS, the blood level did not increase following a decrease in the vesicle size.

### 3.6. *In vivo stability of DPPC / SS liposomes*

After intravenous administration, the fluorescence intensity of calcein in blood circulation was measured. The $F_b$ value indicates the free calcein that was released from the liposomes (free), and the $F_a$ value corresponds to the total calcein that was released from the liposomes and still entrapped in the liposomes (free + liposomal) in the blood. The difference in fluorescence intensity ($F_{lipo} = F_a - F_b$) indicates the proportion of the stable liposomes entrapping calcein in the blood.

The concentration of calcein entrapped in the liposomes in the blood was expressed as a percentage of the injected dose as shown in Fig. 5. This demonstrates that DPPC liposomes were not found in blood circulation after 30 min, but DPPC/SS liposomes that amounted to about 2, 6 and 3% of the injected dose were found in blood circulation even at 360 min for DPPC/SS (7:2, 7:4, 7:7) liposomes after injection, respectively.

### 3.7. *Pharmacokinetic analysis*

The $t_{1/2}$ and AUC values were not significantly different between calcein solution and the DPPC liposomes (groups 1, 2). The $t_{1/2}$ and AUC values of calcein entrapped in DPPC/SS

Table 2
Pharmacokinetic parameters of calcein solution and liposomal calcein after injection via tail vein

| Group | Formulation (molar ratio) | Polycarbonate membrane (nm) | $t_{1/2}$ (min) | AUC ($\mu$mol min ml$^{-1}$) | $V_d$ (ml/kg) | Cl (ml min kg$^{-1}$) |
|---|---|---|---|---|---|---|
| 1 | Calcein solution | | 23.5 ± 5.3 | 0.105 ± 0.033 | 837 ± 230 | 25.3 ± 6.8 |
| 2 | DPPC | 200 | 12.0 ± 2.7 | 0.051 ± 0.024 | 922 ± 319 | 55.4 ± 21.2 |
| 3 | DPPC/SS (7:2) | 200 | 102.9 ± 17.4 | 1.438 ± 0.160 | 264 ± 2 | 1.8 ± 0.2 |
| 4 | DPPC/SS (7:4) | 200 | 107.9 ± 5.3 | 3.109 ± 0.270 | 119 ± 2 | 0.8 ± 0.1 |
| 5 | DPPC/SS (7:7) | 200 | 96.8 ± 19.7 | 1.953 ± 0.356 | 179 ± 18 | 1.3 ± 0.3 |
| 6 | DPPC | 400 | 11.5 ± 3.9 | 0.035 ± 0.012 | 1416 ± 1022 | 78.3 ± 30.1 |
| 7 | DPPC/SS (7:4) | 400 | 60.2 ± 0.2 | 1.172 ± 0.010 | 185 ± 1 | 2.1 ± 0.2 |
| 8 | DPPC/SS (7:7) | 400 | 86.7 ± 0.5 | 1.409 ± 0.103 | 222 ± 15 | 1.8 ± 0.1 |
| 9 | DPPC/Ch (7:2) | 200 | 86.6 ± 15.2 | 1.144 ± 0.189 | 275 ± 46 | 2.2 ± 0.4 |
| *t*-test | 1, 2 | | – | – | | |
| | 1, 3 | | [a] | [a] | | |
| | 1, 4 | | [a] | [a] | | |
| | 1, 5 | | [a] | [a] | | |
| | 1, 9 | | [a] | [a] | | |
| | 2, 6 | | – | – | | |
| | 4, 7 | | [a] | [a] | | |
| | 5, 8 | | – | – | | |
| | 3, 9 | | – | – | | |
| | 3, 4 | | – | [a] | | |
| | 4, 5 | | – | [a] | | |
| | 3, 5 | | – | – | | |

[a] Significant difference. – no significant difference.
Data are expressed as mean ± S.D. ($n = 3$).

and DPPC/Ch liposomes were significantly higher than those of calcein solution after intravenous administration (groups 1, 3; 1, 4; 1, 5; 1, 9). No significant differences were observed in the $t_{1/2}$ values with the DPPC/SS (7:2) and DPPC/Ch (7:2) liposomes (groups 3, 9) and with the ratio of DPPC/SS (groups 3, 4, 5). However, significant differences were observed in the AUC values between the DPPC/SS (7:4) liposomes and the DPPC/SS (7:2 and 7:7) liposomes (groups 3, 4; 4, 5) (Table 2).

## 4. Discussion

Vesicle size, surface charge, liposomal composition, and lipid dose are major determinants for the fate of i.v. administration of liposomes (Abra and Hunt, 1981; Bosworth and Hunt, 1982; Ostro, 1987). However, the characterization of liposomes with SS has not been reported. The present results may indicate that SS within liposomes increases the in vitro and in vivo stability and lengthens the lifetime of liposomes in vivo.

From Fig. 1, this result may indicate that flocculation occurred in the DPPC liposome suspension. According to Stokes' law:

$$v = 2r^2(\rho - \rho_0)g/9\eta_0 \qquad (2)$$

where $v$ is the velocity of sedimentation of the liposomes, $r$ denotes the radius of the vesicle, $\rho$ and $\rho_0$ are the densities of the vesicle and the medium ($\rho_0 = 0.99704$ g cm$^{-3}$ at 25°C), respectively, $g$ represents the acceleration of gravity and $\eta_0$ is the viscosity of the medium (0.890 g m$^{-1}$ s$^{-1}$ at 25°C). Assuming that the $\rho$ value of a floccule of liposomes is equal to that of the original liposomes and that the shape of floccule is spherical, the number of liposomes in the floccule may be estimated. The $\rho$ value may be calculated according to the following: the phospholipid weight ($1.22 \times 10^{-21}$ g) is estimated from the average molecular weight of 734 for DPPC divided by Avogadro's constant ($6.02 \times 10^{23}$) and the phospholipid volume is 105 Å$^3$ (Nojima et al., 1988), thus $\rho$ may be 11.6 g cm$^{-3}$. The rate of sedimentation increases with increasing radius of the liposome vesicle. The radius of the DPPC liposome vesicle may be 342 nm ($r_{cal}$) as calculated using the rate of sedimentation (3.0 cm/2.5 h) and Eq. 2. Considering that the mean radius of the DPPC liposomes was 100 nm after preparation (original, $r_{orig}$), they flocculated into about 40 vesicles as calculated from the ($r^3_{cal}/r^3_{orig}$) value during the period of storage.

The free energy of a particle decreases as its particle size increases, lowering the surface tension between the interacting surfaces. The process of flocculation of liposomes starts with the interaction of the liposomes. The hydrophilic head groups of the DPPC liposomes cannot be close packed on the surface of the liposomes. Therefore, the tendency of contact between the exposed hydrocarbon and the hydrocarbon in each liposome might increase. However, SS in the DPPC/SS liposomes may be packed well and may prevent the interaction of the hydrocarbon portion of each liposome. The stability of the liposome suspension thus may depend on the resistance to film interaction between each liposome.

SS in the DPPC/SS liposomes causes a dramatic decrease in the percentage of calcein leakage in 30% (v/v) rat plasma (Fig. 2). In particular, the DPPC/SS (7:4) liposomes showed the lowest leakage percentage of entrapped calcein, which is approx. 23- and 10-fold lower than that for the DPPC liposomes after 1 and 17 h, respectively. The percentage of calcein leakage may reflect the collapse caused by the interaction of the bilayer of the liposomes with the proteins in plasma.

Allen and co-workers (1991) have shown that inclusion of increasing amounts of Ch up to 50 mol% in phosphatidylcholine liposomes did not decrease significantly the uptake by macrophages. Comparison of SS and Ch regarding the stability was therefore examined at a molar ratio of DPPC and SS or Ch of 7:2. After intravenous administration of DPPC/SS (7:2) and DPPC/Ch (7:2) liposomes, the results indicated that, although the $t_{1/2}$ values were not significantly different, the results of incubation of the DPPC/SS (7:2) and DPPC/Ch (7:2) liposomes in 30% (v/v) rat plasma at 37°C (Fig. 2) indicated that the leakage of calcein from the DPPC/SS (7:2) liposomes is

slower than that of the DPPC/Ch (7:2) liposomes. This corresponded with the result obtained by Muramatsu et al. (1994a,b) that SS may have a greater ability to stabilize the DPPC liposomes than Ch; therefore, the interaction between the liposome and proteins in the blood tends to decrease.

Our study has shown that the blood levels of liposomes are dependent on the amount of SS included in the liposomes. The $t_{1/2}$ values of various DPPC/SS liposomes were not significantly different, but liposomes containing SS exhibited significantly greater blood levels and AUC values (Fig. 3 and Table 2).

The size of the liposomes is also an important factor for extended circulation lifetime. The results indicate that small liposomes remain in circulation for a longer time compared with large liposomes. The $t_{1/2}$ and AUC values increased significantly when the size of the DPPC/SS (7:4) liposomes decreased from 162 to 134 nm (Fig. 4 and Table 2, group 4, 7). This may be due to the fact that the larger vesicles are more rapid cleared than small ones. However, no significant variation in the $t_{1/2}$ value was observed for DPPC/SS (7:7) and DPPC liposomes associated with decreased liposome size (Fig. 4 and Table 2).

The AUC values of DPPC/SS (7:7) and DPPC liposomes were not dramatically affected by the size of liposomes such as occurred with the DPPC/SS (7:4) liposomes. It appears that the clearance of liposomes was not affected by the size of the liposomes, since the liposomes were not stable enough in the blood and collapsed due to the interaction of the bilayer of the liposomes with proteins in the plasma immediately after intravenous administration.

Interestingly, liposomes of the molar composition ratio DPPC/SS (7:4) demonstrated the highest blood level and the longest circulation time. This may be due to the limited loading of SS in the DPPC lipid layer. Above the limited loading, SS might distribute heterogeneously in the DPPC liposomes such as by lateral phase separation. On the other hand, the DPPC/SS (7:7) liposomes demonstrated the same stability in vitro as the DPPC/SS (7:4) liposomes when the liposome suspension was stored at 4°C for 3 months (data not shown). These results suggest that SS rigidifies the bilayer of the DPPC liposomes following the increase in SS but still has a suitable ratio of DPPC to resist plasma-induced leakage while in the blood.

As a result, the present study indicated that SS in DPPC/SS liposomes may stabilize the liposomes by making the DPPC bilayer film rigid, reducing the film interaction between each liposome and suppressing the causes of flocculation. This may induce stability of the DPPC/SS liposome suspension and also prevent the interaction between the liposomes and proteins in the blood.

The rigidity of liposomes containing SS might inhibit opsonization; therefore, the liposomes would tend to remain in the blood circulation. The size of the liposomes is also important for retention of the liposomes in the blood circulation.

## 5. Conclusions

This report indicates that SS stabilizes the DPPC liposomes to a greater extent than Ch which is usually used as stabilizer. The stabilizing effect of SS on DPPC liposomes is greatest at a molar ratio of DPPC and SS of 7:4. Size is also important for extended circulation lifetime. Development of long-circulating liposomes that evade the uptake of RES is in progress.

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