Relation of Rigidity of Membrane and Stability of Dipalmitoylphosphatidylcholine Liposomes with Soybean-Derived Sterols Prepared by Reverse Phase Evaporation Vesicle Method

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A soybean-derived sterol mixture (SS) was used to stabilize liposomes prepared from dipalmitoylphosphatidylcholine (DPPC). The effect of SS on the stability and rigidity of liposomes entrapping calcein was investigated by measuring retentivity of calcein in liposomes *in vitro* and *in vivo*, and by fluorescence anisotropy, respectively. The entrapment efficiency is directly proportional to the total concentration of DPPC and SS. The presence of SS tends to increase the rigidity of the bilayer above its gel–liquid crystalline phase transition temperature of liposomes, and SS tends to fluidize the bilayer below its gel–liquid crystalline phase transition temperature. The relation between the stability of DPPC-liposomes with SS and the rigidity of liposomal membrane was discussed.

Key words dipalmitoylphosphatidylcholine liposome; liposomal membrane rigidity; liposome stability; soybean-derived sterol; fluorescence anisotropy

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-entrapmented dosage forms.[1,2] The stability of liposomes is important when using them as a reservoir for drug; however, their stability still poses a problem. We sought to make the liposomes stable by the addition of a soybean-derived sterol mixture (SS). The SS used in this study was a mixture of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%) and brassicasterol (7.2%). Sterols have been reported to show a function similar to that of cholesterol in membranes.[3] We have reported that SS stabilizes dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicle (MLV) liposomes (molar ratio, DPPC : SS = 7 : 2, DPPC/SS (7 : 2)-MLVs) entrapping calcein by measuring the leakage of calcein *in vitro*, by differential scanning calorimetry and fluorescence anisotropy[4] and *in vivo*.[5] We have also reported that the stabilizing effect was the greatest at a molar ratio of DPPC and SS of 7 : 4 in liposomes prepared by reverse phase evaporation vesicle (REV) method,[6] demonstrating the highest blood level and the longest circulation times. The stability of liposomes depended on the molar ratio of DPPC and SS suggesting that the rigidity of the liposomal membrane is an important factor. In this paper, we describe the relation between rigidity of liposomal membrane and stability of (DPPC/SS-REV liposomes) *in vivo* and *in vitro*.

Materials and Methods

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

Liposome Preparation Liposomes were prepared with 35 μmol DPPC and various molar ratios of SS (DPPC/SS-liposomes, DPPC : SS = 7 : 0—7) according to a REV method as described previously.[6] As a marker, calcein was entrapped in the liposomes. The lipid mixture in chloroform solution was deposited in a pear-shaped flask, and the organic solvent was removed to produce lipid film. This was redissolved in chloroform and isopropyl ether, and mixed with a 1/10 diluted phosphate-buffered saline (1/10 PBS, pH 7.31) containing 20 mM calcein. The mixture was sonicated in a bath type sonicator to give a homogeneous w/o emulsion. The emulsion was then placed on a rotary evaporator and the organic solvent was removed to obtain liposome suspension. The suspension was extruded successively through polycarbonate membranes (Nuclepore, U.S.A.) of 1000, 400 and 200 nm pore size. Nonentrapped calcein was removed by gel filtration of the liposome suspension passed through a Sephadex G-50 column (1.8 × 35 cm, Pharmacia, Sweden) with the 1/10 PBS in all fractions.

Determination of Size Distribution and Lipid Concentration of Liposomes The liposomal size distribution was determined using a Nicomp 370 submicron particle analyzer (Pacific Scientific, CA, U.S.A.). Each DPPC/SS-liposome suspension was quite homogeneous in size distribution and showed a mean diameter of less than 200 nm after extrusion through the 200 nm pores of polycarbonate membrane.

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

Measurement of Entrapment Efficiency of Liposomes The amount of calcein entrapped in the liposomes was determined using a fluorescence spectrometer as described previously.[6] Liposomes were prepared by two methods: (1) the lipid film of 35 μmol DPPC and 10 μmol SS (DPPC/SS 7 : 2) was redissolved in 3.0 ml of organic solvent and 1.0, 1.5, 2.0 ml of 1/10 PBS containing 20 mM calcein, respectively, and (2) the lipid film of DPPC (35 μmol) and SS (0, 10, 20, 35 μmol, respectively) was redissolved in 3.0 ml of organic solvent and 1.0 ml of 1/10 PBS containing 20 mM calcein. The amount of lipid left after extrusion and separation on a Sephadex column was divided by the amount of lipid in the initial preparation before extrusion to yield the lipid recovery. The entrapment efficiency is expressed as the percent of calcein entrapped in liposomes after removal of the free calcein by gel filtration divided by the amount of calcein added.

Measurement of Fluorescence Anisotropy The fluorescence anisotropy of DPH, which is a marker for the fluidity of the lipid bilayer, was measured by the reported method.[4] A solution of DPH in tetrahydrofuran was added to the liposome suspension with incubation for 1 h above the phase transition temperature of liposomes at a final concentration of 0.38 mol%. The samples in PBS were measured at a heating rate of 1.25 °C/min over the range 25—50 °C. The excitation and emission wavelengths used for DPH were 357 and 430 nm, respectively.

Retentivity of Calcein in Liposomes *in Vitro* and *in Vivo* The *in vitro* retentivity of calcein in DPPC/SS-liposomes was examined by storing the liposomes at 4 °C for 4 months. The percentage of calcein retentivity in liposomes was determined from the following equation:

$$\text{percentage of calcein retentivity in liposomes (\%)} = (F^{(t)}_{\text{lipo}}/F_{\text{lipo}}) \times 100$$

where F_{lipo} was the amount of calcein retentivity in the liposomes initially and $F^{(t)}_{\text{lipo}}$ was the amount in the liposomes at time (t) after storage at

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4 °C.

The *in vitro* retentivity of calcein in liposomes is defined as the reciprocal of leakage of calcein from DPPC/SS-liposomes in plasma assessed by incubating the liposome suspension in 30% (v/v) of rat plasma. The *in vivo* retentivity of calcein in DPPC/SS- liposomes was evaluated as the total area under the curve (*AUC*) after injection *via* the tail vein at a dose of 2.5 μmol/kg weight of calcein in mice as described previously.[6]

Results and Discussion

Effect of Lipid Composition and Total Lipid Concentration in 1/10 PBS on Entrapment Efficiency The entrapment efficiency is an important parameter by which to characterize large unilamellar vesicle and is useful in investigating the interaction of lipid components. Generally, higher drug entrapment efficiency of liposomes is believed to be preferable for its clinical use. Figure 1 shows that the entrapment efficiency of liposomes was directly proportional to the total lipid concentration of DPPC and SS. When more lipid is present, more solute can be sequestered within liposomes.[7] The slope of the straight line in the Fig. 1 (0.67) bears this out: the slope of calculated entrapment efficiency against various concentrations of lipids in idealized small unilamellar vesicles with diameters of 200 nm is 0.66.[7] This indicated that SS was included in DPPC-liposomes up to a molar ratio of DPPC : SS = 7 : 7 and did not appear to have a special interaction with DPPC.

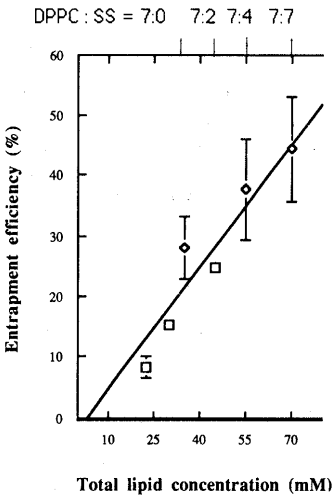

Fig. 1. Effect of Liposome Composition and Total Lipid Concentration in 1/10 PBS on the Entrapment Efficiency of Calcein ($n=3$—4, Mean ± S.D.)

◇, total lipid concentration of DPPC and SS in DPPC/SS (7 : 0—7)-liposome suspension; □, total lipid concentration of DPPC/SS (7 : 2)-liposome suspension. Line was determined by the linear least-squares method using experimental mean values ($Y=0.67X-2.13$, $r=0.97$).

Table 1. Effect of Liposome Composition and Total Lipid Concentration in 1/10 PBS on Entrapment Efficiency

DPPC/SS-liposome composition (molar ratio)	Total lipid concentration (mM)	Lipid recovery (%)	Entrapment efficiency (%)
7 : 0	35.0	85.5 ± 11.1	28.1 ± 5.2
7 : 2	22.5	83.2 ± 19.8	8.2 ± 1.8
7 : 2	30.0	81.4 ± 6.3	15.3 ± 1.4
7 : 2	45.0	90.7 ± 10.9	24.8 ± 0.2
7 : 4	55.0	85.5 ± 11.9	37.7 ± 8.3
7 : 7	70.0	60.9 ± 5.5	44.4 ± 8.7

Data are expressed as mean ± S.D. ($n=3$—4).

As shown in Table 1, the DPPC/SS (7 : 7)-liposomes indicated a high entrapment efficiency (44%) and a low lipid recovery (61%), while the lipid recovery of other DPPC/SS (7 : 0—4)-liposomes was about 81—91%. According to Szoka and Olson *et al.*,[8,9] the polycarbonate membranes used for extrusion have little loss of lipid, so the loss of lipid observed from DPPC/SS (7 : 0, 7 : 2, 7 : 4)-liposomes may be caused mainly by retention of lipid or some large vesicles on the Sephadex column, and the great loss of DPPC/SS (7 : 7)-liposomes was caused by their rigidity during the extrusion. In fact, in our experimental process, extrusion of DPPC/SS (7 : 7)-liposomes through pores 400 nm and 200 nm in diameter was very difficult under the same temperature since the pinch force was not strong enough to cut down the bilayer of liposomes from the high rigidity of liposomal membranes; thus, that the lipid recovery was low (61%).

DPPC/SS (7 : 0)-liposomes appeared to show higher entrapment efficiency than DPPC/SS (7 : 2)-liposomes. This might be due to SS having a condensing effect on the packing state of DPPC in liposomes (Fig. 1 and Table 1).

Rigidity of Liposomal Membrane from Fluorescence Anisotropy The results of the fluorescence anisotropy of DPH provide information on orientational order of the acyl chain of the DPPC bilayers. When the movement of DPH is restricted in the gel state of the lipid, liposomes show higher fluorescence anisotropy. When the fluidity of the lipid layer increases, the anisotropy decreases. To determine the effect of SS on the fluidity of DPPC/SS-liposomes, the fluorescence anisotropy of DPH in these liposomes was measured (Fig. 2). SS decreased the anisotropy in the gel state, and increased it in the liquid crystalline state. SS has an important modulatory effect on the phospholipid phase changes. The sterol interacts strongly with the phospholipids and the result of this interaction is to keep the phospholipid in an "intermediate fluid" condition. Thus, at points where the lipid would normally be above its gel–liquid crystalline phase

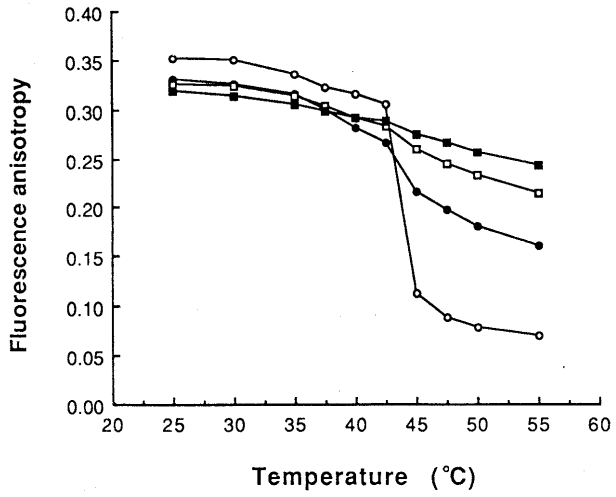

Fig. 2. Temperature Dependence of Fluorescence Anisotropy of DPH in DPPC/SS-Liposomes

○, DPPC/SS (7 : 0); ●, DPPC/SS (7 : 2); □, DPPC/SS (7 : 4); ■, DPPC/SS (7 : 7)-liposomes.

transition temperature, the presence of SS tends to increase the packing and rigidity of the bilayer, while at points where the lipid would be below its gel–liquid crystalline phase transition temperature, SS expands to fluidize the bilayer. The fluorescence anisotropy of DPPC/SS (7:4)-liposomes and DPPC/SS (7:7)-liposomes intersected at about 37 °C. This indicates the rigidity of the membrane of DPPC/SS (7:4)-liposomes changed from lower to higher than that of DPPC/SS (7:7)-liposomes around this temperature.

Retentivity of Calcein from DPPC/SS-Liposomes *in Vitro* and *in Vivo* Generally, the stability of liposomes is thought to be affected by lipid composition and type of liposomes. The results of retentivity of calcein in the DPPC-liposomes after 4 months of storage at 4 °C are summarized in Table 2. SS contained in liposomes suppressed the leakage of calcein from DPPC-liposomes depending on the concentration of SS. The DPPC/SS (7:4)-liposomes are retentive at above 90% for up to 3 months, but calcein showed a decrease there after.

Table 3 shows that the retentivity of calcein in 30% plasma at 37 °C for 17 h depends on the lipid composition. SS contained in liposomes causes a dramatic increase in the retentivity of calcein and the DPPC/SS (7:4)-liposomes showed the highest retentivity. The *AUC* of liposomes after injection *via* the trail vein demonstrated that the DPPC/SS (7:4)-liposomes have the largest value among DPPC/SS (7:0—7)-liposomes, *i.e.* they exhibit significantly higher blood levels.[6] Also, high *AUC* values indicate low uptake of liposomes by the reticuloendothelial system (RES) and systemic degradation.[10] The mechanism of liposomes by RES uptake and systemic degradation is not clearly understood. The interaction of liposomes *in vivo* and *in vitro* with serum components may be different but the rigidity of liposomal membrane is one of the interacting factors. Therefore, the retentivity of calcein in liposomes in plasma incubation and *AUC* of calcein after injection of liposomes indicate the retentivity of liposomes in plasma *in vitro* and *in vivo*, since the free calcein was rapidly eliminated from the blood *in vivo*, *i.e.* the half life of calcein is about 22.4 min.[10]

Table 2. Retentivity of Calcein in DPPC/SS (7:0—7)-Liposomes during 4 Months Storage at 4 °C

DPPC/SS-liposome composition (molar ratio)	Months		
	1	3	4
7:0	88.2±15.8	77.2± 8.0	58.4±3.9
7:2	89.5± 8.7	86.2±15.4	59.4±3.5
7:4	96.6± 4.2	90.1± 7.2	74.0±3.7
7:7	101.6± 2.3	98.2± 2.4	77.9±8.2

The retentivity of calcein (%) was expressed as $(F^{(t)}_{lipo}/F_{lipo}) \times 100$, mean ± S.D. ($n=3$).

Table 3. Retentivity of Calcein in DPPC/SS-Liposomes *in Vitro* and *in Vivo*

DPPC/SS-liposome composition (molar ratio)	Retentivity of calcein at 4 °C for 4 months[a] (%)	Retentivity of calcein in rat plasma at 37 °C for 17 h[b] (%)	*AUC* of calcein after injection[c] (μmol min ml^{-1})
7:0	58.4±3.9	2.3	0.051±0.024
7:2	59.4±3.5	8.8	1.438±0.160
7:4	74.0±3.7	22.7	3.109±0.270
7:7	77.9±8.2	12.0	1.953±0.356

a) From Table 2. *b*) Reciprocal of leakage of calcein from DPPC/SS-liposomes in 30% (v/v) of rat plasma *in vitro* at 37±0.5 °C incubation.[6] *c*) Retentivity of liposomes entrapping calcein *in vivo*; *AUC* of calcein after intravenous injection of DPPC/SS-liposomes entrapping calcein in mice ($n=3$, mean ± S.D.).[6]

Relation of Rigidity and the Stability of Liposomes *in Vitro* and *in Vivo* The retentivity of calcein in plasma *in vitro* and the *AUC* of liposomes may reflect the collapse by the interaction of bilayer of liposomes with protein in plasma. When liposomes are exposed to serum or plasma, they rapidly acquire a coating of plasma proteins. One factor affecting protein interaction is the fluidity and packing of the bilayer. It is well known from surface balance studies of monolayer films that proteins most readily penetrate when the film pressure is low.[13] The fluid-to-solid phase transition increases the packing of lipid molecules, thus tending to exclude protein from penetration into the lipid film. Similarly, the addition of SS to monolayer or bilayer membrane (above the gel–liquid crystalline phase transition temperature) has a condensing effect and tends to retard protein penetration.

The stability and high retentivity of calcein in liposomes *in vitro* and *in vivo* may correspond with the result of rigidity of liposomal membrane, since the fluidity of liposomal membrane is thought to cause calcein leakage from liposomes. We found from Table 3 that the DPPC/SS (7:4)-liposomes demonstrated the highest stability in plasma *in vitro* and *in vivo*, but the DPPC/SS (7:7)-liposomes demonstrated higher stability than DPPC/SS (7:4)-liposomes after storage at 4 °C. This difference in stability may result from the rigidity of liposomes which is affected by temperature. DPPC/SS (7:4)-liposomes appear to have higher rigidity of liposomal membrane around 37—40 °C but lower rigidity at below 25 °C than DPPC/SS (7:7)-liposomes.

We have reprted that the ascending order of fluorescence anisotropy of MLVs is DPPC/SS (7:2) > DPPC/Ch (7:2) > DPPC at 37.5 °C.[4] The fluorescence anisotropy of DPPC/Ch (7:0—7:7)-MLVs[12] was lower than that of DPPC/SS (7:2—7:7)-REV liposomes in Fig. 3 at 33 and 49 °C. The physicochemical properties may not differ between MLVs and REV liposomes and, therefore, DPPC/SS (7:2, 7:4, 7:7)-liposomes show more rigid liposomal membranes than DPPC/Ch (7:2, 7:4, 7:7)-liposomes. This result reflects that the *AUC* values of DPPC/SS (7:2)-liposomes were significantly higher than those of DPPC/Ch (7:2)-liposomes.[6] From these *in vitro* and *in vivo* results, the ability of SS to stabilize DPPC-liposomes is greater than that of Ch, which is usually used as a stabilizer since the effect of SS derives from the stabilization of the gel state of liposomes.[4]

The inclusion of SS in DPPC-liposomes increases the stability of liposomes for storage and as drug carriers, protecting the interaction between liposomes and proteins in blood. The rigidity of liposomal membrane by SS indicated a good relation in the stability of liposomes *in vitro* and *in vivo*.

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