Electron Spin Resonance Studies of Dipalmitoylphosphatidylcholine Liposomes Containing Soybean-Derived Sterylglucoside

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The effects of soybean-derived sterylglucoside (SG) on the fluidity of liposomal membrane composed of dipalmitoylphosphatidylcholine (DPPC) were investigated compared with those of soybean-derived sterol (SS) and cholesterol (Ch) using an electron spin resonance spectrometer. Three kinds of liposomes were prepared in the molar ratio of DPPC/X=7/4, where X is SS, Ch or SG. The fluidity close to the polar head groups increased with an increase of temperature in the DPPC membrane containing SS, Ch and SG in the range 35 to 45 °C. Those near the hydrophobic end changed with an increase in temperature in liposomes containing SS, Ch and SG, which had a fluidizing effect on the DPPC membrane below the transition temperature (Tm, 41.9 °C) and a condensing effect over the Tm. The fluidizing effects of these compounds around 37 °C near the polar head group and the hydrophobic end increased in the following order: Ch<SG≤SS and SS<Ch<SG, respectively. SG increased the fluidity of liposomal membrane dramatically above the Tm (35.4 °C). These results suggest that the high fluidity close to the hydrophobic end of the liposomal membranes around 37 °C, the decrease of Tm, and the sigmoidal nature of fluidity *vs.* temperature are important factors in the effectiveness of liposomes containing SG as a carrier of drugs.

Key words liposome; dipalmitoylphosphatidylcholine; soybean-derived sterylglucoside; ESR spectrum; spin label

Liposomes have been extensively explored as carriers for improving the delivery of various therapeutic drugs.[1—4] When a liposomal drug is administered, it could have different pharmacokinetics than that of the free form.[5,6] In many cases, the toxicity of a free drug can be decreased by the liposomal entrapment. However, the stability of liposomes remains a problem to be solved. Many researchers have investigated the stability of liposomes using cholesterol (Ch), which is mainly contained in animal cells. Plants contain sterols and their glycosides. Sterols have a similar function to Ch in biological membranes.[7,8] Sterols in plants are present as complex mixtures. Soybeans contain sterol (SS) in its oil and sterylglucoside (SG), which remains in the residue after the oil has been extracted.

We previously demonstrated that the effectiveness of dipalmitoylphosphatidylcholine liposomes (DPPC-liposome) containing SG (DPPC/SG-liposomes) is significantly different from that containing SS or Ch in terms of accumulation in the liver,[9,10] and a carrier of drugs administered nasally.[11] The administration of insulin containing in DPPC/SG (7/4, mol)- liposomes showing high fluidity caused a high glucose reduction and long duration of reduction effect, for 8 h. DPPC/SS and DPPC/Ch (7/4)-liposomes showing low fluidity caused low glucose reduction.

We have also reported the relationship between the rigidity of the liposomal membrane and the stability of DPPC/SG-liposomes as measured by differential scanning calorimetry (DSC)[12] and fluorescence anisotropy.[12,13]

In this study, we examined the influence of added SG and temperature on the liposome bilayer fluidity of DPPC using ESR spectroscopy to elucidate its mechanism as a carrier of insulin administered nasally.

Experimental

Materials DPPC was purchased from NOF Co., Ltd. (Tokyo, Japan). Ch was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ryukakusan Co. (Tokyo, Japan) kindly supplied SS and SG. The SG is a mixture of the monoglucosides of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%), as shown in Fig. 1. SS is the aglycon of SG and contains each sterol in the same ratio as in SG. 5- and 16-doxylstearic acids (DSA) were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All other chemicals used were of reagent grade.

Preparation of Liposomes Multilamellar liposomes were prepared according to a standard method[14] as described in a previous study[12]. Briefly, a mixed solution of DPPC (70 μmol), Ch, SS or SG (20 or 40 μmol) and an ESR spin probe were dissolved in chloroform and dried under reduced pressure. The obtained lipid film was then hydrated in 3 ml of pH 7.4 phosphate buffered saline (PBS) (1 : 10 dilution of PBS in distilled water; 137 mM NaCl/2.6 mM KCl/6.4 mM Na_2HPO_4/1.4 mM KH_2PO_4; pH 7.4). It was then mixed by vortexing, followed by sonication in a bath-type sonicator (Honda Electronics, W220R, Tokyo, Japan) and centrifugation at 9500×***g*** for 5 min to remove large particles and form a homogeneous size. The obtained liposomes contained a spin probe at a concentration of 0.6 mol/mol% of total lipids.[15] DPPC-liposomes with SS, Ch or SG were composed of DPPC/SS, DPPC/Ch or DPPC/SG-liposomes at the molar ratio of 7/2 or 7/4, respectively.

ESR Measurements A JEOL JES-TE-200 spectrometer was used to determine ESR spectra at ambient temperature (30—44 °C). The effect of temperature on ESR spectra from the liposomes was measured using a variable temperature controller (JEOL ES-DVT3) at a heating value of 2 °C over the range 30—34 °C and 0.5 °C over the range 35—44 °C, respectively. The

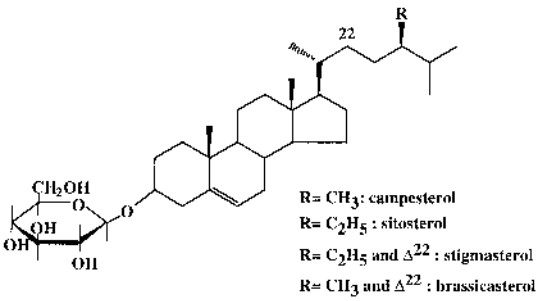

Fig. 1. Chemical Structures of Soybean-Derived Sterylglucoside (SG)

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liposomes were transferred to a glass capillary (20 μl of sample volume). This spectrometer has the function of normalizing all spectra for accurate calculation using manganese dioxide as an internal standard. The conditions for measurement were as follows: microwave power, 4 mW for 5-DSA or 1 mW for 16-DSA; microwave frequency, 9.200—9.255 GHz; field modulation, 100 kHz/0.1 mT; scan time, 4 or 8 min; magnetic field, 328±5 mT; gain, 1×100; response time, 0.3 s. Order parameters, *S*, with the expression given by Gaffney and McConell:[16]

$$S=(T_{//}-T_{\perp}-c)/(T_{//}+2T_{\perp}+2c)\times 1.723$$

where $T_{//}$ and $T_{\perp}$ are the apparent parallel and perpendicular hyperfine splitting parameters of the spectra of 5-DSA, the constant $C=1.4-0.053\times(T_{//}-T_{\perp})$ is an empirical correction for the difference between the true and apparent values of $T_{\perp}$, and the factor 1.723 is a solvent polarity correction factor.[16] These spectra of 16-DSA, the ratio of the low-field peak height to the central one ($h(+1)/h(0)$), can be used as empirical parameters for membrane fluidity.[17,18]

DSC Measurements DSC experiments were performed using a Thermoflex DSC 8230 (Rigaku Denki Co., Tokyo, Japan). The liposome suspension was concentrated by an ultrafilter (USY-5, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The liposome samples were transferred to an aluminum pan. The sample was analyzed by heating at a rate of 4 °C/min over the temperature range of 25—100 °C, using aluminum oxide as a reference. Each transition temperature value from gel to liquid crystalline of the DPPC, DPPC/SS (7/4), DPPC/Ch (7/4) and DPPC/SG (7/4)-liposomes was in the range of 40.0—43.9 °C, 37.9—45.7 °C, 36.8—44.3 °C and 31.4—41.4 °C, respectively. The transition temperature (Tm) was determined from the temperature at a peak.

Results and Discussion

The mean size of the liposomes was 106—111 nm. Ch can be contained up to 50% mol in DPPC-liposomes.[19] SG and SS can be contained up to almost 36%[20] and 50%,[13] respectively.

Steady-state parameters for the membrane fluidity studies were obtained using two different types of ESR probes, 5-DSA and 16-DSA, which have been used as convenient parameters to monitor the rotational freedom of the nitroxide radical group. The nitroxide radical of 5-DSA is located close to the polar head groups of phospholipids in the liposomal membranes, and that of 16-DSA near the hydrophobic end. To determine the effects of SS, Ch and SG on the fluidity of liposomes, ESR spectra in the DPPC/X=7/4-liposomes (X=SS, Ch or SG) were determined.

ESR spectra of 5-DSA and 16-DSA in the DPPC/SG (7/4)-liposomes at various temperatures are shown in Figs. 2 and 3, respectively. As can be seen from Fig. 2, the order parameter decreased with increasing temperature, indicating a decrease in alkyl chain order near the phospholipid head groups, *i.e.*, an increase of fluidity in the liposomal membrane. The order parameter *S* indicates the molar motion of *gauche-trans* isomerization in the lipid bilayer.

The fluidity of the liposomal bilayer near the hydrophobic end was examined using a spin probe of 16-DSA. Three peaks, as shown in Fig. 3, characterized the ESR spectra of 16-DSA in the DPPC/SG (7/4)-liposomes. In these spectra, an increase in the values of ($h(+1)/h(0)$) reflects an increase in the mobility of the nitroxide radical near the hydrophobic end of the acyl chains.

Figure 4 shows that the order parameter *S* of the DPPC/SS (7/4), DPPC/Ch (7/4) and DPPC/SG (7/4)-liposomes at 30 °C and 44 °C was 0.661 and 0.595 (the difference of S values at 30 °C and 44 °C; Δ0.066), 0.689 and 0.626 (Δ0.062), 0.695 and 0.577 (Δ0.118), respectively. These results correspond with the transition temperature range of DPPC/SG (7/4)-liposomes being wide, 31.4—41.1 °C compared with DPPC/SS (7/4) and DPPC/Ch (7/4)-liposomes. The Tm of the DPPC, DPPC/SS (7/4), DPPC/Ch (7/4) and DPPC/SG (7/4)-liposomes were 41.9, 40.0, 41.5 and 35.4 °C, respectively. The DPPC/SS (7/4) and DPPC/Ch (7/4)-liposomes produced small changes in the order parameter at the Tm: 40.0 and 41.5 °C, respectively. The incorporation of SS

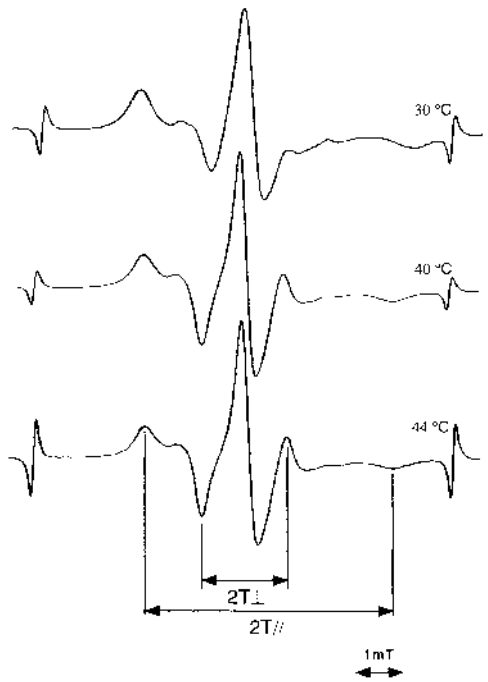

Fig. 2. ESR Spectra at Ambient Temperature (30—44 °C) of 5-DSA in DPPC/SG (7/4)-Liposomes

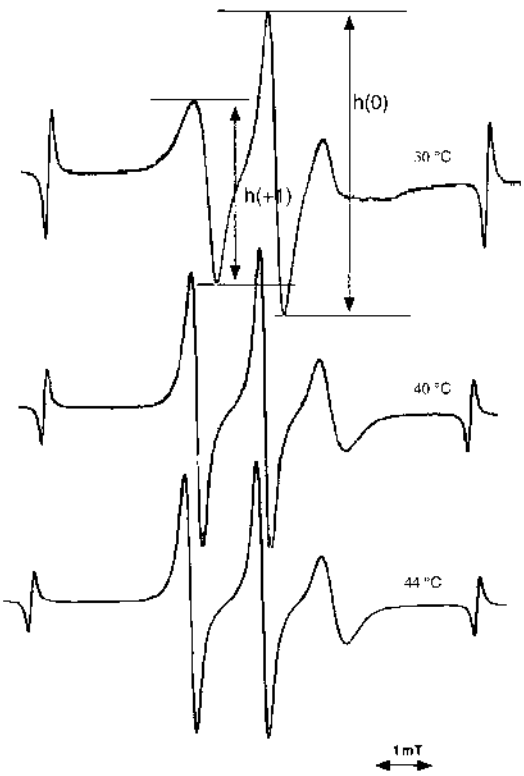

Fig. 3. ESR Spectra at Ambient Temperature (30—44 °C) of 16-DSA in DPPC/SG (7/4)-Liposomes

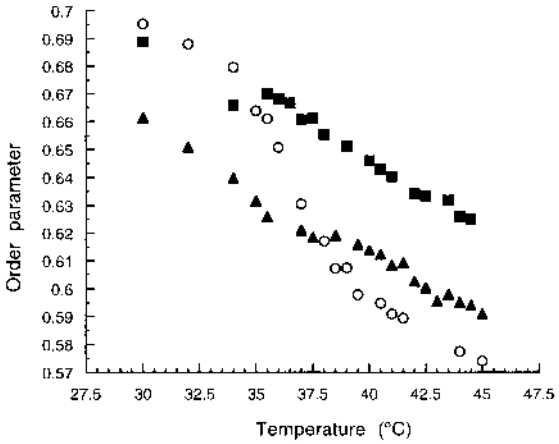

Fig. 4. Temperature Dependence of the Order Parameter S for 5-DSA

(▲), DPPC/SS (7/4)-liposomes; (■), DPPC/Ch (7/4)-liposomes; (○), DPPC/SG (7/4)-liposomes.

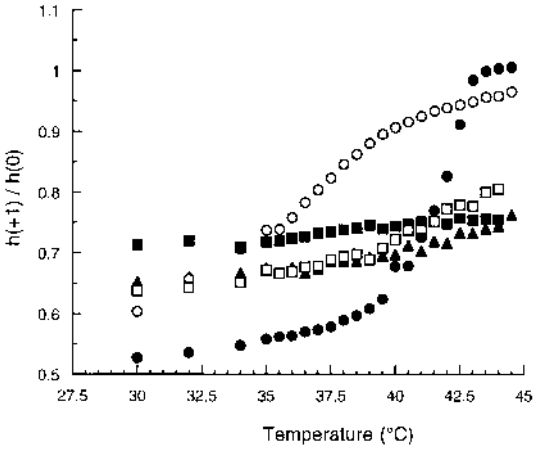

Fig. 5. Temperature Dependence of the Ratio of Peak Height of 16-DSA

(●), DPPC-liposomes (control); (▲), DPPC/SS (7/4)-liposomes; (□), DPPC/Ch (7/2)-liposomes; (■), DPPC/Ch (7/4)-liposomes; (○), DPPC/SG (7/4)-liposomes.

into the DPPC-liposomes resulted in a greater increase of membrane fluidity than with Ch. The incorporation of SG into each sharply increased the membrane fluidity, indicating a decrease in alkyl chain order near the phopholipid head groups above the Tm (35.4 °C). Below the Tm (35.4 °C), SG decreased the membrane fluidity compared with SS and Ch. Around 37 °C, the DPPC/SG (7/4)-liposomes showed a slightly rigid membrane compared with that of DPPC/SS (7/4)-liposomes (Fig. 4). This result corresponded well with the data observed using dansylhexadecylamine (DSHA),[11] although 5-DSA is located close to the polar head groups of the lipid bilayer, and DSHA was located to the polar head regions of the lipid bilayer. The fluidizing effect of these compounds around 37 °C is greater near the polar head group in liposomes in the following order: Ch$<$SG$\leq$SS.

Figure 5 shows the change in the ratio of the low-field peak height ($h(+1)/h(0)$) value as a function of temperature in the presence of the DPPC/SS (7/4), DPPC/Ch (7/4) and (7/2), DPPC/SG (7/4)-liposomes and DPPC-liposomes as a control. A larger ($h(+1)/h(0)$) value means a more fluid membrane. In the case of the DPPC-liposomes, the ($h(+1)/h(0)$) value undergoes a gradual increase with increasing temperature and the abrupt large increase corresponds to the phase transition of gel to liquid-crystalline (40—43.9 °C). The main transition temperature of the control is around 41.9 °C (Tm), corresponding to the melting temperature of the lipid hydrocarbon chains. At the temperature below the Tm (41.9 °C), the fluidity near the hydrophobic end of the liposomal membrane increased on the addition of SS, Ch and SG (fluidizing effect) compared with the control. At temperatures above the Tm (41.9 °C), the fluidity of the membrane of the DPPC/SS (7/4), DPPC/Ch (7/4) and DPPC/SG (7/4)-liposomes, was lower than that of the control (condensing effect). The DPPC/Ch (7/2)-liposomes showed a lower fluidizing effect than the DPPC/Ch (7/4)-liposomes. Many reseachers have reported such a contradictory effect of Ch.[21,22] It seems to be due to the formation of an intermediate gel state brought about by the hydrophobic interaction of Ch with the fatty acyl chains of the saturated phosphatidylcholine, and the formation of a hydrogen bond between Ch and phospholipid molecules.[7]

We reported that based on the fluorescence anisotropy data of 1,6-diphenyl-1,3,5-hexatriene (DPH), which was partitioned deep in the hydrophobic interior in liposomes, the DPPC/SG (7/4)-liposomes decreased the anisotropy of DPH in liposomes more so than did the DPPC/SS (7/4) and DPPC/Ch (7/4)-liposomes in the range 25 to 48 °C.[11] These findings from the fluorescence anisotropy data of DPH and the ESR spectra data of 16-DSA correspond well with the fact that their fluidizing effect around 37 °C is greater near the hydrophobic part in liposomes in the following order: SS$<$Ch$<$SG. However, at the temperature below the Tm (35.4 °C) (Fig. 5), SG slightly decreased the membrane fluidity near the hydrophobic group compared with SS and Ch. This finding from the ESR spin probe was different from that of the fluorescence anisotropy.[11]

The phase transition of pure DPPC-liposomes seems to be more or less retained in the presence of SG (Fig. 5). The phase transition of lipids in the presence of Ch and SS shows a different curve from the sigmoidal nature found in the case of pure DPPC (Fig. 5). SS as well as Ch may be ordered in the DPPC-liposomes by van der Waals force.[23] SS appears to be more efficient than Ch in ordering the acyl chains near the hydrophobic end of the DPPC bilayer above 41.5 °C because the DPPC/SS (7/4)-liposomes showed lower ($h(+1)/h(0)$) values compared with the DPPC/Ch (7/4)-liposomes (Fig. 5). The ESR data showed clearly that SS decreased the fluidity of liposomes more than Ch compared with the result from fluorescence anisotropy above 41.5 °C.[11]

The acyl chain in the lower portion (16-DSA, Fig. 5) of DPPC/SS (7/4) and that in the upper portion (5-DSA, Fig. 4) of DPPC/Ch (7/4)-liposomes were highly ordered at the temperature above the Tm (40.0, 41.5 °C, respectively) compared with the DPPC liposomes. On the other hand, the acyl chain of the lower portion (Fig. 5) and the upper portion (Fig. 4) in the DPPC/SG (7/4)-liposomes was highly disordered at the temperature above the Tm (35.4 °C). In general, a polar head group of DPPC has 10 water molecules (binding water),[24] whereas a glucose residue of SG may have only a few binding waters. The glucose residue of SG projected out of liposomes may change the packing state of the sterol group of SG in the liposomes.

We previously reported that DPPC/SG (7/4)-liposomes are effective carriers for the nasal absorption of insulin compared with DPPC/SS (7/4) and DPPC/Ch (7/4)-liposomes.[11] SG greatly enhanced the permeation of insulin through the nasal mucosa with some effects on lipids in the nasal mucosa.[25] Therefore, the high fluidity close to the hydrophobic end of the liposomal membranes around 37 °C, the decrease in transition temperature and the sigmoidal nature of fluidity *vs*. temperature may be important factors in the release of drug from liposomes containing SG, and/or to stimulate uptake of liposomes, perhaps as a result of the interaction of SG in liposomes on the nasal mucosa.

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References

1) Papahadjopoulos D., Poste G., Vail W. J., Biedler J. L., *Cancer Res.*, **36**, 2988—2994 (1976).
2) Gregoriadis G., Leathwood P. D., Ryman B. E., *FEBS Lett.*, **14**, 95—99 (1971).
3) Weissmann G., Bloomgarden D., Kaplan R., Cohen C., Hoffstein S., Collins T., Gottlieb A., Nagel D. A., *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 88—92 (1974).
4) Patel H. M., Ryman B. E., *FEBS Lett.*, **62**, 60—63 (1976).
5) Juliano R. L., Stamp D., *Biochem. Pharmacol.*, **27**, 21—27 (1978).
6) Muramatsu K., Maitani Y., Nagai T., *Biol. Pharm. Bull.*, **19**, 1055—1058 (1996).
7) Demel R. A., De Kruijff B., *Biochim. Biophys. Acta*, **457**, 109—132 (1976).
8) Schuler I., Duportail G., Glasser N., Benveniste P., Hartmann M. A., *Biochim. Biophys. Acta.*, **1028**, 82—88 (1990).
9) Muramatsu K., Maitani Y., Machida, Y., Nagai T., *Int. J. Pharm.*, **124**, 19—26 (1995).
10) Shimizu K., Maitani Y., Takayama K., Nagai T., *J. Drug Targeting*, **4**, 245—253 (1996).
11) Muramatsu K., Maitani Y., Takayama K., Nagai T., *Drug Dev. Ind. Pharm*., **25**, 1099—1105 (1999).
12) Muramatsu K., Maitani Y., Machida Y., Nagai T., *Int. J. Pharm*., **107**, 1—8 (1994).
13) Qi X. R., Maitani Y., Nagai T., *Chem. Pharm. Bull.*, **44**, 237—240 (1996).
14) Bangham A. D., Miller N. G. A., *J. Methods Membr. Biol*., **1**, 1—68 (1974).
15) Morimoto Y., Hosokawa M., Sato H., Takeuch Y., *Chem. Pharm. Bull.*, **42**, 123—129 (1994).
16) Gaffney B. J., McConnell M., *J. Magn. Reson.*, **16**, 1—28 (1974).
17) Mehlhorn R. J., Keith A. D., "Membrane Molecular Biology," ed. by Fox C. F., Keith A. D., Sinauer Associates, Inc., Stamford, 1972.
18) Glagasigij U., Sato Y., Suzuki Y., *Chem. Pharm. Bull.*, **36**, 1589—1592 (1988).
19) Barry R. L., David A. B., Mathias H., *Biochemistry*, **19**, 1943—1954 (1980).
20) Shimizu K., Maitani Y., Takayama K., Nagai T., *J. Pharm. Sci.*, **85**, 741—744 (1996).
21) Marsh D., *Biol. Magn. Reson.*, **8**, 255—303 (1989).
22) Nagumo A., Sato Y., Suzuki Y., *Chem. Pharm. Bull.*, **39**, 3071—3074 (1991).
23) Cevc G., "Liposome Technology," Vol. I, ed. by Gregory G., CRC Press, New York, 1993, pp. 1—36.
24) Chapman D., "Form and Function of Phospholipids," ed. by Ansell G. B., Hawthorne J. N., Dawson R. H. C., Elsevier Scientific, 1973, pp. 117—142.
25) Ando T., Maitani Y., Yamamoto T., Takayama K., Nagai T., *Biol. Pharm. Bull*., **21**, 862—865 (1998).