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International Journal of Pharmaceutics 300 (2005) 38–47
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Cholesteryl hemisuccinate as a membrane stabilizer in dipalmitoylphosphatidylcholine liposomes containing saikosaponin-d

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Received 25 February 2005; received in revised form 12 April 2005; accepted 10 May 2005
Available online 22 June 2005

Abstract

In the present study, cholesteryl hemisuccinate (CHEMS) was evaluated for use as a membrane stabilizer in dipalmitoylphosphatidylcholine (DPPC) liposomes. Differential scanning calorimetry (DSC) and a calcein release study showed that CHEMS was more effective than cholesterol (CHOL) in increasing DPPC membrane stability. The findings of Fourier transform infrared spectroscopy (FT-IR) also suggested that CHEMS interacts with DPPC via both hydrogen bonding and electrostatic interaction. More importantly, CHEMS did not interact with saikosaponin-d (SSD), a triterpene saponin from Bupleurum species, unlike CHOL. SSD-containing liposomes with DPPC, CHEMS and DSPE-PEG could greatly decrease the hemolytic activity of SSD. This study demonstrated that CHEMS has more stabilization ability than CHOL since CHEMS may exhibit both hydrogen bond interaction and electrostatic interaction with DPPC membrane while CHOL only has hydrogen bond interaction, resulting in stable and low-hemolytic SSD-liposomes.

Keywords: Cholesteryl hemisuccinate; Saikosaponin-d; Saponin; Liposome; Differential scanning calorimetry; Fourier transform infrared spectroscopy

1. Introduction

Saikosaponin-d (SSD) (Fig. 1A), a triterpene saponin from Bupleurum species, has shown corticosterone-like activity (Yokoyama et al., 1984), Na^+-, K^+-ATPase inhibiting action (Zhou et al., 1996), immunoregulatory action (Ushio and Abe, 1991) and anti-platelet activating factor activity (Nakamura et al.,

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; CHEMS, cholesteryl hemisuccinate; CHOL, cholesterol; SSD, saikosaponin-d; DSPE-PEG, methoxypolyethyeneglycol (Mr2000)-distearoylphosphatidylethanolamine; DSC, differential scanning calorimetry; FT-IR, Fourier transform infrared spectroscopy

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doi:10.1016/j.ijpharm.2005.05.005

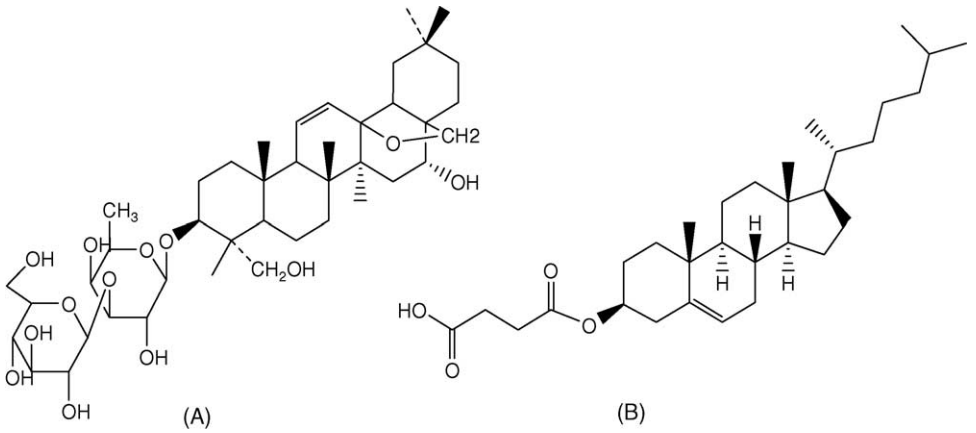

Fig. 1. Structures of saikosaponin-d (A) and cholesteryl hemisuccinate (B).

1993). It has been widely studied as a potential medication in the treatment of nephritis, nephrosis syndrome (Abe et al., 1986) and hepatic fibrosis (Cheng et al., 1999). Furthermore, bupleurum soup and particles for oral administration, the main active constituent of which is SSD, have achieved great success in the treatment of chronic glomerulonephritis and glomerulosclerosis (Zhang, 1993; Cheng, 1994).

SSD and other saikosaponins given in oral dosage form are not readily absorbed in the gastrointestinal tract and easily metabolized by glycosidase to less potent prosaikogenins before absorption occurs (Kida et al., 1998), leading to a dose of 200–300 mg and the need for treatment three times per day for adults. Therefore, the low level (less than 0.01%) of saikosaponins in Bupleurum species and their tendency to transform during separation and purification (Wen, 1993) hinder practical application in the clinic.

Other routes such as intraperitoneal and intramuscular administration have also been explored and are thought to enhance the corticosterone level in serum (Yokoyama et al., 1984; Zhong et al., 1993), however the risk of hemolysis should also be carefully considered. It is widely recognized that the hemolytic activity of SSD is caused by its complex with cholesterol (CHOL) on erythrocyte membrane, leading to membrane disruption and cell lysis. Driven by the need to reduce the hemolytic activity and make possible injections with less SSD, the liposome was chosen as a carrier for the present research because of its non-toxic, enhanced therapeutic efficacy and reduction of drug toxicity (Gregoriads, 1988).

It has been mentioned (Wang, 1992) that sterols with C_3-β-OH (including CHOL) form an insoluble complex with SSD, but, sterols with C_3-α-OH and esterified or glycosidated at C_3-OH do not. It is difficult to prepare liposomes containing SSD (SSD-liposomes), since SSD will form an insoluble complex with CHOL, which is routinely used to stabilize liposomes. Therefore, there is a great need for other membrane stabilizers for the preparation of SSD-liposomes.

Cholesteryl hemisuccinate (CHEMS) (Fig. 1B) is a CHOL-derivative esterified to the 3-hydroxyl group of CHOL and is supposed not to form a complex with SSD. Until now, no toxicity profiles about CHEMS have been reported. On the other hand, it has been found to increase specific immunogenicity of tumor cells by pretreating tumor cells with CHEMS (Skornick et al., 1986). And also, CHEMS was thought to protect against acetaminophen-induced hepatocellular apoptosis (Ray et al., 1996) and carbon tetrachloride-induced hepatotoxicity. It was proved to be a powerful cytoprotective agent against carbon tetrachloride hepatotoxicity in vivo (Fariss et al., 1993). CHEMS can form pH-sensitive fusogenic vesicles when incorporated into phosphatidylethanolamine bilayers and the pH is lowered to 5.5, resulting in H-II phase formation (Ismail et al., 2000; Se'rgio et al., 2004). CHEMS has also been demonstrated to alter acyl chain motion or fluidity in cell membranes (Dumas et al., 1997; Lai et al., 1985). It was proved by fluorescence polarization to be equally effective as CHOL in reducing the acyl chain mobility of DPPC above the phase transition temperature (Massey, 1998) and reported

to be a membrane stabilizer (Zhang et al., 2000). All these attributes contribute to the suitability of CHEMS as a membrane stabilizer in the preparation of SSD-liposomes, which has not been studied in the past.

The mechanism by which CHEMS acts as a membrane stabilizer is not fully understood and little research has been done on drug-loaded liposomes using CHEMS as a membrane stabilizer. Therefore, in the present study, CHEMS was investigated for its membrane stabilization ability using DSC, calcein release and FT-IR measurements. Furthermore, the hemolytic activity of SSD-liposomes using CHEMS as a unique membrane stabilizer was evaluated.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), CHOL, succinic anhydride and methoxypolyethyeneglycol (Mr 2000)-distearoylphosphatidylethanolamine (DSPE-PEG) were purchased from NOF Corporation (Japan). Saikosaponin-d (SSD, purity of 95%) was extracted from the root of *Bupleurum falcatum* by the Department of Materia Medica and Pharmacology, China–Japan Friendship Hospital. A Dialysis membrane (cutoff 8000–14,000) was purchased from Membrane Filtration Products (San Antonio, USA). All other chemicals were of reagent grade.

2.2. Synthesis of CHEMS

CHEMS was prepared according to Kuhn et al. (1975), but with 4-dimethylaminopyridine (DMAP) added as a catalyzer. The molecular weight of CHEMS determined by ESI-TOF-MS was 486.74.

2.3. DSC analysis

Liposome suspensions containing DPPC and different amounts of CHOL, CHEMS and SSD were prepared with 1/10 PBS (pH 7.4) by the film hydration and bath sonication method, with a DPPC concentration of 100 mg/ml. Ten microliters of each liposome suspension (1 mg DPPC) was transferred into a 20-μl DSC aluminum pan and subjected to DSC analysis using a DSC 2010 (Thermal Analysis, Newcastle, USA). The scan rate employed was 0.5 °C/min over the temperature range 20–50 °C, and the reference pan was filled with 1/10 PBS. The transition enthalpies (ΔH, J/g of DPPC) were calculated from the peak areas using the integration program of the TA processor, within an experimental error of ±5%.

2.4. Calcein release study

Calcein-encapsulated liposomes (DPPC-, DPPC/CHOL (10:4)-, DPPC/CHEMS (10:4)-liposomes) were prepared by the reverse phase evaporation vesicle method (Szoka and Papahadjopoulos, 1978). Briefly, 25 mg of DPPC and specific amount of CHEMS or CHOL were dissolved in 15 ml of chloroform/isopropyl ether (2:1, v/v) and mixed with 5 ml of calcein solution (1×10^{-3} mol/l in 1/10 PBS). The mixtures were sonicated by probe sonicator to give a homogeneous emulsion and then placed on a vacuum rotary evaporator until the formation of liposome suspension. The liposomes were then extruded through 200 nm-pore sized polycarbonate film at 45 °C for two times and free calcein was separated by Sephadex G-100. Calcein release from the liposomes was carried out with a dialysis method both in 1/10 PBS and in 30% rabbit plasma at 37 ± 0.5 °C.

2.5. FT-IR analysis

DPPC mixtures with CHOL, CHEMS and SSD (all with a 1:1 molar ratio) were dissolved in chloroform or a chloroform–methanol mixture for SSD. The solvent was evaporated under vacuum, the dried lipid mixture was pressed into thin KBr tablets. The tablets were scanned on a Nicolet 5DX (Thermo Electron Corp., Waltham, USA) from 400 to 4000 cm^{-1}. The separate FT-IR spectrograms were combined over the region between 1800 and 1200 cm^{-1}, which contained the band position of the P=O stretching vibration (V_s P=O), the P=O asymmetric stretching vibration (V_{as} P=O) and the asymmetry flexual vibration of the quaternary ammonium of DPPC.

2.6. Turbidity measurement

Blank liposomes (DPPC/CHOL (10:4)- and DPPC/CHEMS (10:4)-liposomes) were prepared by the film hydration and probe sonication method to

give small unilammelar vesicles (SUVs, average size, 134.6 and 102.9 nm, respectively), with the DPPC concentration at 0.5 mg/ml. An equal volume of SSD suspension of 200 μg/ml or distilled water was added to the liposome suspension, and the relative turbidity ($A_t/A_0 \times 100\%$) of liposomes was measured at 550 nm at room temperature, where A_t and A_0 represent the absorption value at time t and 0 min at 550 nm, respectively.

2.7. Hemolytic activity

SSD-liposomes with serial concentrations of SSD were prepared with DPPC, CHEMS and/or DSPE-PEG by film hydration and probe sonication. A 0.3 ml volume of SSD solution or SSD-liposomes suspension (with a lipid concentration of 2.5 mg/ml and size of about 150 nm) was put into a 5 ml glass tube, and then 2.2 ml of saline and 2.5 ml of 2% rabbit erythrocyte suspension were added. The mixtures were incubated at 37 ± 0.5 °C for 3 h and then centrifuged at 2500 rpm for 10 min. The absorption value of the supernatant was measured at 540 nm by subtracting the blank.

3. Results

3.1. Effect of CHOL, CHEMS and SSD on DPPC membrane by DSC

As illustrated in Fig. 2 and Table 1, the maximal transition temperature from gel to liquid-crystalline (T_m) and the transition enthalpy (ΔH) of the DPPC bilayer was 39.52 °C and 24.24 J/g of DPPC, respectively, addition of CHOL and CHEMS decreased the T_m value slightly and the ΔH value markedly. The T_m values of the five formulations remained at about 39 °C, while the ΔH values of DPPC/CHOL (10:2)-, DPPC/CHEMS (10:1)- and DPPC/CHEMS (10:2)-liposomes were decreased markedly to 5.26, 11.89 and 1.162 J/g of DPPC, respectively.

Table 1
The maximal transition temperature from gel to liquid-crystalline (T_m) and ΔH value of DPPC liposomes with CHOL, CHEMS and SSD (each sample contained 1 mg DPPC)

Composition (molar ratio)	T_m (°C)	ΔH (J/g of DPPC)
DPPC	39.52	24.24
DPPC:CHOL (10:2)	39.28	5.26
DPPC:CHEMS (10:2)	38.28	1.162
DPPC:CHEMS (10:1)	39.34	11.89
DPPC:SSD (10:2)	38.68	23.54

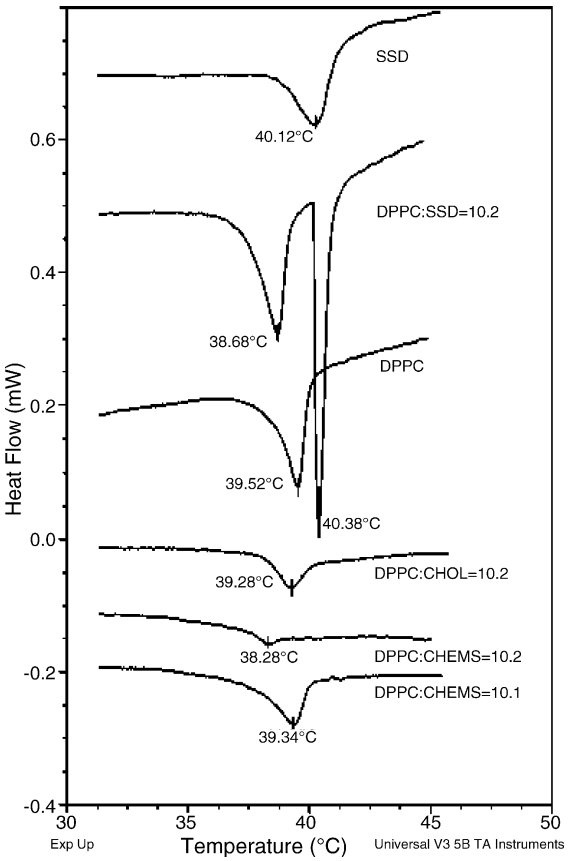

Fig. 2. DSC spectrograms of DPPC liposomes with CHEMS, CHOL and SSD.

We have found that a maximum amount of SSD that can retain in the lipid bilayers of liposomes was less 15% (SSD/total lipid, molar ratio). Therefore, in DPPC/SSD (10:2)-liposome suspension, free SSD surely existed and would form micelles after bath sonication, resulting in the second endotherm peak of 40.38 °C. The value was similar to 40.12 °C in the endotherm of SSD suspension (Fig. 2), confirming that the endotherm of 40.38 °C in DPPC/SSD (10:2)-liposome suspension was the micelles of free SSD. But the endotherm of SSD suspension was broad as compared to the sharp endotherm of 40.38 °C in DPPC/SSD (10:2)-liposome suspension, a reason may

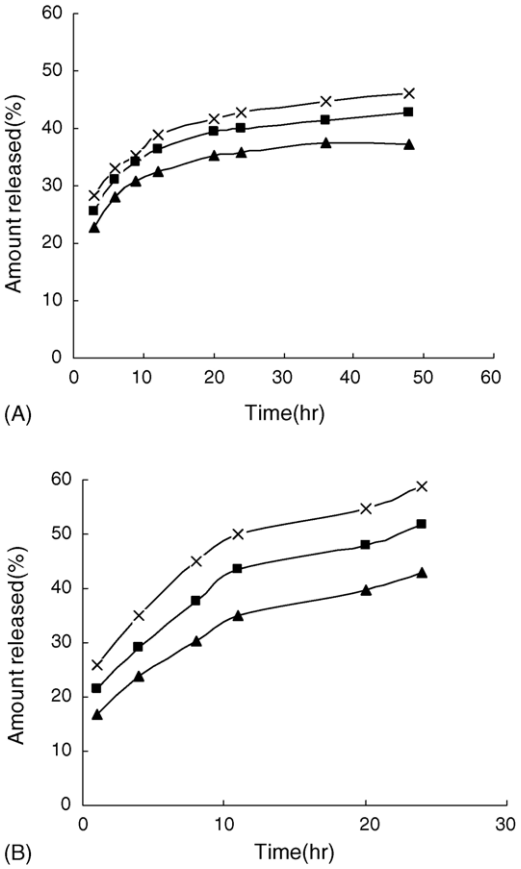

Fig. 3. Calcein release from liposomes at 37 ± 0.5 °C incubated with 1/10 PBS (A) and 30% rabbit plasma (B). (The data are means of two experiments.) (×): DPPC-liposomes, (■): DPPC/CHOL (10:4)-liposomes, (▲): DPPC/CHEMS (10:4)-liposomes.

be surmised that excessive SSD sedimentation might deposit on the base of aluminum pan, which may have interfered the heat conduction and subsequent peak appearance.

3.2. Calcein release study

Calcein release in 1/10 PBS (Fig. 3A) and in 30% rabbit plasma (Fig. 3B) were in duplicate since the experiments were parallel and the variability was very small. The results showed that the release of calcein from DPPC/CHEMS (10:4)-liposomes was slower than that of DPPC/CHOL (10:4)-liposomes, and the release in 1/10 PBS (Fig. 3A) was slower than that in 30% rabbit plasma (Fig. 3B).

3.3. Effect of CHEMS, CHOL and SSD on DPPC by FT-IR

The FT-IR spectrograms of DPPC/CHOL (1:1), DPPC/CHEMS (1:1) and DPPC/SSD (1:1) mixtures were compared with that of DPPC (Fig. 4).

Although the magnitude of the red shift is relatively small compared to the peak width (Asada et al., 2004), addition of CHOL, CHEMS and SSD in DPPC substantially induced the red shift of V_s P=O (from 1247.78 to 1241.99, 1240.07, 1235.21 cm^{-1}, respectively) and V_{as} P=O (from 1469.56 to 1465.71, 1467.64, 1465.71 cm^{-1}, respectively). We know that the asymmetry flexual vibration of the quaternary ammonium of DPPC is a small or side peak at 1670–1640 cm^{-1} (Peng, 1998). We can read from Fig. 4 that CHOL and SSD did not conceal the peak as there is no static electronic interaction between the hydroxyl groups and the quaternary ammonium of DPPC. While in the FT-IR spectrogram of the DPPC/CHEMS (1:1) mixture, the small peak (at 1670–1640 cm^{-1}) was absent, suggesting electrostatic interaction between the carboxyl group of CHEMS and the quaternary ammonium of DPPC.

3.4. SSD complex with CHOL but not with CHEMS

If SSD interacts with CHOL or CHEMS, it will insert lipid bilayers and ultimately disrupt the membrane structure of DPPC/CHOL (10:4)- or DPPC/CHEMS (10:4)-liposomes, causing large lipid aggregates to form and increasing the sedimentation and clarity of the supernatant of the liposome suspension.

To exclude an increase in clarity caused by liposome aggregation and sedimentation, distilled water was added. The results (Fig. 5) indicated there was no change in turbidity, which meant DPPC/CHOL (10:4)- and DPPC/CHEMS (10:4)-liposomes did not undergo sedimentation during the period of the experiment. After addition of SSD to DPPC/CHOL (10:4)- or DPPC/CHEMS (10:4)-liposomes, there was significant sedimentation for DPPC/CHOL (10:4)-liposomes and no turbidity change was observed for DPPC/CHEMS (10:4)-liposomes, which meant that SSD did not interact with CHEMS (Fig. 5).

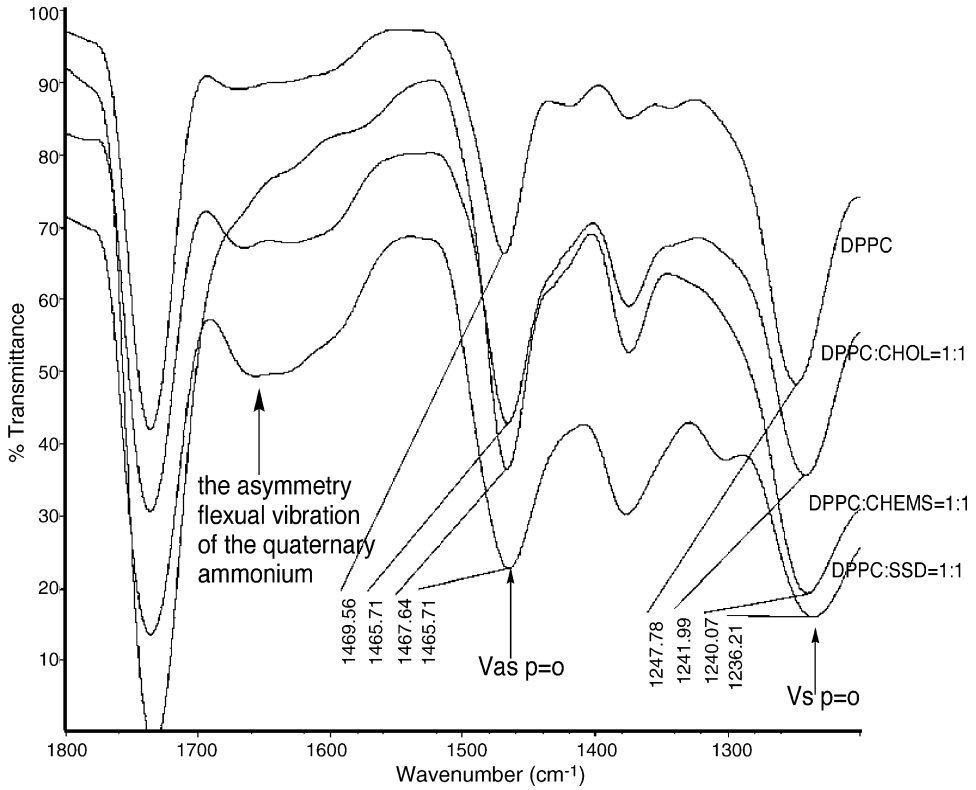

Fig. 4. FT-IR combinational spectrograms of DPPC mixtures with CHOL, CHEMS and SSD (1:1, molar ratio) between 1800 and 1200 cm^{-1}.

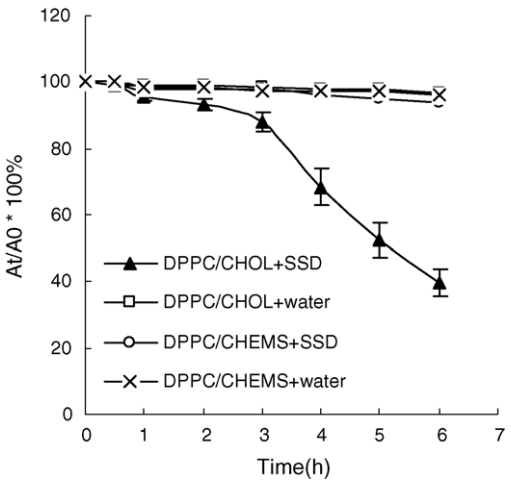

Fig. 5. Relative turbidity curve of DPPC/CHEMS (10:4)- and DPPC/CHOL (10:4)-liposomes (with a DPPC concentration of 0.5 mg/ml) after adding 200 μg/ml SSD suspension or distilled water. (The data are presented as mean ± S.D., $n = 3$.)

3.5. Hemolytic curve of SSD-liposomes

It seemed that DPPC/CHEMS/SSD (10:4:1)-liposomes could reduce the hemolytic activity of SSD in contrast to the SSD solution or the liposomes without CHEMS (DPPC/SSD (10:1)-liposomes) (Fig. 6). The liposomes that incorporated DSPE-PEG (DPPC/CHEMS/DSPE-PEG/SSD (10:4:0.5:1)-liposomes) showed the greatest ability to reduce the hemolytic activity. Thus, the SSD-liposomes should contain DSPE-PEG.

4. Discussion

In the present study, a value of the maximal transition temperature of 39.52 °C was observed for DPPC (Fig. 2), while a more usual literature value of 41 °C was reported. The great difference may be attributed to different lipid concentration used, different running conditions and different machines. We have found that

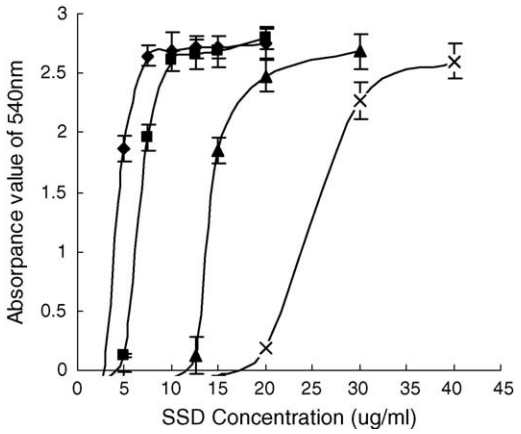

Fig. 6. Hemolytic curve of SSD solution and SSD-liposomes (with a lipid concentration of 2.5 mg/ml) on rabbit erythrocyte in vitro. (The data are presented as mean ± S.D., $n = 3$.) (♦): SSD solution, (■): DPPC/SSD (10:1)-liposomes, (▲): DPPC/CHEMS/SSD (10:4:1)-liposomes, (×): DPPC/CHEMS/DSPE-PEG/SSD (10:4:0.5:1)-liposomes.

a less concentrated liposome suspension of 20 mg/ml of DPPC (10 μl equal to 0.2 mg of DPPC) produced a phase transition temperature of 40.5 °C, which is similar to the literature value. But, lower concentration of DPPC resulted in lower enthalpy value and lower sensitivity, therefore, 100 mg/ml of DPPC were employed during the experiments and the results allowed meaningful comparison since the experimental conditions were strictly controlled as the same for each other.

Addition of CHOL, CHEMS and SSD to DPPC-liposomes decreased the T_m and ΔH values of DPPC (Fig. 2 and Table 1), which accorded with the finding that introduction of a hydrophobic molecular within the lipid bilayers produces a decrease in T_m and ΔH (Mabrey and Sturtevant, 1976). As mentioned in the literature (Yang and Su, 1998; Wang, 1997), when above the phase transition temperature, the membrane fluidity drops when the area of the endothermic peak (ΔH value) decreases and the T_m value increases. Since the ΔH is markedly decreased as compared with the T_m, the reduction of ΔH is much more dominant while the decrease in T_m is negligible in membrane stabilization.

CHEMS could reduce the fluidity of the liposomal membrane according to the ΔH reduction (1.16 J/g for DPPC/CHEMS (10:1)-liposomes compared to 5.26 J/g for DPPC/CHOL (10:1)-liposomes), suggesting that CHEMS possesses more stabilization ability than CHOL. The result was supported by the calcein release study.

The hydroxyl group of CHOL and the sugar moiety of SSD can both form a hydrogen bond with the P=O bond of DPPC (Peng, 1998; Shimizu et al., 1996), which caused the red shift of V_s P=O and V_{as} P=O (Fig. 4). As for CHEMS, the red shift of V_s P=O and V_{as} P=O may be due to the fact that the carbonyl group of CHEMS can form a hydrogen bond with the P–OH bond of DPPC, and therefore affect the vibration of the P=O bond.

CHOL and CHEMS had a similar effect on the red shift of V_s P=O, while SSD had the strongest effect on the red shift of V_s P=O due to abundant hydroxyl groups on the sugar moiety of SSD. But maybe because of the small molecular volume of the hydroxyl group and succinic acid, together with the deep-anchored sterol ring reducing the hydrocarbon movement (Massey, 1998), they may fill the polar headspace of lipid bilayers and thus increase the membrane stability. As for SSD, although it had the strongest hydrogen interaction with the polar head of DPPC, it may disrupt the lipid arrangement due to the large molecular volume of the sugar moiety (Muramatsu et al., 1999), thus increasing the membrane fluidity.

Fig. 7 illustrates the proposed mechanism of interaction mechanism of DPPC with CHOL, CHEMS and SSD. The hydroxyl group of CHOL and the sugar moiety of SSD could form a hydrogen bond with the phosphorus oxygen double bond (P=O) of DPPC, then the electron cloud around the oxygen of P=O will migrate to the hydroxyl group, decreasing the polarity of P=O bond, and causing the red shift of the stretching vibration of P=O.

As for CHEMS, the carbonyl group of CHEMS might have hydrogen bond interaction with the P–OH bond of DPPC, then the electron cloud around the hydroxyl group of P–OH might become much more intense, and the increased electron cloud around the hydroxyl group of P–OH will migrate to the phosphorus atom of P–OH. Therefore, the decrease in the polarity of the P=O bond may cause a red shift. In addition, electrostatic interaction may exist between the carboxyl group of CHEMS and the quaternary ammonium of DPPC.

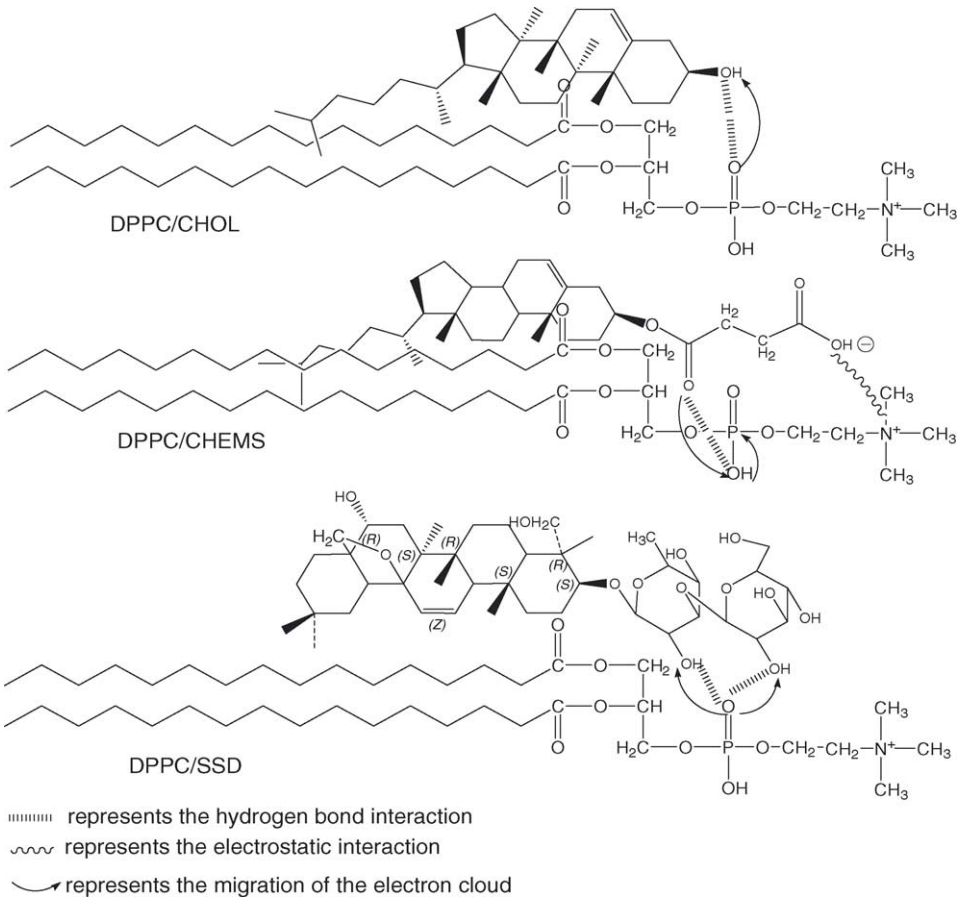

Fig. 7. Schematic diagram of interaction of DPPC with CHOL, CHEMS and SSD.

CHEMS can possess both hydrogen bond interaction and electrostatic interaction with DPPC membrane while CHOL only has hydrogen bond interaction. This difference may make the membrane of DPPC/CHEMS-liposomes more compact and steady than that of DPPC/CHOL-liposomes, being supported by the reduction of ΔH in DSC and low calcein release. In contrast, Massey (1998) reported that CHEMS could extend across one monolayer of the bilayer and partially interdigitate into the opposing monolayer of the bilayer, resulting in less membrane stabilization than CHOL by fluorescence polarization. From our study we hypothesized that the whole succinic group of CHEMS might fit into the polar head of DPPC and the sterol ring might be in the same position as that of CHOL.

CHEMS turned out to be a unique membrane stabilizer in SSD-liposomes due not to interaction with SSD as illustrated in Fig. 5. The reason may be ascribed to the succination of the hydroxyl group of CHOL, blocking recognition by SSD.

Further, CHEMS will find great use in the preparation of liposomes containing cholesterol-dependent hemolytic saponins. The incorporation of SSD into liposomes contained CHEMS could reduce its hemolytic activity (Fig. 6), because: SSD was incorporated in the lipid bilayers of liposomes, and liposomes containing CHEMS were negatively charged due to the presence of carboxylic acid residue and would experience repulsive interactions with erythrocyte membranes. In addition, reduced hemolytic activity of SSD-liposomes was observed on adding DSPE-PEG,

which may further exert a steric hindrance between liposome membranes and erythrocyte membranes.

This study has shown that CHEMS was more effective than CHOL in increasing DPPC membrane stability since CHEMS may possess both hydrogen bond interaction and electrostatic interaction with DPPC membrane while CHOL only has hydrogen bond interaction. Furthermore, CHEMS can be used as a unique membrane stabilizer in liposomes containing SSD. SSD-liposomes contained CHEMS and DSPE-PEG could greatly decrease the hemolytic activity of SSD.

Acknowledgment

This research was supported by a grant (30271520) from the National Science Foundation of China.

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