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Effect of β-Sitosterol in Liposome Bilayer on the Stabilization of Incorporated Retinol

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Abstract In this study, the effect of β-sitosterol (SS) in the liposome bilayer on the stability of incorporated retinol was evaluated. Retinol was incorporated into liposomes consisting of various ratios of soybean phoaphatidylcholine (PC) to SS, while liposomes were prepared as multilamellar vesicles by the dehydration/rehydration method. Retinol was readily incorporated into liposomes with various SS contents, with incorporation efficiencies higher than 98% for all conditions. The incorporation efficiency of retinol increased slightly as the SS content in liposomes increased. Its average particle size also increased as the SS content increased. Mean particle size at PC to SS ratios of 100:0, 90:10, 80:20, 70:30, 60:40, and 50:50 were 12.16, 17.57, 35.00, 40.62, 83.45, and 88.94 μm, respectively. Liposomal retinol degradation in aqueous solution was measured with respect to SS content at various periods of time at four different temperatures of 4, 25, 37, and 50°C, and the stability of the incorporated retinol enhanced as the SS content increased. At 4°C, for example, retinol in the liposomes of 50:50 (PC:SS) remained at 84.42% after storage for 10 days, while in 100:0 (PC/SS) it remained at 42.62%. These results indicate that SS content in liposomes played an important role in the incorporation efficiency of retinol and its stability.

Keywords: liposome, retinol, β-sitosterol, stability

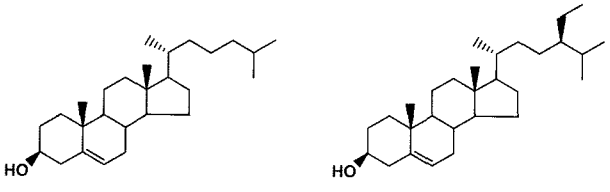
Introduction

Retinol, the common and biologically active form of preformed vitamin A, has been widely used as an ingredient in health formulas and cosmetics. Because retinol is sensitive to air, oxidizing agents, ultraviolet light, and low pH values (1, 2), many studies have attempted to find ways to minimize the effects of these environmental factors on retinol, thereby protecting it from damage. (3-5).

In previous studies, liposomes were shown to protect retinol against an extreme condition (3), and in particular cholesterol (CH) in liposomes plays an important role in stabilizing incorporated retinol (5). CH in the phospholipid bilayer of the membrane has been reported to increase the mechanical stiffness in its physical structure (6). However, excessive CH in higher animal cell membrane may cause many diseases such as heart attacks and Alzheimer's among others (7,8).

Phytosterols, or plant sterols, are commonly found in edible vegetable oils in the form of minor constituents (0.1-0.5%, w/w) (9). Among the wide variety of phytosterols, β-sitosterol, campesterol and stigmasterol are the most common. β-Sitosterol is similar to CH in structure, but has an ethyl group at C-24 (Fig. 1). Phytosterols such as β-sitosterol inhibit the absorption of dietary and endogenous CH and thereby reduce CH concentration in the blood.

In this study, we investigated the effects of â-sitosterol on the incorporation efficiency of retinol into liposomes and the stability of the incorporated retinol.



Cholesterol β-Sitosterol

Fig. 1. Structure of cholesterol and β-sitosterol.

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Materials and Methods

Materials All *trans* retinol, L-α-phosphatidylcholine (PC, 40% pure) isolated from soybeans, and β-sitosterol (SS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade or purer.

Instruments The UV-visible spectra of solutions were viewed using a UV–VIS spectrophotometer (UV 1601, Shimadzu, Japan). The mean size of the liposomes in the aqueous dispersions was estimated with a particle size analyzer (LS230 Small Volume Module, Coulter Co., USA). Liposome pellets were collected by centrifugation and the effects on the incorporation efficiency was examined with a Hitachi preparative ultracentrifuge (SCP 55H, Hitachi Koki Co. Ltd., Japan).

Preparation of liposomes containing retinol The method was based on the dehydration-rehydration procedure (10) with slight modifications. Briefly, retinol was added to phospholipids at a ratio of 0.01:1 (w:w). SS was added proportionally to achieve a ratio of soybean PC to SS on a per weight basis (PC: SS) of 100:0, 90:10, 80:20, 70:30, 60:40, or 50:50. Thus, 130, 117, 104, 91, 78, or 65 mg of PC were mixed with 0, 13, 26, 39, 52, or 65 mg of SS, respectively, in a 50 mL round-bottomed flask containing 1.3 mg of retinol. The mixtures were then dissolved in a

13 mL chloroform/methanol solvent mixture (2:1, v/v). The solvent was evaporated on a rotary evaporator (at 30°C) to deposit a dry lipid film on the wall of the flask. The flask was removed from the evaporator and the residual solvent was removed under nitrogen stream at 4°C. Thirteen milliliters of 10 mM glycine buffer (pH 9.0, containing 0.115 M NaCl) and 0.5 g of glass beads were added to assist in the hydration of the lipids. The solution was then mixed on the rotary evaporator (without vacuum) to hydrate the lipids and to form multilamellar vesicles (MLVs). The solution was centrifuged for 1 hr at 80,000 × *g*, the supernatant was removed and the pellet was washed with 13 mL of 10 mM potassium phosphate buffer (pH 7.0).

Analytical method Retinol in the liposomes was analyzed using a colorimetric assay (11). The liposome solution (0.15 mL) containing retinol was mixed with 0.45 mL of a chloroform/methanol solvent mixture (2:1, v/v). The mixture was centrifuged for 3 min at 4,200×*g*. The aliquot (0.1 mL) of the organic solvent layer was then transferred to a test tube, 1 mL of a 20% $SbCl_3$ solution was added, and the absorbance at 620 nm was measured immediately.

Stability of incorporated retinol in liposomes during storage The aliquots (0.2 mL) of the liposome suspension with the incorporated retinol were placed in glass vials and saturated with oxygen by equilibrating against the atmosphere for 2 hr in the dark. Vials stored in the dark were wrapped in aluminium foil and were stored at various temperatures (4, 25, 37 and 50°C) for 10 days.

Results

Incorporation of retinol into liposomes containing β-sitosterol (SS) The effect of SS on the encapsulation capacity and size of liposomes varies with manufacturing conditions and/or the properties of the encapsulated materials. Retinol was incorporated into the bilayer of the liposomes containing SS at an incorporation efficiency of between 98.02 ± 0.46% and 99.29 ± 0.65% under the experimental conditions (Table 1). Although there was no significant difference ($P<0.05$), the incorporation efficiency of retinol into the liposomes increased slightly as the SS content increased. These results differ from a previous study (12), in which the encapsulation efficiency of ascorbic acid, a water-soluble material, decreased as cholesterol (CH) content in small unilamellar vesicles (SUVs) increased, showing an encapsulation efficiency of only 26.4% at 50:50 (PC:CH). The important factors affecting the encapsulating efficiency may be the properties of encapsulated materials as well as the type of liposomes. In MLV systems, incorporation efficiency of lipid-soluble materials is dependent upon the amount of lipids in the vesicle and is independent of size (13). Upon hydration, the lipids are said to swell, and peel off the support in sheets, generally forming MLVs. In MLVs, the aqueous volume enclosed within the lipid membrane is usually 5~10% of the total volume used for swelling (14). Although water-soluble compounds to be entrapped are very wasteful in MLVs preparation method, lipid soluble compounds can be effectively incorporated into the bilayer phase of the liposomes (15). Therefore, it was supposed that the incorporation efficiency of retinol, a lipid soluble compound, would increase more than the encapsulation efficiency of ascorbic acid, a water-soluble compound. Also, the incorporation efficiency of retinol increased slightly as the pH value of the hydration buffer increased. In the case of CH containing liposomes under the same preparation condition, retinol was incorporated into the bilayer with similar rates and trends (from 95.46 ± 0.27% to 98.51 ± 0.01%, means ± SD) (5).

Mean size of the liposomes also increased as the SS content increased (Table 2). These results are consistent with previous studies which showed that size of liposomes increased as the CH content increased (5, 16). The presence of CH in the lipid bilayer enhances order and rigidity. The fused ring structure of CH is quite rigid by itself, and the presence of CH stabilizes the extended straight-chain arrangement of saturated fatty acids by van der Waals interactions (6). SS may play a similar role with CH in the liposome bilayer due to their structural similarity.

Stability of incorporated retinol in liposomes The effect of SS in MLV bilayer on the stability of the incorporated retinol under alkaline conditions in 10mM phosphate buffer at pH 7 was investigated at four different temperatures (4, 25, 37, and 50°C) for 10 days. As shown at Fig. 2, the stability of the incorporated retinol at all storage temperatures was enhanced as the SS content increased. At 4°C, the incorporated retinol degraded very slowly during storage so that over 84.42% remained at 50:50 (PC:SS) (Fig. 2A), while 42.62% remained at 100:0 (PC:SS) after storage for 10 days. Free retinol in an alkaline buffer has been reported to degrade by about 90%

Table 1. Efficiency of incorporation of retinol into liposomes based on β-sitosterol content

Ratio[1)]	Incorporation efficiency (%)[2)]
100 : 0	98.02±0.46
90 : 10	98.26±0.32
80 : 20	98.64±0.55
70 : 30	98.80±0.59
60 : 40	99.01±0.63
50 : 50	99.29±0.65

Retinol : (phosphatidylcholine + β-sitosterol) = 0.01 : 1(w/w).
[1)]Weight ratio of phosphatidylcholine to β-sitosterol.
[2)]Mean±standard deviation of triplicate measurements.

Table 2. Effect of β-sitosterol content in liposomes on the size of retinol incorporated liposomes

Ratio[1)]	Mean size of liposome (μm)
100 : 0	12.16
90 : 10	17.57
80 : 20	35.00
70 : 30	40.62
60 : 40	83.45
50 : 50	88.94

[1)]Weight ratio of phosphatidylcholine to β-sitosterol.

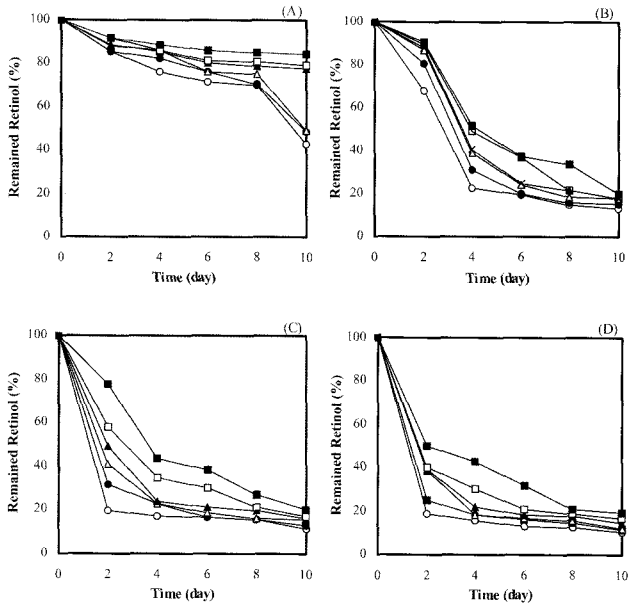

Fig. 2. Stability of retinol in 10 mM potassium phosphate buffer (pH 7.0): the percent of retinol remaining is plotted as a function of storage time. Buffers containing retinol in multilamellar liposomes were stored at (A) 4°C, (B) 25°C, (C) 37°C, and 50°C. Liposomes were composed of phosphatidylcholine and β-sitosterol with ratios (w:w) of 100:0 (○), 90:10(●), 80:20(△), 70:30(▲), 60:40(□), and 50:50(■), respectively.

after one day in storage (3).

Retinol degradation progressed dramatically as the storage temperature increased (Fig. 2B, 2C, and 2D). After four days at 25°C, 51.79% of the incorporated retinol remained at 50:50 (PC:SS), while 22.40% remained at 100:0 (PC:SS) (Fig. 2B). The rate at which the incorporated retinol degraded at 37°C and at 50°C were very similar to the results seen at 25°C (Fig. 2C, 2D). As shown in Fig. 2(C), the retinol remaining after 4 days was 16.94%, 22.50%, 23.01%, 23.62%, 37.76%, and 43.72% at 100:0, 90:10, 80:20, 70:30, 60:40, and 50:50 (PC:SS). After 10 days, the incorporated retinol degraded by approximately 80% under all conditions at 37°C. At 50°C, the stability of the incorporated retinol decreased rapidly during storage (Fig. 2D). The retinol remaining after 4 days in storage was 15.64%, 17.94%, 18.36%, 21.80%, 30.44%, and 42.71% at 100:0, 90:10, 80:20, 70:30, 60:40, and 50:50 (PC:SS).

Discussion

It has been reported that the stability of incorporated retinol in liposomes increases as the CH content in the lipid bilayer increases (5). In this study, we investigated the effect of SS instead of CH on the stabilization of incorporated retinol under four different temperatures. Though the degradation of retinol was faster as the storage temperature increased (Fig. 2), the stability of retinol was enhanced as the SS content in liposomes increased. According to these results, SS plays an important role in the protection of retinol in liposomes.

SS can be easily inserted into the phospholipid bilayer because of its structural and hydrophobic characteristics. While the hydrophobic interaction drives the formation of the bilayer, attractive van der Waals forces constitute the primary interactions between the phospholipid acyl chains and the phospholipid acyl chains and SS. In addition to the van der Waals forces, the interactions between SS and phospholipids may involve hydrogen bonds between the hydroxyl group of SS and the interfacial choline groups of the PC.

Retinol was efficiently incorporated into multilamellar liposomes, with incorporation efficiencies of over 98% for all conditions tested. The length of retinol calculated from the energy minimized structures using SYBYL (Tripos, St Louis, USA) was 15.5 Å, and steric energy was 41.64 $kcalmol^{-1}$, while the thickness of the bilayer in multilamellar liposomes was approximately 40 Å (4). It can be expected that retinol be distributed and stabilized within the hydrophobic region of the liposomes at both the planar interface between lipid leaflets and within each acyl chain region. SS in liposomes may help place retinol in the phospholipid bilayer because of its hydrophobicity.

Stability of the incorporated retinol in liposomes increased as the SS content in the lipid bilayer increased. In recent studies, sitosterol significantly ordered acyl chains of soybean lecithin bilayers (17), and plant sterol (60% β-sitosterol, 27% campesterol and 13% dihydrobrassicasterol) inhibited perturbation (permeability and lipid vesicle aggregation) of the phospholipid bilayer (18). When SS is present in large amounts, it acts as a permeability barrier like CH for the membrane by introducing conformational ordering of the lipid chains (19). CH is known to affect the structure and mesoscopic dynamics of the phospholipid bilayer; a significant ordering of PC chains, a reduced fraction of gauche bonds, a reduced surface area per lipid, less undulations corresponding to an increased bending modulus for the membrane, smaller area fluctuations, and a reduced lateral diffusion of phospholipids as well as CH (20). Thus, CH increases mechanical stiffness of the bilayer and stability of the incorporated materials. Even though SS interacted less favorably than CH with the phospholipids (21), SS played an important role in protecting the incorporated retinol in liposomes which were structurally similar to CH.

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