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Effect of Phase Transition Temperature of Phospholipid on the Stability of Retinol Incorporated into Liposomes

Kyung-Eun Lee, Jin-Ju Kim, Hyun-Gyun Yuk, Ji-Young Jang and Seung-Cheol Lee†

Department of Food Science and Biotechnology, Kyungnam University, Masan 631-701, Korea

Abstract

We investigated the effect of the phase transition temperature (T_c) of phospholipid in liposomes on the stability of incorporated retinol. Two kinds of phospholipid which have different T_c, L-α-dimyristoyl phosphatidyl choline (DMPC, T_c=22°C) and D,L-α-dipalmitoyl phosphatidyl choline (DPPC, T_c=42°C), were used to prepare liposomes. Liposome with retinol was prepared as multilamella vesicles (MLVs) by the dehydration/rehydration method. The incorporation efficiency of retinol into liposomes prepared from DMPC and DPPC were 99.89± 0.08% and 99.97±0.03, respectively. The average size of liposomes from DPPC were greater than that of DMPC. Two kinds of liposomes in phosphate buffer (10 mM, pH 7.0) were stored at 15, 30, and 50°C, and stability of incorporated retinol was analyzed. The stability of retinol in DMPC liposome was decreased, whereas the stability in DPPC liposome was increased as temperature increased, although the overall protection effect of liposome on the incorporated retinol was greater in DMPC liposomes than in DPPC liposomes.

Key words: liposome, DMPC, DPPC, retinol, phase transition temperature, stability

INTRODUCTION

Vitamin A is an essential nutrient that is important for the proper function of a number of biological processes, such as vision, reproduction, cellular growth and differentiation, embryonic development, and immune response (1). Vitamin A readily undergoes degradative reactions that are characteristic of conjugated double-bond systems that result in a decrease, or even a total loss of vitamin activity. These reactions are generally localized in the conjugated isoprenoid side chain and include isomerization to cis-isomers with reduced activity, molecular fragmentation, and photochemical and chemical oxidation. Such degradation reactions can reduce the available vitamin activity of stored and processed foods by as much as 50% (2).

Retinol, the most bioactive form of vitamin A, is a fat-soluble compound that only occurs in animal tissue. It is especially abundant in fish and mammalian liver, milk fat, and egg yolks (3). Due to its hydrophobic character, retinol is usually found in a complex with lipid droplets or micelles in foods (4).

Liposomes are simply vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids). These vesicles form spontaneously when phospholipids are dispersed in aqueous media, giving rise to a population of vesicles which may range in size from tens of nano meters to tens of microns in diameter. These vesicles can be constructed so that they entrap quantities of materials both within their aqueous compartment and within the membrane (5).

Retinol has also been viewed as an interesting molecule for encapsulation into liposomes. The delivery of liposome-incorporated retinol to blood cells has been studied (6). When high doses of retinol are delivered via liposomes, there is reduced blood viscosity and cause less lysis of red blood cells than from retinol that is not complexed in liposomes. Liposomal all-trans retinoic acid has been formulated for treatment of acute promyelocytic leukemia (7,8). In a previous report (4), we demonstrated that the stability of retinol is greatly increased when it is incorporated into the multilamellar form of liposomes.

Phospholipids exist in different phases at different temperatures. Phase transition of phospholipid in bilayers of liposomes occurs with increasing temperature from a tightly ordered gel or solid phase to a liquid-crystal phase where the freedom of movement of individual molecules is higher. The phase state of phospholipids affect their packaging in a liposome, thus the permeability and stability of the liposome is affected (9-13). Each type of phospholipid has its own characteristic phase transition temperature (T_c). Therefore, the T_c of phospholipids must be consistent with the optimal work-

†Corresponding author. E-mail: sclee@kyungnam.ac.kr
Phone: +82-55-249-2684. Fax: +82-55-249-2995

ing temperature of the material to be incorporated into the liposome. The objective of this work was to determine the effect of T_c in liposome on the stability of incorporated retinol.

MATERIALS AND METHODS

Materials

All trans retinal acetate, L-α-dimyristoyl phosphatidyl choline ($C_{14:0}$, DMPC) and D,L-α-dipalmitoyl phosphatidyl choline ($C_{16:0}$, DPPC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

Preparation of liposomes containing retinol

Liposomes containing retinol were prepared by the dehydration/rehydration method (4) with slight modification. A 100 mg quantity of DMPC or DPPC, 1.0 mg of retinol, and 15 mL of chloroform/methanol (2:1) were put into a 50 mL round-bottomed flask. 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 15 mL of 10 mM glycine buffer (pH 9.0, containing 0.115 M NaCl) and 0.5 g of glass beads were added to assist hydration of the lipids. The solution was then mixed on the rotary evaporator (without vacuum) to hydrate the lipids to form multilamellar vesicles (MLVs). The solution was centrifuged for 1 hr at 80,000×g (Hitachi Preparative Ultracentrifuge, SCP 55H, Hitachi Koki Co. Ltd., Japan), the supernatant removed, and the pellet was washed with 15 mL of 10 mM potassium phosphate buffer (pH 7.0) and centrifuged again for 1 hr at 80,000×g. The supernatant was again removed and the liposome pellet containing retinol diluted with 15 mL of 10 mM potassium phosphate buffer (pH 7.0) (4).

Particle size measurement

Mean size of the liposomes in the aqueous dispersions was measured with a particle size analyzer (LS230 Small Volume Module, Coulter Co., USA).

Incorporation efficiency of retinol into liposomes

Incorporation efficiency of retinol was calculated for liposome suspension preparation. Unincorporated retinol was separated by centrifugation at 80,000×g for 1 hr at 4°C. Retinol in supernatant and in the suspension was determined by a colorimetric assay (14). The incorporation efficiency was calculated and expressed at the percentage of retinol incorporated into liposomes.

Analytical methods

Retinol in liposomes was analyzed using a colorimetric assay (14). In summary, 0.15 mL of liposome solution 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 4,220×g. A 0.1 mL aliquot of the organic solvent layer was then transferred to the test tube and 1 mL of a 20% $SbCl_3$ solution was added and the absorbance at 620 nm was measured immediately. Concentration of retinol was determined by comparison of the sample absorbance with a standard curve prepared using pure retinol.

Stability testing of liposomes during storage

Two hundred μL aliquots of the retinol solutions containing 0.5 mg/mL retinol were placed in 1.5 mL glass vials and saturated with oxygen against the atmosphere for two hours in the dark. Vials were wrapped with aluminum foil and vials stored at 15°C, 30°C, and 50°C.

RESULTS AND DISCUSSION

Incorporation efficiency of retinol and particle sizes into liposomes

Two kinds of phospholipid (DMPC, $C_{14:0}$, T_c=22°C; and DPPC, $C_{16:0}$, T_c=42°C) which differ in chain length and phase transition temperature were used to prepare liposomes. Retinol was incorporated into multilamellar liposomes that were composed of DMPC or DPPC at a ratio of 0.01 retinol to 1 phospholipid (wt/wt) at pH 7.0.

The incorporation efficiency of retinol into liposomes of DMPC or DPPC was 99.89±0.08% or 99.97±0.03%, respectively, when retinol was added to lipid at a ratio of 0.01 (wt/wt) retinol to phospholipid (Table 1). In our previous study (4), incorporation efficiency of retinol into liposome composed of soybean phosphatidyl choline was 98.58±0.61% under the same conditions. Though there were some differences in chain length between DPPC ($C_{16:0}$) and DMPC ($C_{14:0}$), and in the saturation degree of between DPPC and DMPC (saturated acyl chain) and soybean phosphatidyl choline (high portion of unsaturated acyl chains), it is difficult to find significant differences in the incorporation of retinol into types of liposomes. However, as shown in Fig. 1, the mean size of liposomes composed of DMPC and DPPC were 7.41 μm and 94.03 μm, respectively. Liposome size

Table 1. Incorporation efficiency of retinol into liposomes prepared with DMPC or DPPC[1)]

Phospholipid	Incorporation efficiency (%)[2)]
DMPC	99.97±0.03
DPPC	99.89±0.08

[1)]DMPC and DPPC mean L-α-dimyristoyl phosphatidyl choline and D,L-α-dipalmitoyl phosphatidyl choline, respectively.
[2)]Mean±standard deviation of triplicate measurements.

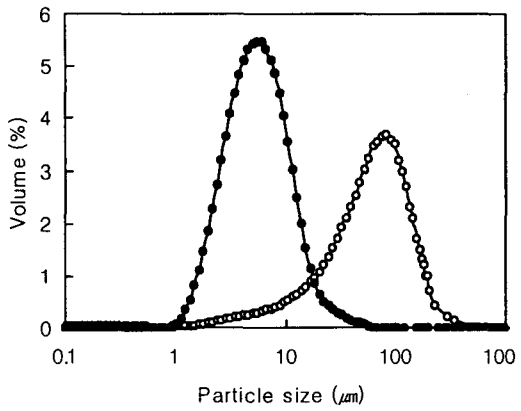

Fig. 1. Size distribution of retinol incorporated in liposome. Liposomes composed of DMPC (●) or DPPC (○) were prepared at a ratio of 0.01 g retinol to 1 g phospholipid and the mixture was hydrated at pH 7.0. DMPC and DPPC mean L- α -dimyristoyl phosphatidyl choline and D,L- α -dipalmitoyl phosphatidyl choline, respectively.

increased with increasing chain length, suggesting that the chain length may affect the intrinsic curvature of the sphere (15). The longer chain of the DPPC increased the mean size of liposomes by more than 10 times than DMPC.

Large unilamellar vesicles (LUVs) can entrap much more aqueous phase than small unilamellar vesicles (SUVs). This would be expected since the surface area increases greatly as particle radius decreases. This means that for small vesicles, a larger proportion of the liposome must be devoted to surface cover (phospholipid) than would be required for large particles. In MLV systems, incorporation efficiency of active materials is dependent upon the amount of lipid in the vesicle and is independent of size (16). Upon hydration, the lipids are said to swell, and peel off the support in sheets, generally to form MLV. In MLVs, the aqueous volume enclosed within the lipid membrane is usually 5 ~ 10% of the total volume used for swelling (5). The MLV preparation method, therefore, very wasteful of water soluble compounds to be entrapped, although the absolute yield of material may be satisfactory for practical purposes. However, lipid soluble compounds can be incorporated into the bilayer phase of the liposomes (17). The size range is a compromise between incorporation efficiency of liposomes (increase with increasing size), liposome stability (decreases with increasing size) and ability to extravasate (decrease with increasing size) (18). In this study, though the size of liposome of DPPC was greater than that of DMPC, the amount of incorporated retinol into liposome was almost equal.

Stability of incorporated retinol into liposome

Retinol solutions at 0.5 mg/mL were prepared in pH 7.0 phosphate buffer. The solutions containing retinol in MLV liposomes were prepared from DMPC or DPPC. These solutions were stored at three different temperatures (15°C, 30°C, and 50°C). The time course of retinol degradation was monitored for ten days and the results are plotted as % retinol remaining versus time (Fig. 2).

The stability of retinol in DMPC liposome was better maintained than in DPPC liposome regardless with temperature. At 15°C, where both DMPC and DPPC are in tightly ordered gel or solid phase, retinol in DMPC liposomes degraded very slowly during storage in the dark with over 94% remaining after ten days (Fig. 2(A)). Retinol in DPPC liposomes degraded faster than in retinol with over 13% remaining after ten days. Free retinol, in our previous report (4), degraded rapidly under the same storage conditions, with only about 10% remaining after one day of storage. After storage at 30°C, which is higher than the T_c of DMPC but below the T_c of DPPC, the protection effect was increased in DPPC liposome while decreased slightly in DMPC liposome (Fig. 2(B)) compared to storage at 4°C. After ten days

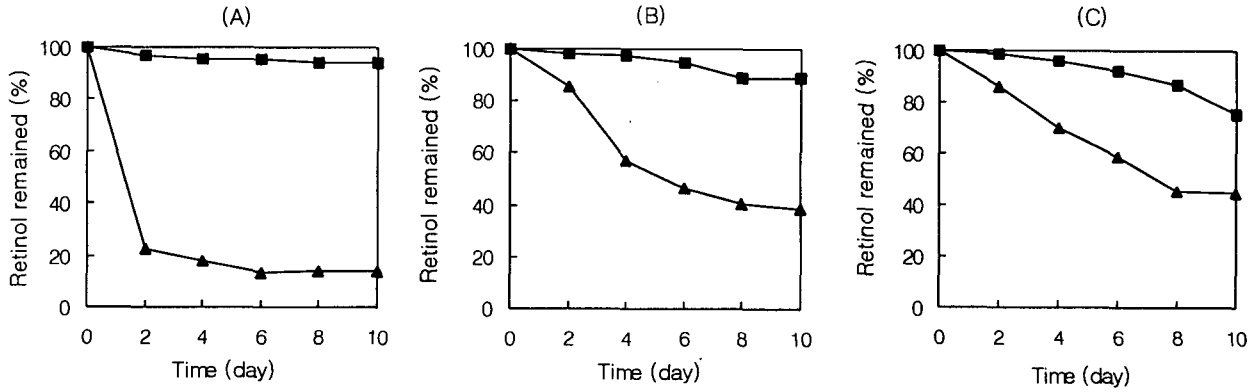

Fig. 2. Stability of retinol in 10 mM phosphate buffer (pH 7.0); the percent retinol remaining plotted as a function of storage time. Buffers containing retinol in multilamellar liposomes were stored at (A) 15°C, (B) 30°C, and (C) 50°C. Liposomes were prepared with DMPC (■) or DPPC (▲). DMPC and DPPC represent L- α -dimyristoyl phosphatidyl choline and D,L- α -dipalmitoyl phosphatidyl choline, respectively.

of storage at 30°C, the retinol remaining in DMPC and DPPC liposome was 88.5% and 38.3%, respectively. Incorporation into liposomes also decreased the rate of retinol degradation at 50°C, which is above the Tc of both DMPC and DPPC (Fig. 2(C)); after one day of storage, free retinol in solution was completely degraded under the same conditions (4) while only 1.42% or 13.93% of the retinol in DMPC or DPPC liposomes was degraded, respectively.

In the case of retinol in soybean phosphatidyl choline MLVs, the protective effect of liposomes was greater at low temperatures than at high temperatures (4). The multilamellar liposomes made from soybean phosphatidyl choline are primarily in the solid gel phase at low temperature (T_c=26.5°C) (19). These results, when rationalized in terms of the effect of temperature on the physical state and structure of the liposome, suggest that solid, gel phase lipids provide the greatest protection against retinol degradation. However, in the present results, retinol incorporation into DMPC liposomes decreased the stability while that in DPPC liposome increased as increasing temperature, although the overall protection effect of liposome on the incorporated retinol was greater in DMPC liposomes than in DPPC liposomes. This means temperature is not the only factor in the structure of the liposomal bilayer. Detailed studies of the effect of temperature on retinol degradation in defined lipid bilayer systems with specific transition temperatures are still needed.

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