Preparation of liposomes encapsulating *Z*-isomerized β-carotene using supercritical carbon dioxide with ultrasonication

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ABSTRACT

Liposomes loading β-carotene were prepared utilizing supercritical carbon dioxide (SC-CO_2) as an alternative to organic solvent. Ultrasonication was supplied which is expected to help liposomal formation. Three β-carotene samples containing different *Z*-isomer content (2.4%, 37%, and 87% of total β-carotene) were prepared by thermal *Z*-isomerization and encapsulated into liposomes. The size, structure and properties of the liposomes were characterized by transmission electron microscopy, UV-Visible spectrophotometry, high-performance liquid chromatography and the dynamic light scattering technique. The particle size range of liposomes was from 90 to 150 nm, and multilamellar vesicles were obtained. β-carotene was incorporated to the lipid bilayer system and dispersed in water. Total amount of encapsulated β-carotene increased by isomerization. Liposomes encapsulating β-carotene ranged in size from 50 to 100 nm, and zeta potential described the high stability of liposomal suspension.

INTRODUCTION

Liposomes are spherical vesicles composed of one or more phospholipid bilayers surrounding discrete aqueous compartments. These vesicles can encapsulate hydrophilic drugs in their aqueous phase and hydrophobic drugs within the bilayer. Liposomes are often classified based on their size and number of bilayers: small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs) [1]. SUVs show a diameter of 20 to approximately 100 nm, and LUVs range in size from 100 to 1000 nm [2]. They are often used as drug carriers in the pharmaceutical, cosmetic and food industry [3]. Conventional methods for liposome preparation usually involve several drawbacks, such as use of organic solvent, multi-stage processes, high energy cost, and

poor stability [4]. In this research supercritical carbon dioxide (SC-CO_2) was utilized as an alternative to organic solvent as it is nontoxic to environment. Besides, the SC-CO_2 phase is gas at normal atmospheric pressure and temperature, and it results in easy separation of CO_2 from products. Furthermore, the low critical temperature (31^{o}C) enables a mild operation temperature which is ideal for processing and encapsulation of thermolabile compounds.

β-carotene, a natural pigment, is composed of eight isoprene units and found in vegetables and fruits such as carrots, pumpkins, and sweet potatoes [5]. Multiple health-promoting properties have been ascribed to β-carotene, assuring an adequate intake is essential for human beings [6]. Once β-carotene is taken into human body, it will be converted into vitamin A which is crucial for normal growth and development. Besides this function, β-carotene also has an antioxidant activity that helps remove free radicals which can cause tissue damage, potentially speeding aging and raising risks of some cancers and other diseases [7]. However, absorption of β-carotene in humans is low because of its hydrophobicity considering their crystalline forms are poorly bioavailable [8]. Besides, β-carotene is sensitive to heat, light and oxygen which further limits its applications in food, nutraceutical and pharmaceutical products [9]. Encapsulation enables β-carotene to disperse in water and prevent the degradation with the barrier function of membranes. Whereas natural β-carotene occurs mainly in their thermodynamically more stable all-*E* configuration, the *Z*-isomers are only present in minor amounts. *Z*-isomerization is induced by heat, and changes physical properties of β-carotene from the crystal state to an amorphous state causing improvement in solubility since *Z*-isomers have relatively higher solubility than the all-*E* isomer [10][11]. Thus, thermal *Z*-isomerization treatment is expected to enhance entrapment amount of β-carotene into liposomes.

MATERIALS AND METHODS

Reagents

Hydrogenated soy phosphatidylcholine (S-10 PLUS) was obtained from Nikko Chemicals Co., Ltd. (Tokyo, Japan), and β-carotene was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All-*E*-β-carotene represented over 90.0% of total β-carotene contained in the product.

Thermal isomerization of β-carotene

β-Carotene containing different *Z*-isomer contents for investigating solubility and physical properties was prepared from β-carotene reference material by thermal *Z*-

isomerization. β-carotene was dissolved in 100 ml of CH_2Cl_2 in a pressure-resistant stainless steel vessel (TVS-1; Taiatsu Techno Corp., Saitama, Japan) [12]. The headspace was purged with nitrogen gas, and the vessel was immediately closed tightly to minimize oxygen exposure, then heated by a water bath of 80°C for 3 hours. After evaporating CH_2Cl_2, CH_3OH was added to dissolve *Z*-isomers preferentially. Then, two different *Z*-isomer contents with or without filtration after the above were obtained.

Preparation of liposomes

A 60 mg of hydrogenated soy phosphatidylcholine and 40 mg of β-carotene were sealed with 60 ml of water in a pressure-resistant cell (inner diameter 20.0 mm, length 250 mm). The cell was places in a water bath of 50°C, and CO_2 was introduced to achieve 10 MP. After approximately 60 min with ultrasonic irradiation (600 W input power, 45 kHz), the pressure was released to obtain the liposomal dispersion.

Particle size and zeta potential

The particle size and zeta potential were determined by Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Worcestershire, UK) using the dynamic light scattering (DLS) technique. Samples were diluted 10 times by water and measured at 25°C with a scattering angle of 173°.

Particle morphology

The surface and lamellarity of liposomes were observed by a transmission electron microscopy (JEM-2100Plus, JEOL Ltd, Tokyo, Japan) at 200 kV. A 2% solution of phosphotungstic acid was made up for negative staining for high resolution electron microscopy of liposomes [13]. Hydrophilic treatment was conducted with AC 100 V and 5 A for 10 seconds on a surface of a carbon-coated copper grid (Micro film NP-C15, Okenshoji Co., Ltd., Tokyo, Japan) by hydrophilic treatment system (DII-29020HD, JEOL Ltd., Tokyo, Japan). A drop of liposome suspension, phosphotungstic acid and water were placed on a parafilm floating on water of 80°C for 5 mins, and the grid was dipped in the liposome suspension for 5 seconds, then the moisture was absorbed with filter papers. After conducting the same treatment with phosphotungstic acid and water in the order which liposome suspension had undergone, the grid was placed in a desiccator for 1 day to dry up, and the samples were examined with TEM.

Absorption spectrum of liposomes loading β-carotene

Absorbance of liposomes loading β-carotene was measured by a UV-Visible

Spectrophotometer (UV-Vis V550, JASCO Corporation, Tokyo, Japan) in the wavelength range of 200-800 nm.

β-carotene extraction

A 1 ml sample was extracted with a mixture of ethanol (3 ml) and n-hexane (4ml) [14]. After the mixture was well shaken, the hexane phase was transferred to a vessel. The extraction was repeated four more times, and the hexane phases were combined in the vessel. After evaporating hexane under reduced pressure at 40ºC, acetone was added to dissolve the extracted β-carotene, and absorbance was measured by high-performance liquid chromatography (HPLC).

Chromatographic Analysis

Reverse-phase HPLC analysis was conducted with a C_{30} column (C30 carotenoid, YMC Co., Ltd., Kyoto, Japan) at 40°C [15][16]. Separation of β-carotene isomers was carried out at a flow rate of 1.0 ml/min using binary mobile phases of MTBE-methanol (11:89, v/v) for 60 mins. Certain chromatographic peaks are identified by comparison to the previous report (Emenhiser *et al*., 1995) on C_{30} columns and UV-visible absorption spectra. The quantification of β-carotene isomers was performed by peak area integration at 456 nm using calibration data gained with an all-*E* β-carotene standard. β-carotene isomer peaks were identified based on HPLC retention time, visible spectral data and the relative intensities of the *Z*-peak. The HPLC system consisted of a controller (LC-NetII/ADC, JASCO Co., Tokyo, Japan), UV/Vis detector (UV-2075Plus, JASCO), pump (PU-2080Plus, JASCO), degasser (DG-980-50, JASCO) and column heater (U-620, Sugai Chemie, Inc., Wakayama, Japan).

RESULTS

Morphology of liposomes

The TEM image of liposomes prepared without β-carotene samples are shown in Figure 1. The liposomes were prepared at 60ºC, 10 MPa, and 45 kHz. The images displayed spherical liposomes with a size range from 90 to 150 nm. The liposomes exhibited MLVs.

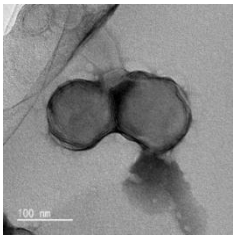

Figure 1. TEM image of morphology of liposomes

Effect of isomerization ratio on absorption spectrums of liposomes loading β-carotene

UV-Vis absorption spectrums of liposomes loading β-carotene are shown in Figures 2, 3 and 4. Figure 2 shows the increase in absorbance in the whole region of 200-800 nm derived from liposomes which have their absorbance peak around 200 nm [17]. However, neither *trans* peak or *cis* peak was observed. Figures 3 and 4 show the absorption of light in the 440-500 nm region for all-*trans* β-carotene and the peak at 340 nm for *cis*-β-carotene [18]. These results indicated β-carotene was incorporated to the lipid bilayer system of liposomes and dispersed in water.

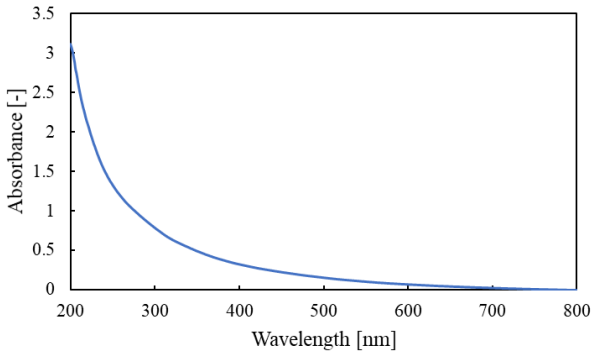

Figure 2. Absorption spectrum of liposomes loading *Z*-isomers 2.4%

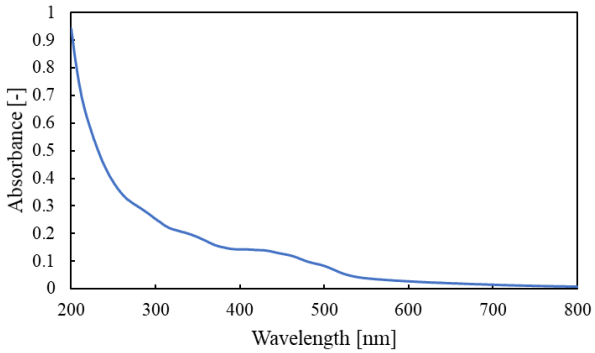

Figure 3. Absorption spectrum of liposomes loading *Z*-isomers 37%

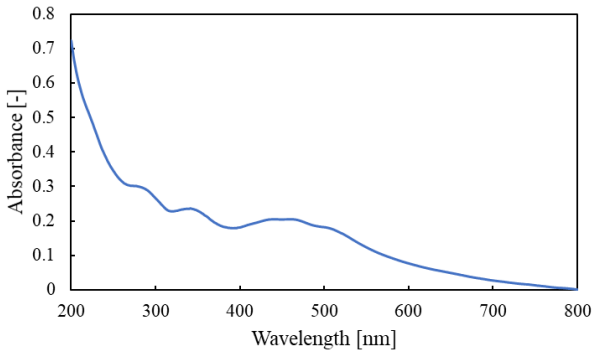

Figure 4. Absorption spectrum of liposomes loading *Z*-isomers 87%

Effect of isomerization ratio on amount of β-carotene encapsulated in liposomes

Total amount of β-carotene encapsulated in liposomes is shown in Figure 5. Along with the increase of isomerization ratio, encapsulated amount of β-carotene increased. *Z*-isomers have a higher solubility in SC-CO_2 than the all-*E* isomer, and amount of β-carotene incorporated to lipid bilayers increased.

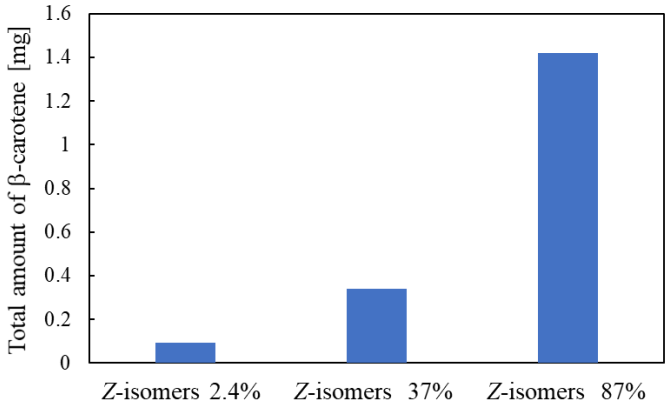

Figure 5. Total amount of β-carotene encapsulated in liposomes

Particle size and zeta potential of liposomes

Particle size and zeta potential of liposomes prepared without β-carotene sample and liposomes loading β-carotene is shown in Figure 6. The size ranged in 50 to 100 nm. One of the factors affecting the diameter of liposomes using this method may be aggregation. Amount of encapsulated β-carotene is considered as another. Furthermore, strong Van der Waals interactions of all-*E*-isomers would have given the change in diameter causing the loss of fluidity of bilayers.

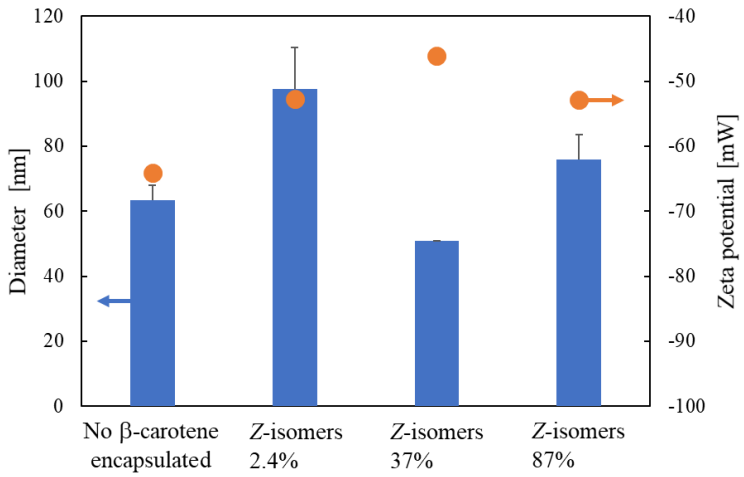

Figure 6. Particle size and zeta potential of liposomes

CONCLUSION

Liposomes loading β-carotene were prepared using SC-CO_2 with ultrasonication and characterized. Using this method, the particle size ranged from 90 to 150 nm, and MLVs were obtained. β-carotene was dispersed in water by encapsulation. Total amount of encapsulated β-carotene increased by isomerization treatment. Liposomes encapsulating β-carotene ranged in size from 50 to 100 nm, and zeta potential described the high stability of liposomal suspension.

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