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Liposomes as Vehicles for Lutein: Preparation, Stability, Liposomal Membrane Dynamics, and Structure

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ABSTRACT: Lutein was loaded into liposomes, and their stability against environmental stress was investigated. Subsequently, these findings were correlated with the interactions between lutein and lipid bilayer. Results showed that the liposomes with loaded lutein at concentrations of 1 and 2% remained stable during preparation, heating, storage, and surfactant dissolution. However, with further increase in the loading concentration to 5 and 10%, the stabilization role of lutein on membrane was not pronounced or even opposite. Membrane fluidity demonstrated that at 1 and 2%, lutein displayed less fluidizing properties both in the headgroup region and in the hydrophobic core of the liposome, whereas this effect was not significant at 5 and 10%. Raman spectra demonstrated that lutein incorporation greatly affected the lateral packing order between acyl chains and longitudinal packing order of lipid acyl chains. These results may guide the potential application of liposomes as carriers for lutein in nutraceuticals and functional foods.

KEYWORDS: *lutein, liposomes, membrane fluidity, Raman spectra, functional food*

INTRODUCTION

Lutein is a prevalent natural carotenoid, found in several vegetables. Among hundreds of carotenoids found in nature, lutein is 1 of 20 carotenoids in the human body. Human are unable to synthesize lutein in the body and, therefore, its presence in human tissues is entirely attributable to dietary intake. Serum levels of lutein have been shown to be inversely related to the risk of ocular diseases, such as macular degeneration and cataract formation.[1,2] Additionally, lutein plays a key role in the prevention of cardiovascular disease,[3] stroke,[4] and lung cancer.[5] The mechanisms by which lutein exerts its health benefits are probably due to the antioxidant activities of its electron-rich conjugated system both by quenching singlet oxygen[6] and by scavenging radicals to terminate chain reactions.[7] Therefore, lutein has become one of the most popular nutritional supplements in the food and pharmaceutical industries.[8] However, its application is limited by its instability toward light, oxygen, and temperature due to the eight conjugated double bonds in its structure. For these reasons, it must be preserved from pro-oxidant elements, which could affect their chemical integrity and decrease their physiological benefits.

Encapsulation, either nano- or microencapsulation, can provide a physicochemical barrier against pro-oxidant elements, improve biological efficiencies, control active components delivery, and prevent side effect apparition.[9] All encapsulation processes should lead to particles with a high encapsulation rate, good polydispersity index (PDI), and long shelf life.[9] Several conventional delivery systems have been applied to improve the stability of lutein, such as nanoparticles,[10] nanoemulsion delivery systems,[11] and liposomes.[12] Among these carrier systems, liposomes have long been received much attention because of their biocompatibility, sustained release potential, and appealing ability to carry both hydrophobic and hydrophilic compounds. They are colloidal systems where phospholipids were dispersed in aqueous solution followed by forming the bilayer membrane of particles through self-assembling. Besides, liposomes at the nanoscale have special properties regarding the penetration and in vivo performance of passive targeting.[13]

However, liposomes are thermodynamically unstable, the particles are easy to occur in aggregation, fusion, phospholipid hydrolysis, and oxidation during storage.[14] It has been shown that the stability of liposomes is sensitive to several technical parameters, especially the liposomal formulations. Varying the formulation parameters can potentially affect the encapsulation quality and stability. Therefore, the optimization of formulation represents a critical step in the development of liposomes before encapsulation of core materials. Previous experiments in our laboratory reported that the addition of cholesterol and Tween 80 can increase the rigidity strength of the lipid membrane and steric stability. Liposomes composed of egg yolk phospholipid, cholesterol, and Tween 80 at a certain fixed mass ratio had relatively strong physical and chemical stability during preparation and storage.[15,16] Additionally, the ethanol injection method to prepare liposomes is simple to scale up and has also found applications in large-scale production methods.[17] Taking these into consideration, it is acceptable to select this formulation to prepare liposomes for encapsulating lutein, except the addition of cholesterol, which may be potentially adverse to human health.

Besides the importance of optimization formulation, we believe that a more crucial aspect is to ascertain the interactions of lutein with lipid bilayer, which can provide the rationale for the liposomes technology for lutein encapsulation. For the past

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three decades, their interactions have been intensely studied to confirm the physiological role of lutein in biological membrane. In general, lutein can orient vertically to the membrane plane through hydrogen bonding between their polar end groups and the membrane's polar region (as depicted in Figure 1). This

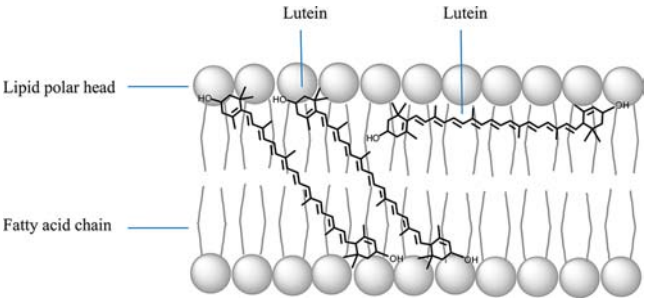

Figure 1. Schematic representation of the main patterns of localization and orientation of lutein molecules in the EYPC membrane.

orientation strongly influences the effects of lutein on the membrane properties, such as thermal parameters, ordering effect, and permeability of the phospholipid bilayer, by means of electron paramagnetic resonance,[18] X-ray diffraction,[19] anisotropy,[20] differential scanning calorimetry,[21] and nuclear magnetic resonance.[22] The most essential finding was the rigidifying effect of lutein on the modulation of the physical properties of membrane and, therefore, it was proposed that lutein acted as a "molecular rivet" to the membrane.[23] Nevertheless, there were some arguments about the modulating effects of lutein as well as its location and interactions with liposomal bilayers. Besides vertical fashion, a certain fraction of lutein has been shown to adopt an orientation in which the hydroxyl group on the ring is horizontal to the membrane plane (Figure 1). This molecular organization may be interpreted in terms of the terminal hydroxyl-containing ring (ε-ring) with relative rotational freedom in structure.[24] As a result, lutein can produce a disrupting effect on membrane structure as apolar carotenoids do.[25] These discrepancies are probably caused by the different experimental conditions such as the lipid used, preparation method of liposomes, and lutein concentration. On the other hand, most of the suggestions aforementioned were obtained from experiments on simple model membrane system. To our knowledge, there is little consideration regarding the interaction and organization of lutein in liposomes composed of mixed lipids including native phospholipid (egg yolk phosphatidylcholine, EYPC) and nonionic surfactant Tween 80.

Herein, a systematic study has been performed to investigate the physical and chemical stability of lutein-loaded liposomes during preparation, storage, heat treatment, and surfactant dissolution. Subsequently, these findings were correlated with the effects of lutein on the membrane dynamic and structural properties. Dynamic light scatter (DLS), zeta (ζ) potential, and fluorescence microviscosity were applied to characterize the physical properties and stability of liposomes. The fluorescence probes, including 1-anilinonaphthalene-8-sulfonate (ANS), 1,6-diphenyl-1,3,5-hexatriene (DPH), and trimethylammonium–DPH (TMA–DPH), were used to evaluate the effects of lutein incorporation on the membrane fluidity, both in the headgroup region and in the hydrophobic core of the lipid. Raman spectroscopy was also carried out to detect the structural changes in the environment of lipid hydrocarbon chains by lutein incorporation. The relationship between lutein organizations inside lipid membrane (localization and orientation) and their effects on liposome stability are discussed.

MATERIALS AND METHODS

Materials. Egg yolk phosphatidylcholine (EYPC) was purchased from Chemical Reagent Plant of East China Normal University (Shanghai, China). Polyoxyethylene sorbitan monooleate (Tween 80) was purchased from China Medicine (Group) Shanghai Chemical Reagent Co. (Shanghai, China). Lutein (98% pure) was purchased from Zhejiang Medicine Co., Ltd. (Zhejiang, China). The fluorescent probe 1-anilinonaphthalene-8-sulfonate (ANS, 98% purity), 1,6-diphenyl-1,3,5-hexatriene (DPH, 98% purity), and trimethylammonium-DPH (TMA-DPH, 98% purity) were purchased from Sigma Chemical Co. (St. Louis MO, USA). All other reagents were of analytical grade.

Preparation of Lutein-Loaded Liposomes. Lutein-loaded liposomes were prepared according to the ethanol injection method:[16] lutein of different weight concentrations was dissolved in 12 mL of warm ethanol at 55 °C together with 6 g of EYPC and 4.32 g of Tween 80. The ethanol solution was rapidly injected using a syringe as a pump into 120 mL of hydration media (0.01 M phosphate buffer solution, 150 mM NaCl, PBS, pH 7.4) at 55 °C in a water bath with a magnetic stirrer. The aqueous phase immediately became milky as a result of liposome formation. The liposomal system was agitated for 30 min and then transferred to a round-bottom flask attached to a rotary evaporator at 55 °C and reduced pressure to remove ethanol. To achieve the nanoliposomes, the liposomal suspension was then submitted to a probing sonication process in an ice bath for 30 min at 240 W with a sequence of 1 s of sonication and 1 s of rest using a sonicator (Sonics & Materials, Inc., 20 kHz). The final sample was sealed in vials (the headspace of the vials was filled witho nitrogen) and kept in the refrigerator (about 4 °C in the dark).

Encapsulation Efficiency and Loading Content. The determination of encapsulation efficiency (EE) was performed by extraction according to our earlier method with a slight modification:[16] aliquots of 0.5 mL of lutein-loaded liposomes and 3 mL of petroleum ether were mixed by vortexing vigorously for 3 min at ambient temperature. The mixed sample was centrifuged at 2000 rpm for 5 min for collecting the supernatant of the centrifuged sample. The above operation was repeated twice. Finally, the collected supernatant was combined together in a tube and diluted to 10 mL with petroleum ether. The free amount of lutein was respectively quantified spectrophotometrically (UV-1600 spectrophotometer; Mapada Instruments Co., Ltd., China) at 440 nm, with petroleum ether as a blank. Each experiment was carried out in triplicate.

The total amount of lutein was expressed relative to the mass of lipids through the lutein initial concentration (IC = $m_{\text{carotenoid}}/m_{\text{lipids}}$, % wt/wt). The amount of lutein loading into the liposomes was calculated as the difference between the total amount used to prepare loading liposomes and that recovered by extraction. The carotenoid encapsulation efficiency (EE, %), loading content (LC, % wt/wt), and retention rate (RR, %) were respectively calculated using the following equations:

$$\text{EE}\ (\%) = \frac{\text{total amount of lutein} - \text{free lutein}}{\text{total amount of lutein}} \times 100$$

$$\text{LC}\ (\%\text{wt/wt}) = \frac{\text{incorporated amount of lutein}}{\text{amount of lipid}} \times 100$$

$$\text{RR}\ (\%) = \frac{\text{incorporated amount of lutein after storage}}{\text{incorporated amount of lutein initially prepared}} \times 100$$

Particle Size and Zeta Potential Analysis. Aliquots of 1 mL of liposomal dispersion were suspended in 10 mL of phosphate buffer to avoid multiple scattering phenomena due to interparticle interaction.[26] The prepared lutein liposomes were taken into polystyrene cuvettes, and z-average diameter (D_z) was recorded by dynamic light scattering (DLS) using a Nano-ZS90 particle size analyzer (Malvern Instruments

Table 1. Loading Content (LC), Encapsulation Efficiency (EE), *z*-Average Diameter Size (D_z), Polydispersity Index (PDI), Zeta (ζ) Potential, and Malondialdehyde (MDA) Amount of Lutein-Loaded Liposomes as a Function of Initial Concentration (IC)[a]

IC (% wt/wt)	LC (% wt/wt)	EE (%)	D_z (nm)	PDI	ζ potential (mV)	MDA (ng/mL)
0			76.47 ± 1.26a	0.17 ± 0.01a	−3.41 ± 0.13a	10.1 ± 0.54a
1	0.92 ± 0.10a	91.98 ± 1.56a	83.53 ± 1.63b	0.23 ± 0.02b	−3.74 ± 0.02b	2.92 ± 0.14b
2	1.82 ± 0.08b	90.86 ± 1.42a	95.65 ± 2.15c	0.25 ± 0.02b	−7.29 ± 0.08c	2.01 ± 0.07c
5	4.36 ± 0.12c	87.23 ± 1.84b	112.50 ± 2.63d	0.27 ± 0.03b	−4.57 ± 0.15d	3.21 ± 0.26b
10	8.20 ± 0.09d	82.64 ± 1.47c	134.82 ± 3.85e	0.32 ± 0.03c	−7.18 ± 0.12c	4.63 ± 0.53d

[a]Results are expressed as mean value ± standard deviation (n = 3). Different letters represent a significant difference ($P < 0.05$).

Ltd., Malvern, UK) with a He/Ne laser (λ = 633 nm) and scattering angle of 90°. Zeta (ζ) potential measurements of the pure liposomes and lutein liposomes were performed by checking the laser Doppler electrophoretic mobilities using the same Nano-ZS90 particle size analyzer. The mobility u was converted into ζ potential utilizing the Smouluchowski relationship: $\zeta = u\eta/\varepsilon$, where η and ε were the viscosity and permittivity of the solution, respectively.[27] Each sample was analyzed at least three times.

Lipid Peroxidation Measurements. Malonaldehyde (MDA), a final product of fatty acid peroxidation, reacted with thiobarbituric acid (TBA) to form a colored complex that had a maximum absorbance at 535 nm. The MDA concentration was detected spectrophotometrically by the TBA reaction following the method of an earlier study.[28] Five milliliters of solution containing TBA (15%, w/v), trichloroacetic acid (0.37%, w/v), and hydrochloric acid (1.8%, v/v) was added to 1 mL of liposomal sample and mixed followed by heating at 100 °C for 30 min. Afterward, the mixture was cooled rapidly with an ice bath, centrifuged for 5 min at 2000 rpm, and filtered. The absorbance of the filtrate was measured by spectrophotometer at 535 nm ($A_{535\ nm}$). The concentration of MDA was calculated using the equation[29]

$$\text{MDA (ng/mL)} = \frac{A_{535\text{nm}} \times 4.15 \times 1000}{W}$$

where 4.15 is the conversion coefficient of milliliter liposomes containing malonaldehyde (μg/mL) and W is the lipid content per volume (mg/mL).

Resistance against Surfactant. The stability of the liposomes against the surfactant Triton X-100 was evaluated with an aqueous solution of 1% Triton X-100.[30] The Triton X-100 solution (1 mL) was added to the liposomes (5 mL) at 5 min intervals at 25 °C. The absorbance was measured by spectrophotometer at 500 nm, and the change of absorbance was recorded as $A/A_{0,\ 500\ nm}$, where A was the absorbance of samples at a predetermined time after Triton-100 addition and A_0 was the initial absorbance of samples.

Microviscosity of Liposomal Membrane. A weighed amount of ANS probe was dissolved in ethanol solution, and the final concentration was 6×10^{-3} mol/L. The DPH stock solution was prepared as follows: amounts of DPH powders were dissolved in tetrahydrofuran, and the final concentration of DPH was adjusted to 2×10^{-3} mol/L. The obtained stock solution was kept at 4 °C in the dark. Aliquots of DPH stock solution (250 μL) were added to the 25 mL volumetric flask with the dilution of buffer solution (0.01 M phosphate buffer solution, 150 mM NaCl, PBS, pH 7.4). The final concentration of DPH was 2×10^{-5} mol/L. The DPH−PBS was prepared freshly and used at room temperature. For the preparation of TMA−DPH stock solution, the TMA−DPH powders were dissolved in tetrahydrofuran/water (1:1, v/v) and the concentration was adjusted to 10^{-2} mol/L. Aliquots of stock solutions (250 μL) were taken to 25 mL volumetric flasks followed by the PBS dilution, namely, TMA−DPH−PBS solution, in which the final TMA−DPH concentration was 10^{-4} mol/L. When the liposomal fluidity was determined as a function of temperature or heating time, aliquots of DPH−PBS solution and liposomal samples were mixed in a 10 mL tube and then heated in the water bath. The heating conditions were set as follows: heat treatment in the range of 25−80 °C, temperature interval of 5 °C, and heating time of 10 min at each test temperature (a); heat treatment at 80 °C in the range of 0−40 min, time interval of 5 min (b).

The microviscosity of liposomal membrane was measured by a fluorescence spectrometer (Hitachi F-7000, Japan) equipped with excitation and emission polarization filters and with a cell controlled at 37 °C. The excitation and emission wavelengths were, respectively, for ANS, λ_{ex} = 337 nm and λ_{em} = 480 nm; for TMA−DPH, λ_{ex} = 365 nm and λ_{em} = 430; and for DPH, λ_{ex} = 358 nm and λ_{em} = 425. The slit widths for both excitation and emission were 5 nm. The fluorescence intensities were measured by the following protocol: the samples were excited first by vertical polarized light, and two polarizing parts of horizontal $I_{0,0}$ and vertical $I_{0,90}$ were obtained. To record the two polarizing parts of horizontal $I_{90,0}$ and vertical $I_{90,90}$, the samples were then excited by horizontal light. The microviscosity (η) was calculated according to the equation[31]

$$\eta = \frac{2P}{0.46 - P}$$

where P is the fluorescence depolarization, $P = (I_{0,0} - GI_{0,90})/(I_{0,0} + GI_{0,90})$, and G is the grating correction coefficient,[32] $G = I_{90,0}/I_{90,90}$.

Raman Spectra Analysis. Raman spectra were recorded using a portable laser Raman spectrometer RamTracer-200-WF-B (OptoTrace Technologies, Inc., USA) equipped with a 785 nm near-infrared frequency stabilized laser source. The laser output power was 334 mV, and collection time was 20 s. Ordinary Raman spectrum was baseline-corrected, and the Raman intensities were measured as peak height.

S_L responds to changes in both the lateral packing of the chains and the *trans/gauche* population ratio. S_T could quantitively measure the number of phospholipid hydrocarbon chain segments in the *all-trans* conformation and gauche rotation, reflecting the degree of the longitudinal order of liposomes.[33] These parameters were normalized so that S = 1 indicated the highest possible order and S = 0, no order (not necessarily the lowest possible).[34] The parameters were calculated from the following equations:[34]

$$S_L = \frac{I_{CH_2(\text{sample})} - 0.7}{1.5} \quad I_{CH_2} = I_{2890}/I_{2850}$$

$$S_T = (I_{1133}/I_{1090})/1.77$$

Statistical Analysis. Data are presented as a mean value with its standard deviation indicated (mean ± SD). The experiments were done in triplicate. Statistical significance ($P < 0.05$) was determined using one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Lutein Loading. The carotenoid LC and EE in liposomal systems were analyzed separately by extraction and centrifugation. The loading content of lutein was very close to the initial concentration (IC), as shown in Table 1, indicating that the added amount of lutein can be effectively incorporated into liposomal membrane within the studied concentration. The EE was as well maintained at a high level; the values exceeded 80% even though the concentration was increased to 10%. The strong inserting ability of lutein into liposomal membrane was due to the fact that lutein can well fit into the membrane architectures in a vertical manner as well as their good lipid solubility.[20] Herein, it was interesting to consider a feature structure in the lutein molecule, a double bond in the e-ring

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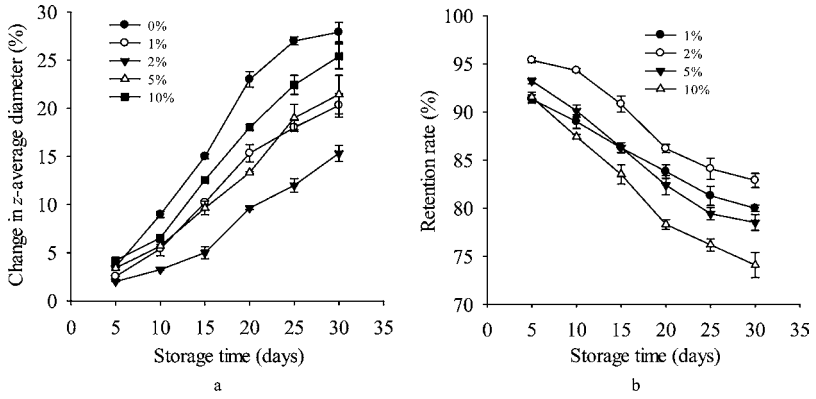

Figure 2. Change in z-average diameter (ΔD_z) (a) and retention rate (RR) (b) of pure liposomes and liposome loading different concentrations of lutein during storage at 4 °C in the dark as a function of time. The weight ratio of EYPC/Tween 80 was 1:0.72. Each point represents the mean value ± standard deviation (n = 3).

located between C4 and C5. It endows the entire ring of lutein with relative rotational freedom around the C6−C7 single bond, suggesting that lutein can adopt an orientation in which the hydroxyl group on the ring is either vertical or horizontal to the membrane plane.[24] The equilibrium between both locations was partially subjected to external forces. When lutein liposomes underwent mechanical forces, such as extrusion stress,[35] the molecules tended to be the parallel location in the headgroup region. After the extrusion, they relaxed into the perpendicular position and the particle increased against its size. Thus, the flexible choice of location may partially explain why lutein displayed high EE.

DLS data represented that the particle size was significantly increased with lutein incorporation (P < 0.05). Only 1% incorporation concentration can increase the particle size by around 10%, as compared with that of lutein-free liposomes. A similar observation was recorded in an earlier paper,[35] showing that EYPC liposomes containing lutein had the largest diameter compared with β-cryptoxanthin and zeaxanthin liposome. It was explained that lutein can span the membrane with their polar end groups extending toward the polar regions of the membrane bilayer, spanning the membranes in a parallel fashion.[36] Additional information by means of linear dichroism proved that the presence of lutein in the hydrophobic core of membrane formed with EYPC increased its thickness from 2.26 to 2.33 nm, due to the fact that the membrane was smaller than the distance between opposite polar groups in lutein.[23] On the other hand, it should be noted that the particle size even increased by 75% after the inclusion of lutein of 10% concentration. The great increase of particle size indicated that besides spanning effect, lutein of high concentration could influence the mechanical properties of EYPC membrane so that it was difficult to disperse the multilamellar liposomes to form unilamellar particles using ultrasonic treatment. The PDI is a measure of dispersion homogeneity, ranging from 0 to 1. Values between 0 and 0.3 indicate a relatively homogeneous dispersion.[37] Although the incorporation of lutein broadened the size distribution of particle, the PDI values remained below or very close to 0.3, suggesting that the dispersion of liposomal suspension was relatively homogeneous and acceptable during preparation.

The zeta (ζ) potential is an important and useful index to predict and control the stability of colloidal suspensions. In general, ζ potential values above +30 mV or below −30 mV are an indication of stability and enhanced uniformity through causing strong repulsion forces among particles to prevent aggregation.[37] Table 1 reveals that the incorporation of lutein can affect the surface charge of liposomal membrane (P < 0.05). Because the lutein molecule was neutral, it was speculated that the differences of charge by lutein incorporation may be due to the effects of lutein on the membrane structural properties. However, the liposomal particles showed a very slight negative charge. This observation implied that the charges of particles were not sufficiently large to repel one another and thus overcome the natural tendency to aggregate. Despite that, the liposomal suspension was considered to be relatively stable owing to the presence of Tween 80, which is often used as an emulsifier in food, and its tolerance is only 0.1−1 (g/100 g) by FAO/WHO. Our earlier experiments have demonstrated that Tween 80 can bring the steric repulsion and increase the resistant ability of particles to aggregation or fusion.[15,16]

On the other hand, EYPC may undergo peroxidation during the preparation procedure due to the presence of polyunsaturated acyl chains in lipid molecules. The final product of peroxidation, MDA, can be detected spectrophotometrically by the TBA reaction. The MDA amount after initial preparation was determined, and results are displayed in Table 1. A small quantity of MDA was generated in liposomal suspension loading lutein in comparison with pure liposomes. The most significant antioxidant effect was observed at the concentration of 2%, followed by 1, 5, and 10%. The inhibition of lipid peroxidation was the consequence of the combination of two mechanisms: lutein degradation by making themselves available for reactions with oxygen instead of lipids and the effects of lutein on the membrane properties. For the latter, lutein can limit molecular oxygen penetration into the lipid bilayer by restricting the molecular motion of lipid.[36]

Storage Stability. Liposomes are thermodynamically unstable; the particles have a high tendency to degrade or aggregate, thus leading to the leakage of entrapped compounds during storage.[14] To evaluate the shelf life of liposomal samples, the retention rate (RR) and change in z-average diameter (ΔD_z) were determined during storage at 4 °C. It was found that pure liposome exhibited a great increase in particle size; its D_z increased further to 97.8 nm after 30 days with ΔD_z values of 27.9% (Figure 2a). In the case of lutein-loaded liposomes, the increase percentages of D_z after 30 days of storage were around 20.3, 15.32, 21.4, and 25.4%, and corresponding lutein concentrations were 1, 2, 5, and 10%, respectively. This result suggested that lutein incorporation suppressed particle

aggregation in a concentration-dependent manner. At the same time, the encapsulated lutein underwent different degrees of leakage (Figure 2b). After 30 days of storage, the greatest retention rate was observed at 2% with 82.9% RR value, followed by 80.1% (1%), 78.5% (5%), and 74.1% (10%), respectively. It appeared that the lipid bilayer can strongly retain the lutein of low concentrations, while displaying a weak retaining capacity when the concentration was increased above 5%. This trend was well in agreement with the variation of particle size.

The PDI, ζ potential, and MDA values were determined after 30 days of storage, and results are represented in Table 2. The

Table 2. Polydispersity Index (PDI), Zeta (ζ) Potential, and Malondialdehyde (MDA) Amount of Lutein-Loaded Liposomes after 30 Days of Storage at 4 °C in the Dark[a]

IC (% wt/wt)	PDI	ζ potential (mV)	MDA (ng/mL)
0	0.35 ± 0.01a	−3.01 ± 0.13a	16.1 ± 1.43a
1	0.24 ± 0.03bc	−4.26 ± 0.02b	4.47 ± 0.32b
2	0.23 ± 0.02b	−7.08 ± 0.12c	3.61 ± 0.16b
5	0.28 ± 0.03c	−5.21 ± 0.21d	4.61 ± 0.28b
10	0.34 ± 0.02a	−7.62 ± 0.37e	6.18 ± 1.02c

[a]Results are expressed as mean value ± standard deviation (n = 3). Different letters represent a significant difference (P < 0.05).

liposomal suspension without lutein became nonhomogenous after storage (PDI > 0.3) corresponding to the serious aggregation of particles. In contrast to this, only a slight increase of PDI was observed for lutein-loaded liposomes; the values were below 0.30 except those loading 10% lutein, suggesting a relatively homogeneous system. Besides, storage hardly changed the surface charge of liposomes. It was important to note that after 30 days of storage, the MDA amount generated in lutein-free liposomes increased considerably, whereas it was less apparent for liposomes containing lutein. Liposomes loading 2% lutein had the lowest MDA, whereas those loading 10% lutein, the highest. This result can partially explain the different retaining abilities of lipid bilayer to lutein at different loading concentrations, because lipid oxidation can affect the membrane permeability and consequently the leakage of encapsulated compounds.[38]

Thermal Stability. To investigate the temperature resistance of lipid bilayer loading different concentrations of lutein, the particle size, ζ potential, and membrane fluidity were monitored during the heating period. Figure 3a shows that the z-average diameter (D_z) of pure liposomes increased slightly at low temperature, but significantly from 60 °C, the particle size was increased by 34.13% after heat treatment at 80 °C. It was believed that the energy input by temperature was the main factor to the aggregation and fusion of liposomes. For lutein-loaded liposomes, particle size varied slightly. After heat treatment at 80 °C, the smallest increase percentage of D_z was observed at the concentration of 2% (3.80%), followed by 1% (16.61%), 5% (19.20%), and 10% (22.78%).

ζ potential was referred to the electrical potential at the surface of shear, which is the region at which the fluid becomes mobile and is coincident with or just beyond the outer Stern surface.[39] Thus, the change of ζ potential may be related to the structural change of liposomal membrane. The ζ potential values of each liposome practically unchanged at low temperatures (<60 °C), whereas an increase was observed at higher temperature (Figure 3b). It was further found that a charge inversion of pure liposomes and liposomes loading lutein of 5 and 10% appeared from 60 and 70 °C, respectively. Previous study revealed that the ζ potential of lipid nanoparticle decreased with increasing energy input by light and temperature, resulting from the crystalline reorientation of the lipid.[40] The charge inversion may be also related to the reorientation of lipid molecules. In general, the phosphate group tended to be exposed on the membrane surface due to its strong polarity, and the liposomal particles showed weak negative charge. As the increase of temperature, the mobility of lipid molecules was increased and more positively ammoniums in choline groups may move to the surface of membrane. Thus, the ζ potential slightly increased until charge inversion at high temperature. In contrast to this, liposome loading lutein of 1 and 2% underwent a little variation of surface charge instead of charge inversion, even if the temperature increased to 80 °C. It was speculated that lutein restricted the motion freedom of lipids through the hydrophobic and hydrophilic interactions and inhibited the structural change of lipid bilayer.

Membrane microviscosity was a temperature-dependent factor. A high level of membrane microviscosity (also named rigidity) was supposed to prevent particle aggregation effectively. When temperature increased, microviscosity decreased, leading to the destabilization of the particles.[37] Herein, the fluorescence probe DPH, which can reflect the average packing order of lipid bilayer,[41] was introduced to assess the effects of temperature on the membrane fluidity. Figure 4a

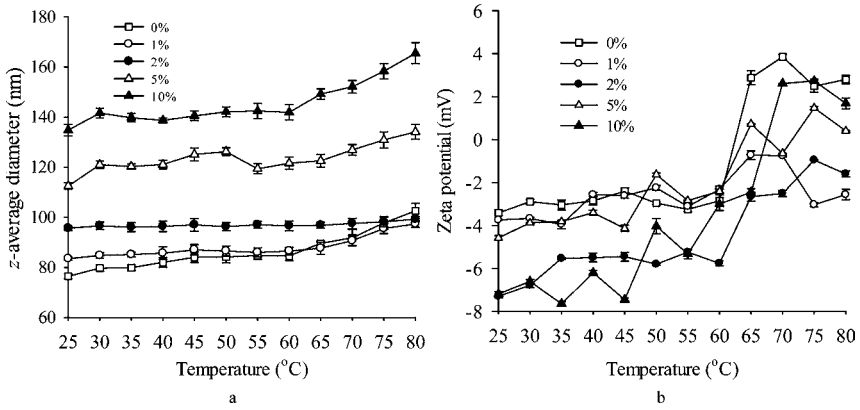

Figure 3. Temperature dependence of z-average diameter (a) and zeta potential (b) in EYPC/Tween 80 (1:0.72, w/w) liposomes loading different concentrations of lutein. Each point represents the mean value ± standard deviation (n = 3).

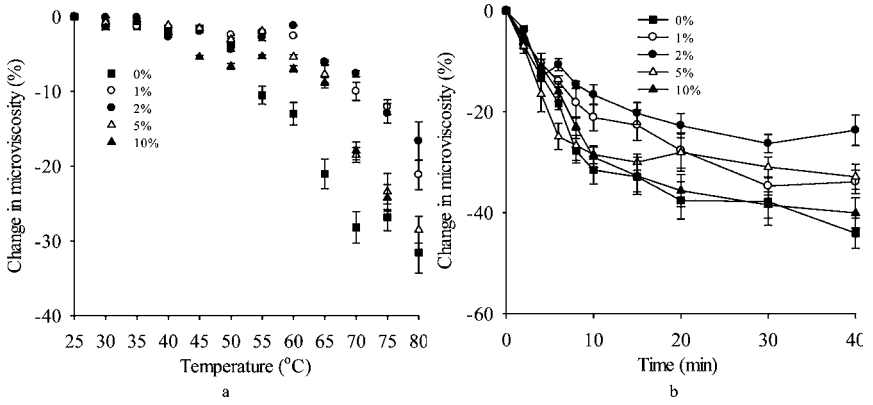

Figure 4. Microviscosity variation in liposomes as a function of temperature (the heating time at each test temperature was 10 min) (a) and as a function of heating time at 80 °C (b). The weight ratio of EYPC/Tween 80 was 1:0.72. Each point represents the mean value ± standard deviation (n = 3).

shows that the membrane fluidity of pure liposomes greatly decreased when the temperature increased above 50 °C. By contrast, lutein-loaded liposomes maintained the membrane fluidity at a high level. After heat treatment at 80 °C, the microviscosity of liposomal membrane decreased by 31.54% (0%), 21.16% (1%), 16.65% (2%), 28.50% (5%), and 29.01% (10%), respectively. On the other hand, Figure 4b shows that when the heating time was prolonged at 80 °C, a fast and significant decrease of microviscosity occurred for each liposome within 10 min (Figure 4b). The decrease percentages of microviscosity after 10 min were 31.54% (0%), 21.16% (1%), 16.65% (2%), 28.52% (5%), and 29.00% (10%), respectively. In combination with the DLS data, it was concluded that the small change in membrane fluidity of lutein-loaded liposomes was directly responsible for the slight aggregation and fusion of particles as well as ζ potential variation during heating.

Resistant Ability against Surfactant. To evaluate the lutein incorporation on the rigidity of lipid membrane, the resistance of liposomal particles against surfactant Triton X-100 was investigated. The turbidity change of liposomal suspension at 500 nm ($A/A_{0,\ 500\ nm}$) indicated the solubilization process of lipid bilayer.[30] It was observed that the transmittance of pure liposomes dropped dramatically; the change of absorbance value even decreased to around 28% at 30 min (Figure 5). It was suggested that plenty of Triton X-100 penetrated into the bilayer and initiated the solubilization process. Whereas the absorbance of lutein liposomes gradually dropped with Triton X-100 addition. Besides, liposome loading 1 and 2% lutein possessed higher durability against X-100 solubizization than those loading 5 and 10%. At 30 min, the highest value of $A/A_{0,\ 500\ nm}$ was around 47% observed for liposomes loading 2% lutein, followed by 45% (5%), 44% (1%), and 38% (10%), respectively. These results further confirmed that lutein played a rigidifying effect on the liposomal membrane in a concentration-dependent manner.

Effects of Lutein Incorporation on Membrane Fluidity. The microviscosity is considered to be inversely proportional to membrane fluidity. A high degree of microviscosity represents a higher structural order or lower membrane fluidity. ANS binds to phospholipids in such a way that the anionic sulfonate group is oriented toward the hydrophilic headgroup of the lipid. It serves as a marker for the molecular movement on the exterior membrane surface.[42] TMA−DPH was used to monitor fluidity of superficial region including the surface and glycerol side chain region of the membrane.[41] Figure 6 shows

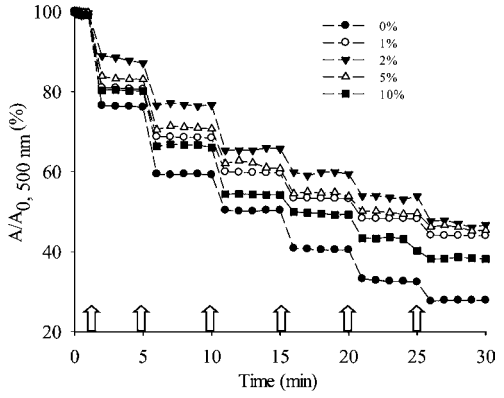

Figure 5. Changes in relative absorbance ($A/A_{0,\ 500\ nm}$) of liposomal suspension by the addition of Triton X-100 at 25 °C to pure liposomes and lutein-loaded liposomes. A_0 was referred to the initial absorbance of liposomal suspension. A was referred to the absorbance of liposomal suspension after the addition of an aqueous solution of 1% Triton X-100. The arrows at the x-axis represent the time (5 min intervals) when Triton X-100 was added to the suspension. The absorbance was measured at 500 nm.

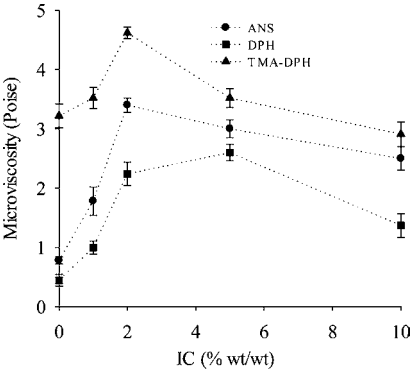

Figure 6. Microviscosity (η) change of EPYC/Tween 80 (1:0.72, w/w) liposomes by increasing the weight concentration of incorporated lutein. Each data point is expressed as the mean value ± standard deviation (n = 3). Dotted lines were added to guide the eye and have no physical meaning.

that the variation of microviscosity for both ANS and TMA−DPH exhibited a similar trend. Incorporation of lutein in a small concentration (1 and 2%) can increase the microviscosity of the environment surrounding both probes. This indicated

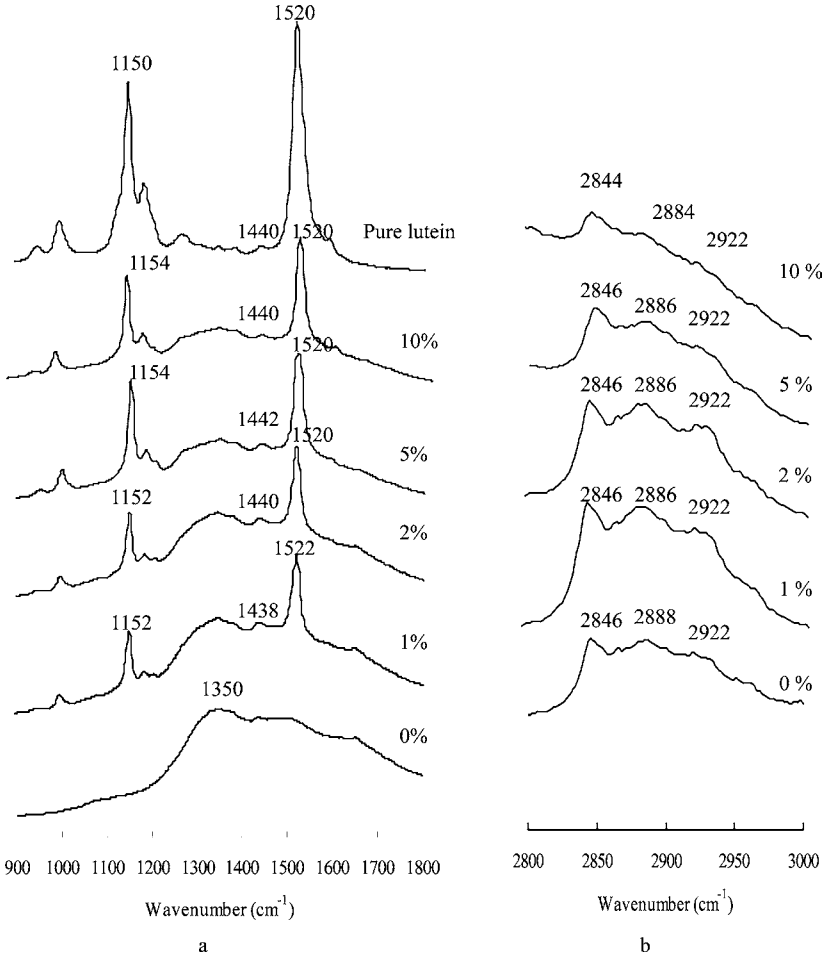

Figure 7. Raman spectra in the range from 900 to 1800 cm^{-1} (a) and from 2800 to 3000 cm^{-1} (b) of pure liposomes and liposomes loading different weight concentrations of lutein: 1, 2, 5, and 10%.

that the incorporation of lutein induced an enhancement of membrane rigidity, both in the surface region and in the glycerol side chain region. However, with further increasing concentration to 5 and 10%, lutein displayed high fluidizing properties at these two regions. DPH is preferentially oriented in the deep regions of the lipid bilayer, and the values reflected the average packing order of lipid bilayer.[41] It was observed that the microviscosity for DPH progressively increased as lutein concentration was increased to 5%, followed by a decrease at higher concentration. Fluorescence measurements demonstrated that at low concentrations (1 and 2%) lutein displayed less fluidizing properties both in the polar headgroup region and in the hydrophobic core of the lipid. It was believed that the restrictions to the motion freedom of lipids, due to the hydrophobic and hydrophilic interactions with different groups of lutein molecules, gave rise to the high membrane microviscosity (low fluidity).[23] At high concentrations (5 and 10%), the fluidity in these regions exhibited a great increase, especially in the membrane surface and glycerol side-chain region.

Effects of Lutein Incorporation on the Structural Properties of Lipid Bilayer. Raman spectroscopy has been proved as a powerful technique to detect changes in the environment of lipid hydrocarbon chains by other molecules incorporation. The change of *trans* and *gauche* conformations by hydrocarbon chains of lipids and that of the order parameters for the longitudinal interaction within chains and for the lateral interaction between chains suggest the character of structural changes of liposomes.[43] Raman spectroscopy has already been used to investigate the interactions of some carotenoids with lipid membrane, including violaxanthin,[44] zeaxanthin,[33,44] and β-carotene.[45] In the present study, the structural changes of EYPC/Tween 80 liposomes by lutein incorporation of different concentrations were investigated by observing the Raman spectra ranging from 900 to 1800 cm^{-1} and from 2800 to 3000 cm^{-1}.

There existed two strong characteristic peaks in Raman spectra of lutein molecules at around 1160 and 1520 cm^{-1}, which were assigned to C—C and C═C stretching modes in the polyene chain respectively. As shown in Figure 7a, the characteristic C—C stretching band of lutein tended to shift to higher wavelengths with increasing lutein concentrations: 1150 cm^{-1} (pure lutein), 1152 cm^{-1} (1%), 1152 cm^{-1} (2%), 1154 cm^{-1} (5%), and 1154 cm^{-1} (10%). Herein, the nonvariation of variable angle vibration in CH_2 near 1444 cm^{-1} was used as an internal standard against which the C═C intensity of the carotenoid was compared. The calculated intensity ratios I_{1152}/I_{1440} and I_{1520}/I_{1442} are displayed in Table 1. Both ratios hardly varied after lutein incorporation of 1 and 2%, whereas they showed significant increase when the concentration was further increased to 5 and 10%. This phenomenon might reveal some changes in acyl chain packing and conformational order, which arose from the incorporation of lutein into the liposomal membrane.

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The C−C stretching mode at 1130 cm^{-1} is associated with chains in the *all-trans* conformation, whereas the feature at 1100 cm^{-1} is derived from hydrocarbon chains containing *gauche* rotations. The more *all-trans* bonds there are, the larger the longitudinal order parameter of liposomes is.[33] For *gauche* rotamers, the result is the opposite. Unfortunately, it was difficult to observe the corresponding bands from the Raman spectra due to the strong C−C characteristic bands of lutein. The calculated parameter S_T is displayed in Table 3, and it was

Table 3. Order Parameters of Liposomes Loading Different Concentrations of Lutein and Variation Percentage As Deduced from Raman Spectra

	IC[a] (% wt/wt)				
	0	1	2	5	10
I_{1152}/I_{1440}		0.925	1.044	1.53	1.489
I_{1520}/I_{1440}		1.525	1.562	1.843	1.950
S_L	0.176	0.191	0.192	0.163	0.154
$(S_L - S_{L,0})/S_{L,0}$		8.523%	9.090%	−7.386%	−12.500%
S_T	0.611	0.660	0.647	0.663	0.638
$(S_T - S_{T,0})/S_{T,0}$		8.020%	5.892%	8.511%	4.419%

[a]EYPC/Tween 80 (1:0.72, w/w) liposomes. The IC was the weight percentage of lutein initially prepared against the lipids (% w/w).

revealed that lutein incorporation increased the longitudinal order of liposomes regardless of the concentration. This observation was probably due to the decreased activation energy of rotational diffusion of fatty acid induced by spatial constraints of lutein.[46]

The strong bands located around 2850 and 2890 cm^{-1} derived from the symmetric ν_{as} (CH_2) and antisymmeteic ν_{as} (CH_2) C−H stretching vibrations of the methylene groups of the acyl chains of lipids, respectively. The Raman spectra of pure liposomes and lutein-loaded liposomes in the region 2800−3000 cm^{-1} are shown in Figure 7b. The bands of the antisysymmeteic CH_2 exhibited a blue shift as the lutein concentration was increased, and the location was at 2888 cm^{-1} (0%), 2886 cm^{-1} (1%), 2886 cm^{-1} (2%), 2886 cm^{-1} (5%), and 2884 cm^{-1} (10%), respectively. It was noted that at 10% concentration, the bands of the antisymmeteic C−H stretching vibration at 2884 cm^{-1} lost considerable intensity, becoming a shoulder on the bands of symmetric C−H stretching vibration. The order parameters S_L reflecting the state of lateral packing of the liposomes are summarized in Table 3. Incorporation of lutein at 1 and 2% led to an increase in S_L indicating an enhancement of lateral packing of lipid molecules. Nevertheless, S_L exhibited a small but not significant decrease with further increasing lutein concentration to 5 and 10%. The change tendency was consistent with the fluorescence microviscosity measurements (Figure 6).

In general, lutein can be anchored in the opposite polar membrane zones, owing to the fact that the polar OH groups are located on the opposite side of the rigid-shaped molecules. Linear dichroism spectroscopy[47] revealed that lutein was oriented at an angle of 67° with respect to the normal axis to the plane formed by the EYPC membrane. The direct consequence of the vertical orientation was the strong rigidifying effects of lutein, as evidenced by nuclear magnetic resonance[48] and electron paramagnetic resonance (EPR) studies.[46] Our fluorescence experiments and Raman spectra also confirmed this effect of lutein when the incorporated concentration was 1 and 2%. Besides, the influence of lutein on the membrane dynamics and structure strongly affected the stability of liposomes. Lutein modified the lipid headgroup region and decreased the fluidity. This can explain why particles maintained integrity and stability during storage and heating. The hydrophobic interactions with rigid rod-like molecules of lutein are the main reason for the structural stability of lipid bilayer. On the other hand, lutein could increase the high penetration barrier of membrane against small molecules (e.g., oxygen) by both decreasing membrane permeability and increasing hydrophobicity in the center of the bilayer.[46,49] This effect was most probably a direct reason for the low level of lipid peroxidation during preparation and storage and their ability against surfactant solubilization. In any case, the lesser fluidity and enhanced order of lipid bilayer, owing to lutein incorporation, led liposomes to be less susceptible to external environmental stress, such as oxygen penetration, high temperature, and surfactant dissolution. In addition, when lutein adopted a vertical manner, increasing more incorporated molecules can induce more significant rigidifying effect on liposomal membrane. That was why liposome loading 2% lutein possessed higher stability than those loading 1% lutein.

Nevertheless, the rigidifying effects were not pronounced or even opposite when lutein was encapsulated at the concentration of 5 and 10%. This may be correlated with the variation of orientation inside liposomal membrane. As mentioned earlier, lutein can orient horizontally to the membrane plane due to the presence of its rotational terminal rings in structure. The horizontal manner led lutein to act more like apolar carotenoids due to the shield of its OH groups from the water interface. X-ray diffraction[25] was used to demonstrate that the horizontal manner of lutein induced a somewhat high perturbation to lipid bilayers formed by palmitoylphosphatidylcholine (POPC). Similarly, this finding may explain the perturbation of lipid packing of liposomes composed of EYPC and Tween 80. It was very likely that when concentration was >2%, a certain fraction of lutein molecules preferentially localized in the headgroup region, parallel to the membrane plane. Furthermore, the population of the horizontal fraction increased with the increase in the concentration of lutein in the lipid bilayer. Such a hypothesis tended to be supported by the stronger modifying effect of lutein on the membrane surface region than hydrophobic region at the concentrations of 5 and 10% (Figure 6). With these points taken into consideration, it was understandable that liposomal membrane became more fluidized and the lateral packing of lipid was perturbed. As a result, the stabilizing effect of lutein on liposomes was not significant. The fluorescence microviscosity and Raman spectra did not show any evidence for dynamics and structure perturbation of liposomes composed of mixed lipids by the incorporation of lutein at 1 and 2%.

Conclusions. In this study, the preparation, characterization, and stability of lutein-loaded liposomes were investigated. The results showed that the incorporation of lutein can maintain the particle integrity and enhance the stability of liposomes during preparation, storage, heating, and surfactant dissolution. These effects were highly dependent on the incorporated concentration. Fluorescence probes and Raman spectra provided direct evidence that the stabilization role of lutein was correlated with its capacity to affect the lipid membrane dynamics and structure. Lutein at concentrations of 1 and 2% preferentially located in the opposite polar membrane zones vertically to the membrane plane. This molecular organization inside the lipid bilayer endowed the liposomal

membrane with high rigidity and order of lipid hydrocarbon chain. However, the lipid bilayer displayed poor stability and weak retaining ability to lutein when the concentration was increased to 5 and 10%. A probable explanation was that a certain fraction of lutein molecules adopted a parallel orientation due to its rotational terminal ε-ring. These findings may guide the potential use of liposomes as vehicles for lutein in nutraceuticals and functional foods.

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Notes

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