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Preparation of 9Z-β-Carotene and 9Z-β-Carotene High-Loaded Nanostructured Lipid Carriers: Characterization and Storage Stability

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ABSTRACT: *Cis* (*Z*)-β-carotenes with 25.3% 9*Z*-β-carotene were prepared for nanostructured lipid carriers (NLCs). The optimal conditions for NLC preparation using an orthogonal experimental method were as follows: the total lipid concentration was 9% (w/v), the surfactant concentration was 1.4% (w/v), the solid to liquid lipid ratio was 3:1 (w/w), and the homogenization pressure was set at 500 bar for three cycles. Under these conditions, the encapsulation efficiency (%) of the NLC was 95.64%, and the total β-carotene in NLCs was 2.9 mg/mL, which was significantly higher than those reported by others. The proportion of total *Z*-β-carotenes was as high as 53.3%, the particle size was 191 ± 6.46 nm, and the polydispersity index was 0.2 ± 0.03. Storage stability results indicated that the β-carotene-loaded NLC stabilizes both 9*Z*-β-carotene and total β-carotene from leakage and degradation during 21 days of storage at pH 3.5−7.5 at low temperatures (4 °C), especially for the more bioactive 9*Z*-β-carotene. The technique with an improved ratio of 9*Z*-β-carotene, loading capacity, water solubility, and bioaccessibility of the β-carotene NLC provides an effective strategy for β-carotene applications in functional foods or beverages and in nutraceutical preparations.

KEYWORDS: *9Z-β-carotene, nanostructured lipid carriers, loading capacity, stability, bioaccessibility*

INTRODUCTION

β-Carotene is one of the most important carotenoids that occurs widely in vegetables (e.g., yellow pepper, carrots, and pumpkins) and fruits (e.g., mangoes) with a deep orange-yellow color.[1,2] As a cyclic carotenoid containing 11 conjugated double bonds, β-carotene exhibits provitamin A and antioxidant activities and many human health benefits including reducing the risk of developing chronic diseases (e.g., cancer, cardiovascular diseases) and age-related degenerative diseases.[3,4] However, applications of β-carotene in food and beverage systems and in pharmaceutical formulations are still challenging because of its high hydrophobicity, chemical instability, and high crystallinity, and consequently its low bioavailability.[5,6]

Like most carotenoids, β-carotene exists predominantly in all-*trans* configuration in unprocessed fruits and vegetables.[7,8] However, higher ratios of 9*Z*-, 13*Z*-, and 15*Z*-β-carotenes (25% of total β-carotene) were present in human tissues.[9] Studies showed that *trans-cis* isomerization not only improved the solubility in solvents of all-*E*-β-carotene, but also boosted its physiological functions such as antioxidant and anticancer activities.[10,11] 9*Z*-β-Carotene showed higher antioxidant activity than all-*E*-β-carotene against external oxidants and other reactive oxygen species in different tests.[12] Even though the antioxidant activities of β-carotene isomers were found similar in a peroxyl radical ($ROO^{\bullet}$) scavenging test,[3] according to the electroaccepting and electrodonating powers, they followed a decreasing order of 9,13′-dicis >13*Z*- ≳ 9*Z*- ≳ 15*Z*- > 7*Z*- > all-*E*- > 11*Z*-β-carotene.[13] Moreover, a diet containing the alga *Dunaliella bardawil* rich in 9*Z*-β-carotene (50%) was found to be more effective than that containing all-*E*-β-carotene in inhibiting atherogenesis in low-density lipoprotein receptor knockout mice[14] and in further inhibiting the progression of established atherosclerosis in old male apoE-deficient mice.[15]

The abovementioned observations indeed point to the fact that *cis* isomers such as 9*Z*-β-carotene could be a more effective functional ingredient than its all-*E*-β-carotene counterpart. A technology that can increase the ratio of *Z*-β-carotene will therefore lead to the development of β-carotene products with enhanced health benefits. Several methods have been developed to obtain *Z*-β-carotene; however, most of these methods are still limited to low conversion efficiency and a long reaction period or require complicated removal of the catalyst after the reaction.[16−18] On the other hand, the pigment extract from *D. bardawil* is shown to contain 38% 9*Z*-β-carotene, and the extract from *D. salina* contains 3.2% 9*Z*-β-carotene and 10% all-*E*-β-carotene.[19] However, the cultivation of *D. bardawil* requires high salinity and intense illumination, and the purification process also costs significantly, making it uneconomical to produce from this natural source. An efficient method to prepare 9*Z*-β-carotene with a high ratio is necessary.

One of the weaknesses of the encapsulation technology for carotenoids is the loading capacity (LC), which is strongly

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affected by the solubility of carotenoids in the oil phase; thus the quantity of the oil phase is a key determinant factor.[20] It has been reported that lipid nanoparticles improve drug absorption and bioavailability because of their nanosize diameter and enhancing effect of lipids.[21,22] Nanostructured lipid carriers (NLCs) have attracted considerable attention as an effective delivery system with sustained-release effects and capability for industrial-scale production.[23] The NLC system is generally from a mixture of solid and liquid lipids and is normally first heated until melted down and then cooled to form a large amount of imperfect crystals and amorphous lipid cores and incorporate more drugs.[24] Schjoerring-Thyssen et al. reported solid lipid nanoparticles (SLNs) loaded with a high concentration of all-*E*-*β*-carotene (37.5%, w/w in the lipid phase) with *Z*-isomers; however, they used a concentration of surfactant as high as 10% of the SLN system and a temperature as high as 165 °C, which are not good for the encapsulation and stability of *β*-carotene inside the SLN product.[25] Moreover, a surfactant with high concentration was used in most studies. Tween 80 was used at 3% (w/v) in the NLC containing *β*-carotene[26] and lycopene,[27] and at as high as 10−40% (w/v) in a milk-fat-based NLC for *β*-carotene.[28] On the other side, Ono et al. developed an NLC containing *Z*-*β*-carotene, while the major *Z*-isomer was a 13*Z*-isomer, a less stable *cis*-isomer than 9*Z*-*β*-carotene. In this system, the *β*-carotene loaded was less than 1 mg/mL, and the NLC was less stable than the all-*E*-isomer.[6]

The objectives of the present study were therefore to establish an effective method to produce 9*Z*-*β*-carotene and to develop an NLC system using *β*-carotene with a high ratio of 9*Z*-isomer to improve the water-solubility, functionality, stability, and bioavailability of a *β*-carotene-containing product with a smaller amount of surfactant. The physicochemical properties, that is, the LC, morphology, particle size, and polydispersity index (PDI), of the 9*Z*-*β*-carotene high-loaded NLC were determined. In addition, the effects of temperature and pH on the retention and stability of both *β*-carotene isomers and the NLC were also investigated during storage. The pH and the homogeneous pressure were chosen according to the processing conditions in the beverage industry. Results of this study will provide fundamental information on the production, stability of *Z*-*β*-carotene, and application of *Z*-*β*-carotene loaded-NLCs in functional foods and/or pharmaceutical products.

MATERIALS AND METHODS

Materials. All-*E*-*β*-carotene (HPLC >96%) was purchased from Sigma-Aldrich (Shanghai, China). Glyceryl monostearate (GMS), Tween 80, citric acid, iodine, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Medium-chain triglycerides (MCTs) were obtained from Shanghai General Pharmaceutical Co., Ltd. (Shanghai, China). HPLC-grade solvents, including acetonitrile, methanol, and methyl *tert*-butyl ether (MTBE), were purchased from TEDIA High Purity Solvents (Ohio, USA). Distilled and deionized water was used for the preparation of solutions. All the other chemicals were of analytical grade.

Rapid Conversion of all-*E*-*β*-Carotene to the 9*Z*-Isomer. The isomerization was performed according to a previous report with slight modifications.[29] All-*E*-*β*-carotene was dissolved in ethyl acetate (1 mg/mL) and heated at 50 °C for 2 h, or heating at 50 °C for 2 h with an I-TiO_2 catalyst or I_2 (5%, I_2/*β*-carotene, w/w). The reaction with I_2 was terminated by adding $Na_2S_2O_3$ solution (1 mL, 1 mol/L) to each vial to wash out the I_2 by vortexing. After reaction, samples were centrifuged, filtered through a 0.22-*μ*m membrane, and analyzed by high-pressure liquid chromatography (HPLC) at 450 nm. The method has been previously studied and confirmed to be repeatable.[30] The isomerization products were reproducible, and the ratio of *Z*-isomers was stable during the reaction.[29] All experiments were performed in triplicate.

Analysis and Identification of *β*-Carotene Isomers. *β*-Carotene isomers in the isomerized product and *β*-carotene-loaded NLCs were separated and analyzed using a polymeric C_{30} column (YMC Carotenoids, 250 mm × 4.6 mm, 2.6 *μ*m, Phenomenex Inc., Torrance, CA, USA) according to a previous report with slight modifications.[31] The column temperature was set at 25 °C. The detection wavelength was 450 nm, the injection volume was 20 *μ*L, and the flowing rate was 1.0 mL/min for a total run time of 30 min. The binary mobile phase consisted of A: 25% methanol mixed with 75% acetonitrile (v/v) and B: 100% MTBE. The solvent gradient was as follows: 0−20 min, 100−50% A; 20−30 min, 50% A. Peaks were detected at 450 nm. The DAD scan range was 250 to 600 nm. Different *β*-carotene isomers were identified through matching retention times and UV/vis spectral data with those of the standard and data reported in the literature and further identified using a high-pressure liquid chromatography-mass spectrometry (HPLC-MS) system.[32−34] Quantification was performed using a calibration curve of the all-*E*-*β*-carotene standard. Concentrations of *Z*-*β*-carotenes were expressed in all-*E*-*β*-carotene equivalents.

A Waters HPLC system (Alliance 2695) coupled with an Esquire 6000 ion-trap mass spectrometer (Bruker-Daltronics, Bremen, Germany) was used to perform the HPLC-MS analysis. The optimized APCI conditions were as follows: APCI, normal mode; spray voltage, 4.0 kV; nebulizer, 45 psi; desolvation temperature, 300 °C; vaporizer temperature, 400 °C; flow rate of desolvation gas, 5.0 L/min; and scanning range, m/z 310 to 650.

Optimization of the Preparation of the 9*Z*-*β*-Carotene High-Loaded NLC. The 9*Z*-*β*-carotene high-loaded NLC was prepared by high-pressure homogenization without organic solvents according to previous reports with modifications.[27,35] Briefly, *β*-carotene with a high ratio of 9*Z*-isomer (5%, w/w total lipids) was added to the mixture of GMS (melting point: ∼81 °C) and MCTs at 85 °C under the protection of nitrogen. An aqueous phase (100 mL) containing Tween 80 was also preheated at 85 °C and mixed with the above lipid phase, stirred for 1 min, and then sheared by high-shear homogenization (IKA Instruments, Germany) at 16000 rpm for 2 min. After pre-emulsification, the mixture was subjected to high-pressure homogenization (ATS Instruments, Canada) under different conditions. After that, the dispersion was cooled down in an ice bath for 20 min to obtain a 9*Z*-*β*-carotene high-loaded NLC. The samples were made freshly and kept at 4 °C in a refrigerator before analysis.

A systematic investigation into the optimization and effects of the various factors including lipid concentration, percentage of the surfactant, ratio of solid to liquid lipids, and homogeneous conditions on the particle size, PDI, and encapsulation efficiency (EE) of the 9*Z*-*β*-carotene high-loaded NLC was performed. The orthogonal experimental design was applied for the optimization of NLC preparation (Table S1). The EE of the NLC was set as the evaluation index. A brief description of the experimental design is as follows:

Effect of Total Lipid Concentration. Five lipid concentrations (2.5%, 5.0%, 7.5%, 10.0%, and 12.5%, w/v) were tested under the following fixed conditions: homogeneous pressure of 500 bar for three cycles; 1.2% (w/v) Tween 80 as the aqueous phase; and a solid−liquid lipid ratio at 3:1 (w/w). The particle size and PDI of each sample were monitored.

Effect of Surfactant Concentration. The nonionic surfactant Tween 80 with a hydrophilic−lipophilic balance number of 15 was suitable to stabilize the O/W solutions and had been widely used in NLC and beverage products.[36] The critical micelle concentration of Tween 80 was 13−15 mg/L, which was good for it to stabilize the emulsion in lower concentration. The surfactant concentrations of Tween 80 were therefore selected as 0.4, 0.8, 1.2, 1.6, and 2.0% (w/v) according to reported studies.[26,27,35] These emulsions were analyzed for the particle size, PDI, and EE under the same pressure and

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number of cycles as stated above. The total lipid concentration was 10% (w/v), and the ratio of solid−liquid lipids was set at 3:1 (w/w).

Ratio of Solid to Liquid Lipids. Different ratios of solid to liquid lipids (1:1, 2:1, 3:1, 4:1, and 5:1, w/w) were selected to investigate their effects on the particle size, PDI, and EE under the same pressure and number of cycles as above. Tween 80 was 1.2% (w/v), and the total lipid concentration was 10% (w/w).

Effects of Homogeneous Pressure. The concentration of the surfactant was set at 1.2% (w/v). Other conditions were the same as those described in the "Effect of surfactant concentration" section. The homogeneous pressure was set at 200, 300, 400, 500, and 600 bar each for three cycles. The particle size and PDI of the NLC were investigated.

Characterization of the 9*Z*-β-Carotene High-Loaded NLC. *Particle Size and PDI Measurements.* The particle size and PDI of the NLC were determined using a Zetasizer Nano-ZS90 (Malvern Instruments, Worcester, UK). The appearances of the 9*Z*-β-carotene high-loaded NLC were compared at 1, 10, 100, and 1000 times dilutions. The original preparation was diluted by 1000 times with deionized water for the measurement of the particle size and PDI. Intensity distribution was analyzed to evaluate the grain size. Three separate measurements were taken for PDI and particle diameter data collection.

Transmission Electron Microscopy (TEM). The morphological characteristics of the 9*Z*-β-carotene high-loaded NLC was determined by TEM. Deionized water was added to dilute the NLC by 1000 times. One drop of the diluted sample was placed on carbon-coated copper grids and dried at room temperature.[37] The sample was dyed using phosphotungstic acid and observed using a FEI Tecnai G2 F20 S-TWIN TEM at 200 kV (FEI Company, Hillsboro, USA).

Fourier Transform Infrared (FTIR) Spectroscopy. All-*E*-β-carotene powder, the freeze-dried 9*Z*-β-carotene high-loaded NLC, and a blank-NLC were scanned using a Fourier transform infrared (FTIR) spectrophotometer (Nicolet Instruments, USA), in a frequency range between 4000 and 400 cm^{-1} at 2 cm^{-1} measuring resolution by the KBr pellet method.[35]

Total β-Carotene Loading in the NLC. The free amount of total β-carotene in the NLC was analyzed following a reported method with slight modifications.[23,35] A mixture of 100 μL of the 9*Z*-β-carotene high-loaded NLC and 3 mL hexane was vortexed for 1 min at room temperature and centrifuged at 2500 × *g* for 2 min. The upper organic phase was collected, and the aqueous phase was extracted two times more. The organic phase was pooled, filtered using a 0.22-μm membrane, and subjected to HPLC analysis as described above. The total amount of β-carotene in the NLC was evaluated in the same way as described above with ethyl acetate as the solvent.

The retention percentage (RP, %) of β-carotene isomers, EE (%), and LC (%) were calculated according to the following equations:

$$\mathrm{EE}(\%) = \frac{\text{total amount of } \beta\text{-carotene} - \text{free } \beta\text{-carotene}}{\text{total amount of } \beta\text{-carotene}} \times 100 \quad (1)$$

$$\mathrm{LC}(\%) = \frac{\text{incorporated amount of } \beta\text{-carotene}}{\text{total of the used lipid}} \times 100 \quad (2)$$

$$\mathrm{RP}(\%) = \frac{\text{amount of encapsulated } \beta\text{-carotene isomer after storage}}{\text{initial amount of encapsulated } \beta\text{-carotene isomer}} \times 100 \quad (3)$$

Degradation of β-Carotene. Degradation of total β-carotene can be affected by the conditions of 9*Z*-isomer preparation, the process of shearing during pre-emulsification, and homogenization during the preparation of the 9*Z*-β-carotene high-loaded NLC.

A. Percent degradation of total β-carotene during the isomerization of all-*E*-β-carotene:

$$\mathrm{A} = \text{Degradation I}\ (\%) = \frac{C_0 - C}{C_0} \times 100 \quad (4)$$

where c_0 and c (m/v) are the concentrations of total β-carotene before and after the isomerization reaction, respectively.

B. Percent degradation of total β-carotene during pre-emulsification:

$$\mathrm{B} = \text{Degradation II}\ (\%) = \frac{m_0 - m}{m_0} \times 100 \quad (5)$$

where m_0 and m (g) separately represent the amount of total β-carotene before and after the mixing and shearing process during the preparation of the NLC.

C. Percent degradation of total β-carotene during homogenization:

$$\mathrm{C} = \text{Degradation III}\ (\%) = \frac{M_0 - M}{M_0} \times 100 \quad (6)$$

where M_0 and M (g) represent the amount of total β-carotene before and after homogenization, respectively.

The overall percent degradation of total β-carotene during the entire preparation process of the 9*Z*-β-carotene high-loaded NLC was calculated based on the following equation:

$$\text{Degradation}\ (\%) = 1 - (1 - \mathrm{A}) \times (1 - \mathrm{B}) \times (1 - \mathrm{C}) \quad (7)$$

Storage Test of β-Carotene Isomers and the 9*Z*-β-Carotene-Loaded NLC. The particle size, PDI, and retention of 9*Z*-β-carotene and total β-carotene of the NLC were assessed during storage under different temperature and pH conditions.

Effects of Temperature. The freshly made NLC was filled with nitrogen gas and stored in the dark for 21 days, at 4 °C, 25 °C, and 37 °C. The original isomerized β-carotene product with a high ratio of 9*Z*-isomer was used as the negative control (NC). All experiments were performed in triplicate.

Effects of pH. The stability of the 9*Z*-β-carotene high-loaded NLC was examined at pH 3.5, 4.5, 5.5, 6.5, and 7.5, a pH range of most food systems, in disodium hydrogen phosphate-citric acid buffer solutions. A total amount of the 1 mL NLC dispersion was mixed with 2 mL of the buffer solution, filled with nitrogen, and then stored in a refrigerator at 4 °C for 21 days. The retention percentages of total β-carotene and 9*Z*-β-carotene were analyzed by HPLC in different time intervals during storage. All experiments were performed in triplicate.

Morphology of the NLC after Storage. The morphology of the 9*Z*-β-carotene high-loaded NLC was examined at the end of the storage test, using an inverted biological microscope at 1000× magnification (ZEISS Axio Vert A1, Carl Zeiss Microscopy, Germany).

Statistical Analysis. All experiments were performed in triplicate, and the data were presented as mean value ± standard deviation (mean ± SD). One-way analysis of variance followed by Duncan's multiple range test was used to analyze data. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using SPSS (Version 18.0, Chicago, IL, USA).

RESULTS AND DISCUSSION

Preparation of *Z*-β-Carotene. Refluxing all-*E*-β-carotene with an iodine-doped TiO_2 catalyst in ethyl acetate for 2 h significantly enhanced the conversion rate, resulting in 25.3% 9*Z*-β-carotene and 47.3% of total *Z*-β-carotene after the reaction (Table 1A), significantly higher than 0.39% 9*Z*-β-carotene by heating in ethyl acetate at 50 °C for 2 h. The UV/

Table 1. Ratios of β-Carotene Isomers in Isomerization Product (A) and NLC Suspension (B)

ratio (%)	15*Z*	13*Z*	9*Z*,13*Z*	All-*E*	9*Z*	total *Z*-β-carotene
A	3.0	14.0	5.0	52.6	25.3	47.3
B	3.7	17.2	5.4	46.7	27.0	53.3

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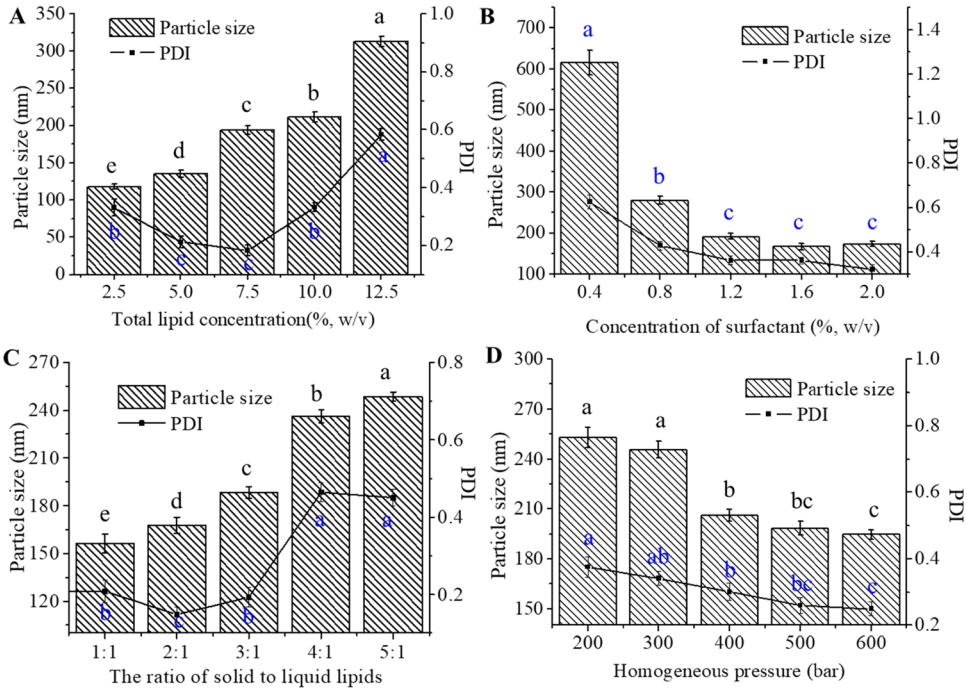

Figure 1. Influence of total lipid concentration (A), surfactant concentration (B), the ratio of solid to liquid lipids (C), and homogenization pressure (D) on the particle size and the PDI of 9*Z*-*β*-carotene high-loaded NLC. Data were means ± SD of three replicated treatments. Different letters indicated a significant ($p < 0.05$) difference for the particle size (in black) or PDI (in blue).

vis spectrum and Q-ratio were used to identify the isomerized products.[38] HPLC-MS was applied to further identify the *β*-carotene isomers.[39] The protonated molecular ion of each *β*-carotene isomer was 537 $[M + H]^+$.[39] The percentage of the *Z*-*β*-carotene isomers in the mixture was in the following order: 9*Z* > 13*Z* > 9*Z*, 13*Z* > 15*Z*-*β*-carotene as shown in Table 1A. This isomerization product had the same total content of *Z*-*β*-carotenes as that from *D. bardawil* extract and a higher ratio of 9*Z*-*β*-carotene than that *from D. salina* extract.[19]

I_2 alone had a similar catalytic effect to I-TiO_2 on both the reaction rate and the isomer composition at equilibrium (data not shown); however, because of the lack of reusability of iodine and ease in separation and recycling of iodine-doped TiO_2, the latter was selected as the catalyst for the laboratory process and potentially for industrial production of the *Z*-*β*-carotene isomers. The percent degradation of total *β*-carotene after isomerization (degradation I) was 12.6%, which was similar to that of astaxanthin after isomerization.[29,39] The catalyst I-TiO_2 could be reused five times, and the composition of the isomerization products was consistent after each reaction under the same conditions. This method has been validated and confirmed to be reproducible.[30]

Optimization of 9*Z*-*β*-Carotene High-Loaded NLC Preparation. *Total Lipid Concentration.* Figure 1A shows that as the total lipid concentration increases, the particle size of the NLC also increases, to above 300 nm at the highest concentration of 12.5% used in the study. This is similar to reports by others.[37] Increased lipid concentration promotes collision and aggregation among the molecules that ultimately results in a larger particle size.[40] Meanwhile, the PDI of the NLC decreased at first and then increased with the increasing lipid concentration. The PDI was the lowest (0.2 ± 0.03) at an intermediate lipid concentration of 7.5%, at which the average particle size was 194 ± 5.43 nm.

Concentration of the Surfactant. The particle size of the NLC gradually decreased as the concentration of surfactant increased (Figure 1B); however, the decrease was only significant at concentrations lower than 1.2%. The changes of the particle size were not significant at higher surfactant concentrations ($p > 0.05$). A similar phenomenon was reported by others. For example, the particle size of the ifosfamide-loaded NLC showed a negative correlation with the increase in the surfactant from 0.25 to 1%.[41] It was also found that once the surfactant reached certain concentrations, such as 1.2% for Tween 80 in this study, the surfactant concentration could no longer affect the particle size.[36] The PDI showed a similar trend to that of the particle size. The EE also increased gradually with increased surfactant concentration (Figure S1A), but stopped at 94.23% when the latter reached 1.2%. Elevated surfactant concentration increases the surfactant adsorbed on the interface and improves the strength of the oil−water interface film formed on the surface of the NLC, resulting in a beneficial inhibition of the precipitation of *β*-carotene and a better dispersion of the particles in water.[37] At surfactant concentrations higher than 1.2%, the strength of the oil−water interface film on the surface of the NLC reached the maximum, which explains why there was no significant further improvement in EE (Figure S1A).

Ratio of Solid to Liquid Lipids. As the ratio of solid lipid to liquid lipid increased, the particle size and PDI of the NLC increased gradually (Figure 1C). When the ratio reached 3:1, the particle size and PDI reached 188 ± 5.79 nm and 0.2 ± 0.03, respectively. Once the ratio exceeded 3:1, the particle size and PDI both increased significantly ($p < 0.05$). A PDI of 0.5 means that the dispersion of NLC was unstable. A further increase in solid lipid to liquid lipid ratio was therefore not beneficial in lowering the particle size or enhancing the stability of the system.

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When the ratio of solid lipid to liquid lipid was 3:1, the EE reached the highest (94%, Figure S1B). In fact, any higher or lower ratios led to EE less than 90%, suggesting that a balance between the content of solid and liquid lipids is important for obtaining the highest EE in the NLC. Imbalanced ratios are not beneficial for the maximum formation of lipid carriers and can have imperfect crystals. A balanced ratio allows for a large amount of space to accommodate more bioactive substances and the ability to avoid leaking.[23]

Effects of Homogeneous Pressure. As shown in Figure 1D, the particle size of the NLC showed an inverse association with the homogenization pressure between 200 bar and 600 bar, similar to other reports.[42] When the homogenization pressure was at 500 bar, the particle size was 198 ± 6.65 nm. However, there was no significant change in the particle size beyond 500 bar. The PDI showed a similar trend to the particle size (Figure 1D). When the homogenization pressure was 500 bar, the PDI was 0.26 ± 0.03, and the particles of the NLC were dispersed uniformly.

As excessive pressure may increase the particle size. The higher the pressure was, the more intense was the collision between particles, resulting in agglomeration of the particles, increased particle size, and poor stability of the carrier system.[43] The temperature of the solution could also increase during the increment of the pressure, which affects the emulsification effect of the surfactant and intensifies the degradation of β-carotene. For these reasons, the optimal homogenization pressure of the present study was set at 500 bar. Additionally, the homogenization time was also tested for the optimization of NLC preparation (data not shown). Homogenization for three cycles was set for the optimal particle size and PDI of the NLC.

Based on the preliminary single-factor tests, the optimized preparation of the 9*Z*-β-carotene high-loaded NLC was carried out at total lipid concentrations of 6, 7.5, and 9% (w/v), at surfactant concentrations of 1, 1.2, and 1.4% (w/v), and at the ratios of solid to liquid lipids of 5:2, 3:1, and 7:2, respectively (Table S1). The results of the orthogonal experiment showed that the influence of the three factors on the EE of the NLC followed the order of: the solid lipid to liquid lipid ratio > the surfactant concentration > total lipid concentration (Table S2). As shown in Table S2, the final conditions for the optimal NLC preparation were as follows: the total lipid concentration was 9% (w/v), the surfactant concentration was 1.4% (w/v), the solid to liquid lipid ratio was 3:1 (w/w), and the homogenization pressure was set at 500 bar for three cycles. Under these conditions, the EE of the NLC was 95.64%, which was higher than the EE (91.2%) of the β-carotene-loaded NLC based on palm stearin and palm olein in a single factor test reported by others.[44] Moreover, the overall percent degradation of total β-carotene was 35.2% after the preparation of the NLC as calculated using equation 7, and the temperature and homogeneous pressure during the production could be the major reasons for the degradation of total β-carotene.[37] The NLC can be scaled up in food production systems by autoclaving, spray-drying, and lyophilizing because the particle diameter is below 200 nm.[20,23]

Characterization of the 9*Z*-β-Carotene High-Loaded NLC. *Isomeric Profiles of β-Carotene in the NLC.* The chromatogram and ratios of β-carotene isomers in the 9*Z*-β-carotene high-loaded NLC are presented in Figure 2 and Table 1. The proportion of the all-*trans*-β-carotene isomer decreased from 52.59% in the isomerized product to 46.74% in the 9*Z*-β-carotene high-loaded NLC, and that of 9*Z*- and total *Z*-isomers increased from 25.31% and 47.41% in the isomerized product to 27.02% and 53.26% in the NLC, respectively (Table 1). The result suggests that the homogenization treatment of the NLC increased the content of the desirable 9*Z*-β-carotene, 13*Z*-β-carotene, and total *Z*-isomers during the processing.

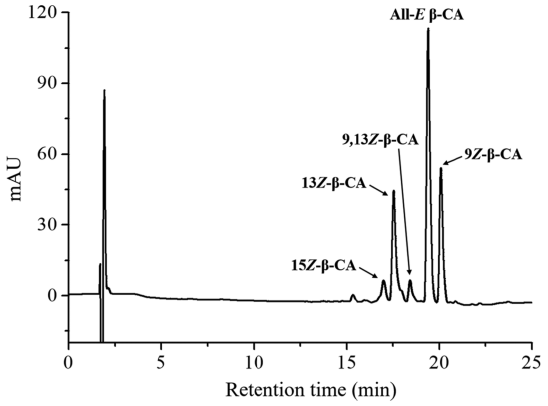

Figure 2. HPLC chromatogram of β-carotene isomers in the 9*Z*-β-carotene high-loaded NLC. CA was short for carotene.

Particle Size, PDI, and 9Z-β-Carotene Load of the NLC. The 9*Z*-β-carotene high-loaded NLC had a good aqueous solubility with a bright orange-yellow color and good color stability at different dilutions (Figure 3A). Similar preparations from milk fat were reported to be in yellowish color with thick texture.[28] The 9*Z*-β-carotene high-loaded NLC of the present study contained 2.9 mg/mL of β-carotene, far much higher than that of similar preparations with high oleic sunflower oil (25 μg/mL) obtained by the solvent displacement method and the reported β-carotene nanosuspension (~1 mg/mL).[6]

The mean particle size and the PDI are key characteristics for nanodispersions that determine the physical stability, solubility, biological performance, release rate, and chemical stability of a preparation.[20,45] The particle size distribution of the NLC is shown in Figure 3B. The 9*Z*-β-carotene high-loaded NLC showed a uniform particle size distribution with an average particle size of 191 ± 6.46 nm and PDI of 0.2 ± 0.03, indicating potential higher bioavailability (bioaccessibility) than conventional emulsions (1−10 μm).[46]

Morphology of the 9Z-β-Carotene-Loaded NLC. TEM reflects the apparent morphology and dispersion of β-carotene-loaded particles. Particles of the 9*Z*-β-carotene high-loaded NLC were found to be spherical and uniform in shape (Figure 3C), which was consistent with particle size distribution shown in Figure 3B.

FTIR Analysis. The FTIR spectrum (Figure 4A) of all-*E*-β-carotene powder revealed the absorption bands at 3027 cm^{-1} (=C−H stretching vibration), 2950 cm^{-1} (asymmetric methyl stretching vibration), 2916 cm^{-1} (C−H stretching vibration), 2860 cm^{-1} (symmetric methylene stretching vibration), 1560 cm^{-1} (aromatic ring skeletal stretching vibration), and 965 cm^{-1} ($R_1HC{=}CHR_2$ wagging vibration).[47] FTIR spectra of the 9*Z*-β-carotene high-loaded NLC (Figure 4B) and the blank NLC (Figure 4C) were almost the same, and the characteristic peaks of β-carotene isomers were not present. The results indicated that β-carotene isomers were well incorporated into the NLC.

Storage Test of the 9*Z*-β-Carotene High-Loaded NLC. The particle size and PDI of NLC stored at 25 °C were close to those stored at 4 °C during the 21 days storage period, and

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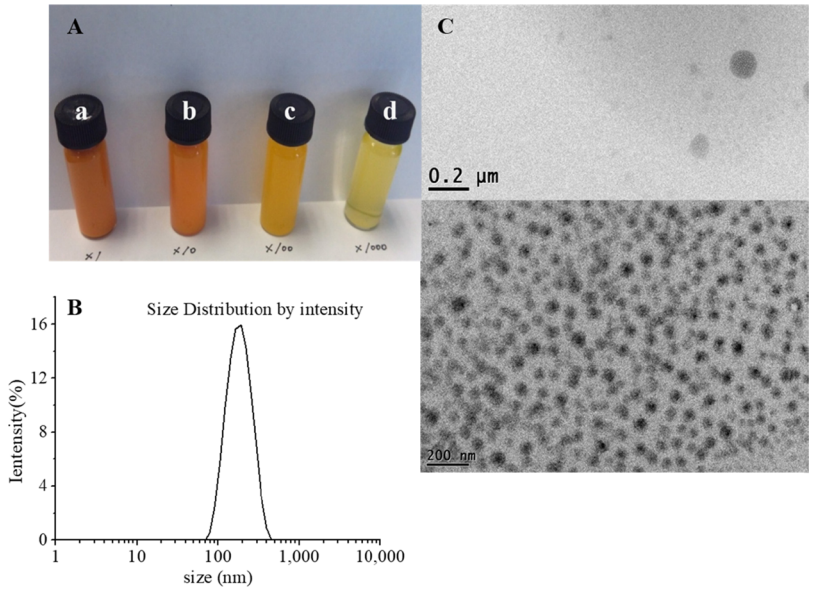

Figure 3. Characterization of the 9*Z*-*β*-carotene high-loaded NLC. A: Appearances of the NLC with different dilution multiples containing 2.9 mg/mL (a), 0.29 mg/mL (b), 0.029 mg/mL (c), and 0.0029 mg/mL (d) of total *β*-carotene; B: Size distribution of the 9*Z*-*β*-carotene high-loaded NLC; C: TEM of 9*Z*-*β*-carotene high-loaded NLC in *μ*m and nm scales.

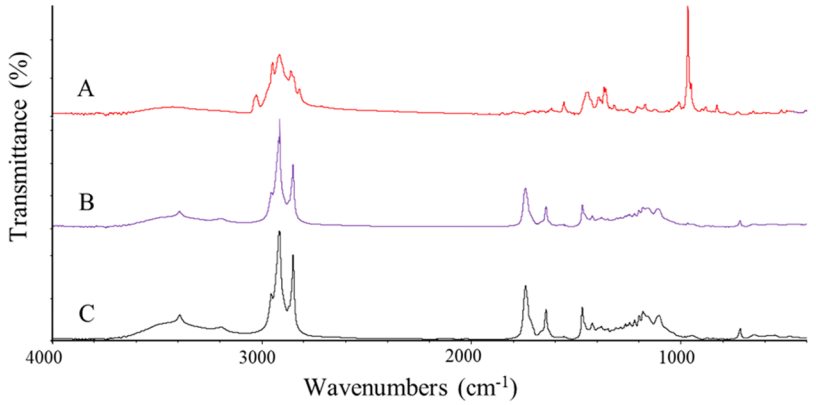

Figure 4. FTIR spectra of all-*E*-*β*-carotene powder (A), 9*Z*-*β*-carotene high-loaded NLC (B), and blank-NLC (C).

the NLC remained stable and uniform at both temperatures (Figure 5A,B). The morphology of the NLC stored at 4 °C showed that the particles were intact, and little agglomeration was found after the storage (Figure S2). However, the particle size and PDI of the system increased significantly at 37 °C, from 168 ± 4.24 nm to 594 ± 5.37 nm and 0.2 ± 0.02 to 0.6 ± 0.01, respectively, and the system became less stable over the same period ($p < 0.05$) (Figure 5A,B). This may be attributed to the higher kinetic energy at 37 °C than the other two temperatures tested for the carrier system. The high kinetic energy accelerates the collision of the nanoparticles and thereby increases the probability of agglomeration between nanoparticles.[48] These data suggest that the *β*-carotene-NLC should be stored at lower temperatures such as 4 °C and 25 °C, which are common conditions for the storage of foods and beverages.

The retention of 9*Z*-*β*-carotene and total *β*-carotene in the NLC was compared with that of the isomerized product under different storage conditions. In general, the retention percentages of 9*Z*-*β*-carotene and total *β*-carotene in the NLC were higher than those of the nonformularized isomerized *β*-carotene powder at all temperatures over the 21 days period (Figure 5C,D). Both 9*Z*-*β*-carotene and total *β*-carotene in either the NLC or the isomerized powder were most stable at 4 °C compared with those at other tested temperatures (25 °C and 37 °C), suggesting again that the storage temperature had a great influence on the stability and degradation of compounds in the NLC.[37,49] The slopes of the retentions of 9*Z*-*β*-carotene and total *β*-carotene in the NLC and the isomerized product among the treatments during storage were determined (Table S3). Fitting results showed that there were significant differences for the retention rates of 9*Z*-*β*-carotene stored in different temperatures (Table S3). The original percentage of 9*Z*-*β*-carotene in the isomerized product decreased to half in 54 days at 25 °C during the storage by the fitting equation (Table S3); however, the original amount of 9*Z*-*β*-carotene was reduced to half in 101 days under the same conditions with the protection of NLC, which was almost twice that of 9*Z*-*β*-carotene in the isomerized product. There were also significant differences of the retention rates of total *β*-carotene stored at different temperatures in each system. However, there was no significant difference between the retention rate of total *β*-carotene in the NLC stored at 37 °C and that of total *β*-carotene in the

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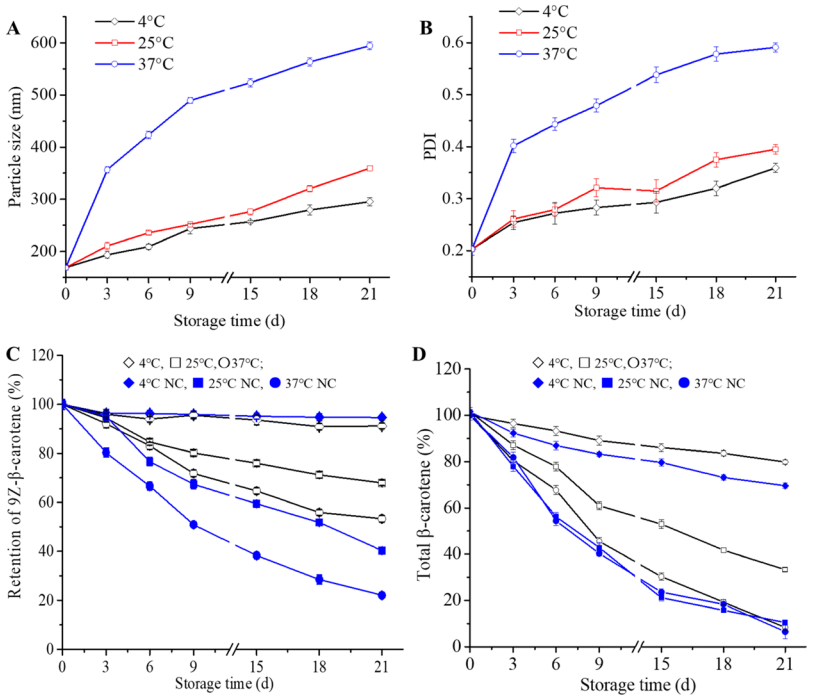

Figure 5. Effect of temperature on the particle size (A) and PDI (B) of the 9Z-β-CA high-loaded NLC and the retention of 9Z-β-CA (C) and total β-CA (D) in the 9Z-β-CA high-loaded NLC and isomerized product (NC). CA was short for carotene. Each value represented the mean ± SD (n = 3). The degradation kinetics of β-carotene is shown in Table S3.

isomerized product stored at 25 °C. All the results indicated a protective effect of the NLC. 9Z-β-Carotene could be efficiently protected by this formulation at lower temperatures during the tested storage period of the present study.

The differences in retention between 9Z-β-carotene and total β-carotene (Figure 5C,D) and the final balance among different isomers may be attributed to the relatively higher stability of 9Z-β-carotene than that of other cis-isomers[13,50] and degradation and transformation of all-E-β-carotene to 13Z-, 15Z-, and the 9Z-isomers, among which the 13Z-isomer is known to be relatively less stable.[3] The retention of 9Z-β-carotene was similar to what has been reported for lycopene.[35]

The effects of pH on the particle size and PDI of the NLC are shown in Figure 6. In general, the PDI was lower than 0.33, and the particle size distribution remained uniform around 300 nm after storage for 21 days at pH 3.5 − 7.5 (Figure 6). In fact, these parameters did not vary significantly during the 21 days

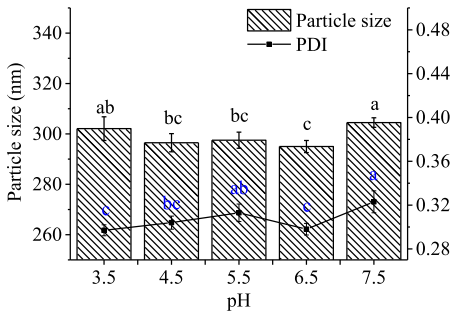

Figure 6. Effects of pH on the particle size and PDI of the 9Z-β-carotene high-loaded NLC after storage of 21 days. Data were means ± SD of three replicated treatments. Different letters indicated a significant (p < 0.05) difference for the particle size (in black) or PDI (in blue).

period (data not shown). There was no significant trend of the PDI or particle size of the NLC stored from 3.5 to 7.5. The overall result indicated that the NLC suspension was relatively more stable under acidic conditions than in an alkaline environment. This supports the use of the NLC product in beverages, which are mostly kept under acidic conditions.

pH also showed little effects on the retention of β-carotene isomers; however, the retention decreased over a period of 21 days at pH 3.5 but was relatively stable in a neutral environment (pH 6.5 and pH 7.5) (Figure 7). The retention

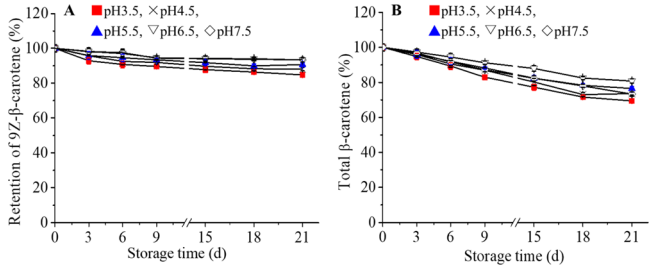

Figure 7. Effects of pH on the retentions of 9Z-β-carotene (A) and total β-carotene (B) in the 9Z-β-carotene high-loaded NLC during the storage in the dark at 4 °C for 21 days. Each value represented the mean ± SD (n = 3).

of 9Z-β-carotene decreased by 7.94% after 21 days of storage at pH 6.5 and pH 7.5, whereas it was by 15.61% at pH 3.5 (Figure 7A). A similar trend was found for the total β-carotene. At the end of the 21 days storage, percent retention of total β-carotene was reduced by 20% under neutral conditions, but by 30.58% at pH 3.5 (Figure 7B). Mehrad et al. used the color fading of β-carotene SLNs as an indicator for the chemical degradation of β-carotene and also found that degradation was faster at pH 3.5 than at neutral pH.[51] A similar result was

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found in our previous study on the stability of astaxanthin isomers that it was significantly affected by highly acidic conditions, that is, pH 2.0 and 3.5.[29] Our results suggest that the 9Z-β-carotene high-loaded NLC would be stable when used in common food systems. The stability of β-carotene isomers under the tested pH may be due to the protection in intact nanoparticles of the NLC (Figure 6).

In this study, Z-β-carotenes with 25.3% 9Z-β-carotene were prepared for the first time as an NLC. Using an orthogonal experimental method, the following optimal conditions were obtained for the NLC preparation: total lipid concentration, 9% (w/v); surfactant concentration, 1.4% (w/v); solid to liquid lipid ratio, 3:1 (w/w); and homogenization pressure, 500 bar for three cycles. Under these conditions, the EE of the NLC was 95.64%, and the total β-carotene in the NLC was 2.9 mg/mL which was significantly higher than that reported by others. The proportion of total Z-β-carotenes was as high as 53.3%, the particle size was 191 ± 6.46 nm, and the PDI was 0.2 ± 0.03. The processing method also helped to maintain the profiles of the β-carotene isomers loaded in the NLC without causing any major conformational transformations. Storage stability analysis indicated that the β-carotene-loaded NLC stabilizes both 9Z-β-carotene and total β-carotene from leakage and degradation during 21 days of storage at pH 3.5 − 7.5 at low temperatures (4 °C), especially for the biologically more interesting 9Z-β-carotene. The results on the improved ratio of 9Z-β-carotene, LC, water solubility, and bioaccessibility of the β-carotene NLC provide an effective strategy for β-carotene applications in functional foods or beverages and in nutraceutical preparations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c02342.

The orthogonal experimental design (Table S1), results of the orthogonal array design for three variables (Table S2), retention rates of 9Z-β-CA and total β-CA in the 9Z-β-CA high-loaded NLC and isomerized product (Table S3), influence of surfactant concentration and the solid to liquid lipid ratio on the EE of the 9Z-β-carotene high-loaded NLC (Figure S1), and morphology of the 9Z-β-carotene high-loaded NLC stored for different time intervals (Figure S2) (PDF)

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Notes

The authors declare no competing financial interest.

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