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Transparent Dispersions of Milk-Fat-Based Nanostructured Lipid Carriers for Delivery of β-Carotene

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ABSTRACT: Nanostructured lipid carriers (NLCs) are possible vehicles to incorporate lipophilic bioactive compounds in transparent functional beverages. In this work, anhydrous milk fat (AMF) and Tween 80 were used to prepare NLCs using a phase-inversion temperature method, and β-carotene was used as a model lipophilic bioactive compound. The phase-inversion temperature decreased from >95 to 73 °C, when NaCl increased from 0 to 1.0 M in the aqueous phase. At 0.8 M NaCl and phase inversion by heating at 90 °C for 30 min, transparent NLC dispersions were observed at AMF levels higher than 10% (w/w), corresponding to particles smaller than ∼25 nm. The NLC dispersions were dilution- and dialysis-stable and maintained turbidity and particle size during 90 days of storage at room temperature. The degradation of β-carotene encapsulated in NLCs was much reduced when compared to its encapsulation in the soybean-oil-based nanoemulsion.

KEYWORDS: *Nanostructured lipid carriers, phase-inversion temperature method, anhydrous milk fat, transparent dispersion, β-carotene, storage stability*

INTRODUCTION

There are numerous lipophilic bioactive compounds of significance to food, pharmaceutical, and other consumer products.[1] However, low solubility and poor stability are two major concerns when incorporating these bioactive compounds in products that possess an aqueous continuous phase. Thus, a suitable delivery system is needed to physically distribute them in product matrices with maintained chemical stability during storage, in either the presence or absence of hydrophilic and lipophilic antioxidant compounds.[2]

The lipid core of oil-in-water (O/W) emulsions is commonly used to encapsulate lipophilic bioactive compounds. Furthermore, nanoemulsions, with droplet diameters smaller than about 200 nm, have the advantages of good physical stability during storage and a translucent or even transparent appearance.[3,4] Nanoemulsions have also shown the enhanced bioavailability of encapsulated bioactive compounds, such as β-carotene.[5] If the oil body of nanoemulsions can be prepared from lipids present in the solid state at typical application conditions, so-called solid lipid nanoparticles (SLNs), the degradation of encapsulated bioactive compounds can be much reduced.[6] This is because the solid lipid matrix reduces the mobility of encapsulated compounds diffusing to the particle surface, where most degradation occurs.[6] A variety of SLN systems have been developed as effective delivery agents.[7−9] However, there are some potential problems associated with the SLN systems containing lipid structures with perfect crystals, including low drug loading, drug expulsion,[10] and physical instability caused by the possible transformation of crystallized lipids to the more thermodynamically stable polymorphic form.[11] To overcome these disadvantages, nanostructured liquid carriers (NLCs) have been developed by adopting lipid blends, in most cases, solid and liquid lipids, to form imperfect crystals, amorphous lipid cores, or a nanocompartment of liquid lipids.[12]

SLNs and NLCs are usually formed by high-energy methods, such as high-pressure homogenization (HPH) above or below the melting temperature of lipids.[8,12,13] High-energy methods are effective in reducing particle size but demand high capital and operating costs. Degradation of sensitive compounds during processing is another concern. Conversely, low-energy methods, such as those involving phase inversions, because of changes in temperatures or compositions, do not require specialized equipment and high mechanical energy.[3] In particular, the phase-inversion temperature (PIT) method has been used to prepare transparent nanoemulsions of lemon oil.[14] Because heating lipids above the melting temperature is needed to dissolve lipophilic compounds before preparing SLNs and NLCs, it is logical to form transparent SLN/NLC dispersions using the PIT method.

The phase behavior of water−oil−surfactant systems as a function of surfactant properties, temperature, and composition is affectively described by the hydrophilic lipophilic deviation (HLD; eq 1 for non-ionic surfactants).[15] Negative and positive HLD values indicate the possibility of forming oil-in-water (O/W)- and water-in-oil (W/O)-type emulsions, respectively, while a bicontinuous phase behavior corresponds to a HLD value of 0.[16] For an emulsion with a fixed composition, a fine emulsion with a small particle size can be formed during the process of inversion between W/O and O/W emulsions because of changes in the temperature.[17,18] Partial inversion was observed for transformation of turbid coarse O/W emulsions of lemon oil emulsified by polyoxyethylene (20) sorbitan monooleate (Tween 80) to transparent nanoemulsions after heating at 90 °C for 30 min[14]

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$$\mathrm{HLD} = \alpha - \mathrm{EON} - k\mathrm{ACN} + bS + \Phi(A) + C_{\mathrm{T}}(T - T_{\mathrm{ref}}) \quad (1)$$

where α is a characteristic parameter of the hydrophobic part of the surfactant, EON is the number of ethylene oxide groups per surfactant molecular, ACN is the number of carbon atoms in the alkyl tail of the molecule (or equivalent), $\Phi(A)$ is a function of the alcohol (added as a co-surfactant) type and concentration, S is the salinity of the aqueous phase in weight percent of NaCl or equivalent salt, T is the temperature (°C), T_{ref} is generally taken at 25 °C, and k, b, and C_{T} are constants characteristic of the surfactant type and electrolyte.[15] The value of temperature coefficient C_{T} is typically larger for an ethoxylated non-ionic surfactant than an ionic surfactant.[19]

The first objective of this work was to study the formation of transparent dispersions of NLCs using the PIT method. Anhydrous milk fat (AMF) was chosen as the solid lipid because it is a blend of lipids with various chain lengths and is a commercially available dairy ingredient. Variables in NLC formation were studied for thermal treatment conditions, salinity, and composition. The second objective was to study the prevention of degradation for β-carotene, a model lipophilic bioactive compound, when encapsulated in NLCs.

MATERIALS AND METHODS

Materials. AMF was kindly donated by Land O'Lakes, Inc. (St. Paul, MN). Tween 80, NaCl (purity > 99.5%), ethanol, hexane, and deionized water were purchased from Fisher Scientific (Pittsburgh, PA). Soybean oil was a product of Kroger Co. (Cincinnati, OH). β-Carotene (predominantly in the *trans* form) was purchased from MP Biomedicals, LLC (Solon, OH). Potassium persulfate, 6-hydroxy-2,5,7,8-tet-ramethychroman-2-carboxylic acid (Trolox), and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were products of Sigma-Aldrich Corp. (St. Louis, MO).

Preparation of NLC Dispersions. *Preparation of Surfactant Solutions.* NaCl and Tween 80 were dissolved in deionized water at concentrations of 0−1.00 M and 5−40% (w/w), respectively. The mixtures were stirred overnight to ensure complete dissolution and heated to 60 °C before use.

Preparation of Coarse Emulsions. Preparations of coarse emulsions and NLC nanodispersions were studied on the basis of a PIT method detailed previously,[14] with some modifications. To form a coarse emulsion, AMF was heated to 60 °C for complete melting, followed by mixing with warm (60 °C) aqueous phase under agitation at 1000 rpm using a magnetic stirring plate. The AMF concentration in coarse emulsions was 1−15% (w/w). For encapsulation studies, β-carotene was dissolved in warm AMF at a concentration of 6.25 mM before emulsification. To compare impacts of the structure of the lipid body (solid fat versus liquid oil) on the stability of encapsulated β-carotene, a control nanoemulsion was prepared using soybean oil at conditions comparable to the treatment using AMF.

Thermal Treatment for the Preparation of NLCs. The coarse emulsions were transferred to 4 mL glass vials that were heated for 30 min in a water bath maintained at a constant temperature (75, 80, 85, 90, or 95 °C) without stirring. After thermal treatment, these samples were first cooled at ambient conditions with hand shaking until a homogeneous appearance, followed by quenching in an ice bath under static conditions.

Turbidity Measurement. Turbidity of samples was measured for absorbance at 600 nm (Abs_{600}) using an ultraviolet−visible (UV−vis) spectrophotometer (Unicam, Cambridge, U.K.). Tween 80 solutions at corresponding concentrations were used as controls.

Particle Size Determination. The particle size of samples was measured using a Delas Nano particle analyzer (Beckman Coulter, Fullerton, CA). To avoid multiple scattering effects, all samples were diluted to an appropriate concentration in deionized water prior to analysis. The volume-length mean diameter was calculated as follows

$$d_{4,3} = \sum n_i d_i^4 / \sum n_i d_i^3 \quad (2)$$

where n_i and d_i are the number and diameter of the ith group of particles, respectively.

Viscosity Measurement. Rheological properties of dispersions were studied with an AR 2000 rheometer (TA Instrument, New Castle, DE) using a Searle setup (bob outer diameter of 28 mm and cup inner diameter of 30 mm). After the sample was loaded, heated, and equilibrated at 95 °C for 2 min, a temperature sweep with a fixed shear rate of 100 $1/s$[15] was applied from 95 to 25 °C at 3 °C/min. A thin layer of soybean oil was applied on the top of the sample to minimize water evaporation.

Differential Scanning Calorimetry (DSC). DSC (model Q2000, TA Instruments, New Castle, DE) was used to study melting and crystallization properties of AMF and NLCs during heating and cooling. The transparent NLC dispersions with or without β-carotene prepared with 15% AMF and 45% Tween 80 in 0.8 M NaCl as the aqueous phase were used to generate detectable endothermic and exothermic peaks. A sample corresponding to 5−8 mg of AMF or 15−20 mg of NLC dispersion was placed in an aluminum pan and hermetically sealed. An empty pan was used as a reference. The thermal profile was studied using a heating and cooling cycle, following the steps of holding at −15 °C for 5 min, heating from −15 to 90 °C at 5 °C/min, and cooling from 90 to −15 °C at 20 °C/min. The heat flow of the samples was recorded using the instrument software.

Atomic Force Microscopy (AFM). The shape and dimension of particles were characterized using NanoScope IIIA Multimode AFM (Veeco Instruments, Inc., Santa Barbara, CA). The NLC dispersion was first diluted to 10 ppm of AMF using deionized water. A total of 2 μL of the diluted sample was dropped on a freshly cleaved mica disk and dried under ambient temperature (21 °C) overnight. A resonant frequency of about 71.0 kHz and a scan rate of 1.0 Hz were applied. Images were collected at the tapping mode and analyzed using the instrument software.

X-ray Diffraction (XRD). Crystallinity of pristine β-carotene, AMF, and β-carotene-loaded NLCs was studied by XRD (Panalyticla, Westborough, MA). NLC dispersions were freeze-dried before being spread on the glass plate and pressed to make a smooth layer. The diffraction spectrum was acquired at 2°/min for a 2θ range of 5−55°. The Cu Kα radiation (λ = 1.54 Å) was generated at 30 kV and 10 mA.

Antioxidant Properties. The antioxidant properties of the emulsions were estimated using an ABTS method.[20,21] This method measures the relative ability of antioxidant substances to scavenge the ABTS^+, which is referenced to Trolox, an antioxidant standard. To generate ABTS^+, 7 mM ABTS and 2.45 mM potassium persulfate were dissolved in ethanol and allowed to stand in the dark at room temperature overnight. The ABTS^+ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. A total of 2 μL of Trolox or emulsion was mixed with 2 mL of the ABTS^+ solution, and the absorbance of the mixture at 734 nm was measured after mixing for 15 min. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of the concentration of Trolox and emulsion. The antioxidant properties were obtained by referring to a standard curve established from Trolox and expressed as millimolar Trolox equivalents per milliliter.

Quantification of the β-Carotene Concentration. The β-carotene concentration in the emulsion was determined following a literature method.[22] A total of 20 μL of an emulsion sample was extracted using a mixture composed of 2 mL of ethanol and 3 mL of hexane. After hand shaking for 2 min, the upper organic phase was collected. The remainder aqueous phase was extracted repeatedly until the top phase became colorless. All hexane extracts were pooled into a 10 mL flask and added with hexane to a total volume of 10 mL. The absorbance at 450 nm was measured using a UV−vis spectrophotometer (Unicam, Cambridge, U.K.). The concentrations of β-carotene in emulsions were obtained by referring to a standard curve established from standard solutions with different amounts of β-carotene in hexane.

Storage Stability of NLCs and Encapsulated β-Carotene. *Physical Stability of NLCs.* Selected NLC dispersions without β-

carotene and the corresponding dispersions after dialysis treatment were stored for 90 days at ambient temperature (21 °C). The dialysis of NaCl-containing emulsions was conducted using a membrane with a molecular weight cutoff of 3500 Da (Fisher Brand, Pittsburgh, PA), which was immersed in bulk distilled water for 48 h that was exchanged with fresh water every 6 h. A total of 2 mL of dispersions was included in 4 mL capped glass vials, and the Abs_{600} and particle size in individual vials were measured periodically using the methods described above.

Chemical Stability of β-Carotene. The stability of β-carotene in NLCs and comparable nanoemulsions prepared with soybean oil was characterized for changes of both antioxidant properties and β-carotene content during room temperature (21 °C) storage for 16 days. Samples in 4 mL sealed clear glass vials were exposed to light-emitting diode (LED) fluorescent light during storage. Individual vials were sampled every 2 days for assays of β-carotene concentration and antioxidant property as above. Antioxidant properties of NLCs and soybean oil nanoemulsions without β-carotene were also determined and used as controls.

Statistical Analysis. All samples were prepared in duplicate. All measurements were repeated at least twice. Results from all measurements were reported for means and standard deviations. Significant differences were analyzed with a least significant difference ($p < 0.05$) mean separation method, assisted using Statistical Analysis Software (version 9.2, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Influence of Salinity and Temperature on NLC Formation. The first group of experiments was used to study the temperature and NaCl concentration that are important parameters of HLD (eq 1). Furthermore, on the basis of our preliminary trials, emulsions with high surfactant concentrations can form gels at high NaCl concentrations during thermal treatments, while a low surfactant/oil ratio typically results in phase separation. Therefore, this group of samples was prepared with 1% (w/w) AMF and aqueous phase with 15% (w/w) Tween 80 and 0−1.0 M NaCl. After heating at 80−95 °C for 30 min and cooling to room temperature, the Abs_{600} is shown in Figure 1. Emulsions with a NaCl concentration lower than 0.4 M had no significant change in Abs_{600} after heating, while those containing 0.6−1.0 M NaCl showed significant reduction in Abs_{600} at a heating temperature of 85−95 °C. Figure 1 also indicates that a lower NaCl concentration was required to transform turbid emulsions to transparent emulsions when heated at 95 °C than at 90 °C.

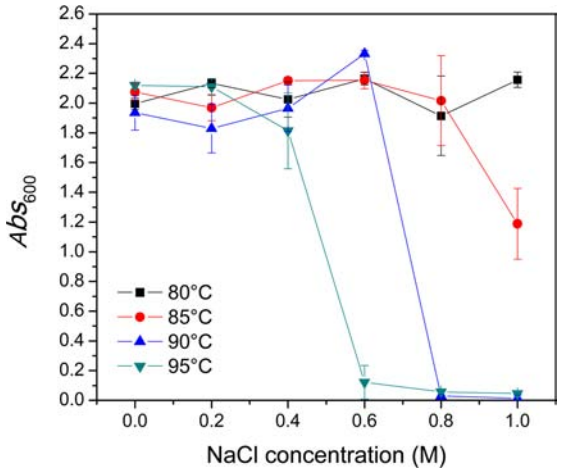

Figure 1. Absorbance at 600 nm (Abs_{600}) of dispersions containing 1% (w/w) AMF and aqueous phase with 15% (w/w) Tween 80 dissolved in 0−1.0 M NaCl solutions after heating at a temperature between 80 and 95 °C for 30 min and cooling to room temperature. Error bars are standard deviations from duplicate samples.

Figure 1 suggests that the temperature and NaCl concentration strongly control phase inversion. Monitoring viscosity changes of emulsions during cooling is a convenient method to characterize phase behavior and determine the PIT.[23,24] Figure 2 shows viscosities of samples during cooling from 95 to 35 °C and the determined PIT. Using the emulsion formed from 0.80 M NaCl as an example, the sample viscosity was small at high temperatures. When the temperature decreased to about 77 °C, the viscosity increased quickly and reached a maximum value at 65 °C. Upon further cooling, viscosity decreased until reaching about 55 °C, followed by an increase with a further decrease in the temperature. Typically, there are two viscosity maxima during cooling, corresponding to formation of fine W/O and O/W emulsions, and the temperature corresponding to the lowest viscosity between the two maxima is treated as the PIT.[23] For systems with a large water fraction as the case of the present study, the viscosity maximum above the PIT is usually not observed,[15] possibly because of the formation of multiple water-in-oil-in-water (W/O/W) emulsions instead of W/O emulsions. The PIT of systems with a high water content can be approximated by the temperature at which the viscosity starts to increase during cooling, e.g., 76 °C for the system containing 0.8 M NaCl. As presented in Figure 2, emulsions containing a higher NaCl concentration had a lower PIT. According to eq 1, HLD is larger at a higher NaCl concentration, requiring a lower temperature (PIT) to reach a HLD value of zero, i.e., phase inversion. The remaining experiments to form NLCs were conducted at a NaCl concentration of 0.8 M and thermal treatment of 90 °C for 30 min.

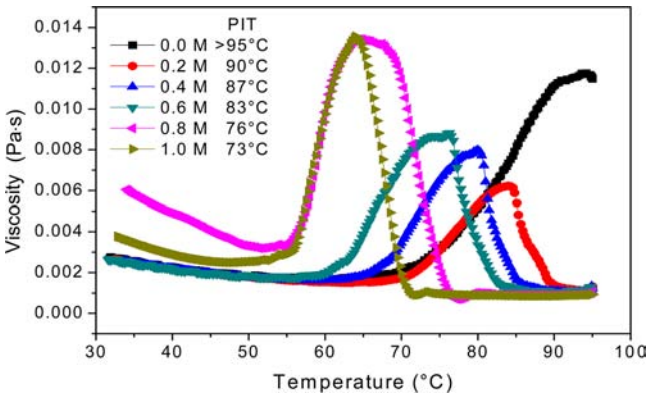

Figure 2. Effects of the NaCl concentration on the viscosity of emulsions during cooling from 95 to 35 °C. Emulsions contained 1% (w/w) AMF and an aqueous phase with 15% (w/w) Tween 80 dissolved in 0−1.0 M NaCl solutions. Values of the PIT determined from viscosity data are listed in the legend.

Influence of the Surfactant/Oil Ratio on NLC Formation. The impact of composition on NLC formation was studied for emulsions with 1.0−15.0% (w/w) AMF in the aqueous phase with 10.0−40.0% (w/w) Tween 80 and 0.80 M NaCl. Samples with low oil concentrations were transparent before heating [1, 1−3, and 1−7% (w/w) AMF for the treatments with 20, 30, and 40% (w/w) Tween 80 in the aqueous phase, respectively], because micelles of Tween 80 are capable of dissolving some lipids, forming O/W micro-

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emulsions. These samples remained transparent and had low Abs_{600} after thermal treatment. Turbid emulsions with 2−6, 4−10, and 8−14% (w/w) AMF in samples with 20, 30, and 40% Tween 80 in the aqueous phase, respectively, became transparent after thermal treatment, while turbid emulsions were still observed after heating samples with higher AMF concentrations. Some samples with high AMF concentrations even underwent phase separation, with creaming observed after 10 min. These observations are demonstrated visually in Figure 3A for emulsions with 30% (w/w) Tween 80 in the aqueous phase. Consistent with these observations, values of Abs_{600} and $d_{4,3}$ of samples after cooling to room temperature are shown in panels A and B of Figure 4, respectively. Generally, transparent samples are evidenced by small Abs_{600} (<0.1 absorbance units) and $d_{4,3}$ (< ~23 nm) because of the inability of small NLCs in scattering visible light. All samples with the lowest amount of Tween 80 [10% (w/w) in the aqueous phase] were turbid before and after heating. Sub-micrometer-sized particles (<400 nm) were observed for samples with 8−10, 12−15, and 15% (w/w) AMF in the aqueous phase with 20, 30, and 40% (w/w) Tween 80, respectively. The standard deviations of $d_{4,3}$ and widths of size distributions (the latter not shown) were large for these treatments, possibly because of the deficiency of surfactants required for phase inversion at the studied conditions. The high polydispersity may also play a role. Conditions enabling formation of NLCs are summarized in Figure 3B for this group of treatments, in the form of a phase diagram.

Our results generally agreed with those by Rao and McClements.[14] In their research, propylene glycol was used in the aqueous phase to enhance the inversion of transparent nanoemulsions with lemon oil. In contrast, propylene glycol was not observed to enhance the formation of transparent NLC dispersions in our preliminary studies. This may be due to the lower solubility and higher melting temperature of AMF than those of lemon oil.

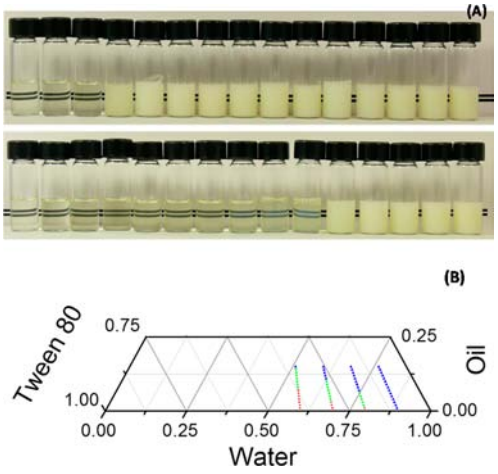

Figure 3. (A) Appearance of example emulsions before (top) and after (bottom) heating at 90 °C for 30 min. Emulsions before heating were prepared with 1.0−15.0% (w/w) AMF and an aqueous phase with 30% (w/w) Tween 80 dissolved in a 0.8 M NaCl solution. The AMF concentration increased by 1% (w/w) successively for samples ordered from left to right. (B) Partial phase diagram showing conditions [×100% (w/w) for each constituent] of forming microemulsions (red squares), transparent NLC dispersions (green circles), and turbid or phase-separated emulsions (blue triangles) at 25 °C formed by the PIT method. Dispersions were prepared as in panel A, with the Tween 80 concentration being 10−40% (w/w).

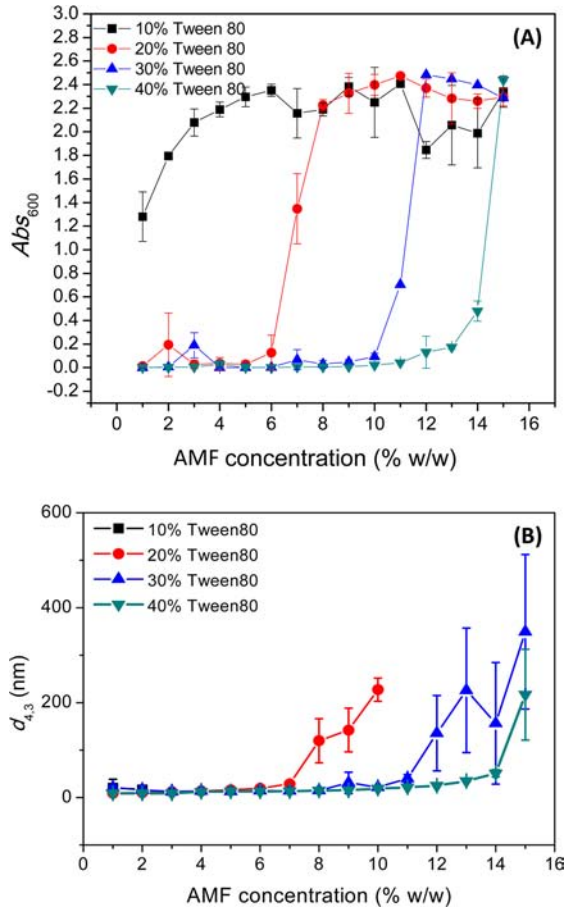

Figure 4. (A) Absorbance at 600 nm (Abs_{600}) and (B) volume-length mean diameter ($d_{4,3}$) of dispersions after heating at 90 °C for 30 min and cooling to room temperature. Emulsions were prepared with 1.0−15.0% (w/w) AMF and an aqueous phase with 10−40% (w/w) Tween 80 dissolved in a 0.8 M NaCl solution. Samples that exhibited creaming within 10 min after thermal treatment are not plotted in panel B. Error bars are standard deviations from duplicate samples.

It is worth noting that the stability of surfactant-based systems upon dilution is an important attribute. For the systems with lemon oil emulsified with Tween 80,[14] samples containing relatively high surfactant concentrations after thermal treatment exhibited increased turbidity after dilution. The observations were attributed to the instability of microemulsion subjected to compositional changes. In the present study, all transparent samples remained stable upon dilution at room temperature. The differences between the two studies perhaps are related to the interface of lemon oil/water being more fluidic, while the interface between solid lipid core and water is more rigid in our study. Droplets composed of solid lipids also are less susceptible to emulsion instability mechanisms, such as coalescence and Ostwald ripening. The structure and storage stability of NLCs are presented in the following sections using emulsions prepared with 10% (w/w) AMF and an aqueous phase with 30% (w/w) Tween 80 in 0.8 M NaCl, after heating at 90 °C for 30 min.

Structure of NLCs Studied by AFM, XRD, and DSC. *AFM.* Figure 5A displays an AFM image of NLCs in a transparent dispersion. Particles were discrete and mostly spherical. When compared to particle size distribution (Figure

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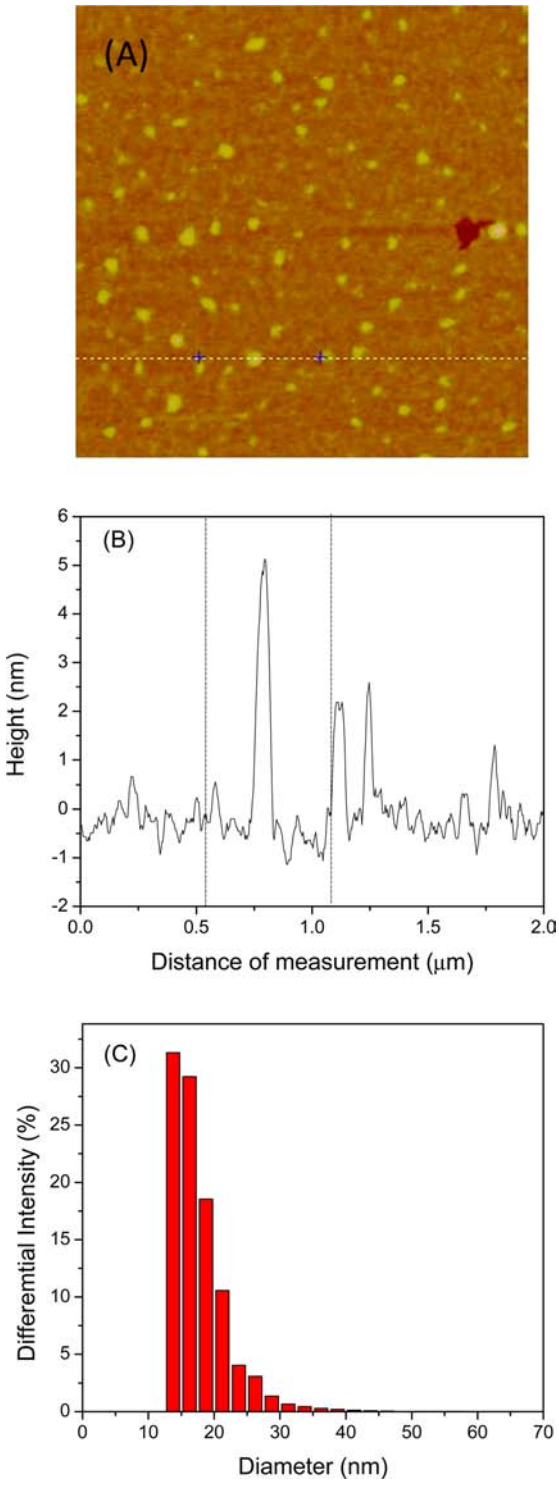

Figure 5. (A) AFM topographic image with a dimension of 2 × 2 μm and (B) height distribution of particles along the measurement line. Particle size distribution of NLCs from light scattering is plotted in panel C for comparison. The NLCs were prepared by heating at 90 °C for 30 min using an emulsion prepared with 10% (w/w) AMF in an aqueous phase with 30% (w/w) Tween 80 dissolved in a 0.8 M NaCl solution.

5C), particle heights measured by AFM (<5 nm; Figure 5B) were much smaller, as reported in other studies.[25−27] The difference may be due to the underlying theory for the two methods. An assumption used in dynamic light scattering (DLS), "infinite dilution",[28] is hard to fulfill. If interactions among particles occur, common for emulsion systems, the interaction may contribute toward the diffusion coefficients obtained from DLS. Thus, the estimation of the particle size obtained by the Stokes−Einstein equation may be overestimated. Additionally, the dimension occupied by particles may decrease after drying the NLC dispersion for AFM.

XRD. XRD is a convenient technique not only for characterizing NLCs[29,30] but also for confirming the encapsulation of lipophilic compounds,[31] especially β-carotene, which is highly crystalline.[32,33] AMF also has crystalline structures below its melting temperature.[34] Because AMF is composed of a mixture of lipids, its crystalline structures are dependent upon the temperature and cooling rate.[35,36] Figure 6

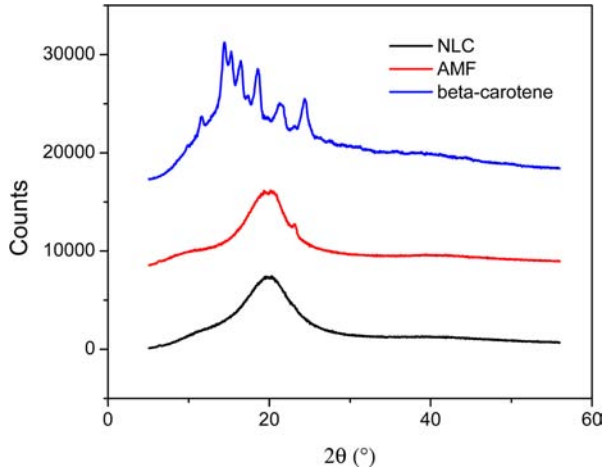

Figure 6. XRD patterns of pristine β-carotene (blue), AMF (red), and β-carotene-loaded NLCs (black). NLCs were prepared by heating at 90 °C for 30 min using an emulsion prepared with 10% (w/w) AMF in an aqueous phase with 30% (w/w) Tween 80 dissolved in a 0.8 M NaCl solution.

shows the XRD spectra of β-carotene, AMF, and β-carotene-loaded NLCs. β-Carotene showed several sharp peaks, indicating crystallinity, while AMF possesses smaller and broader peaks, indicating that AMF has mixed morphology at room temperature. It has previously been suggested that amorphous morphology is dominant, but the crystalline structure is also present in AMF at room temperature.[37] For NLCs loaded with β-carotene, because Tween 80 is a noncrystalline compound at ∼20 °C,[38−40] the smooth XRD curve of the β-carotene-loaded NLCs and its similarity to the spectrum of AMF suggest that β-carotene was embedded in NLCs.[41] Detailed structures of AMF crystals however were not studied in the present work, because it requires temperature-controlled small-angle XRD.[42−44]

DSC. Thermograms of AMF and NLCs during the heating and cooling cycle are shown in Figure 7. Bulk AMF with and without β-carotene showed similar thermograms. During heating, multiple melting temperatures of AMF were observed at about 8.7, 15.3, and 24.1 °C, with the last peak extending to 36.4 °C (Figure 7A). During the subsequent cooling process, the crystal formation started at around 17.6 °C and the crystallization of bulk AMF occurred mostly at around 5.0 °C (Figure 7C). These results indicate that β-carotene does not affect the crystallization and melting temperatures of AMF but may reduce the formation of lipid crystals. The DSC results of bulk AMF agree with previous studies.[45,46] Multiple melting

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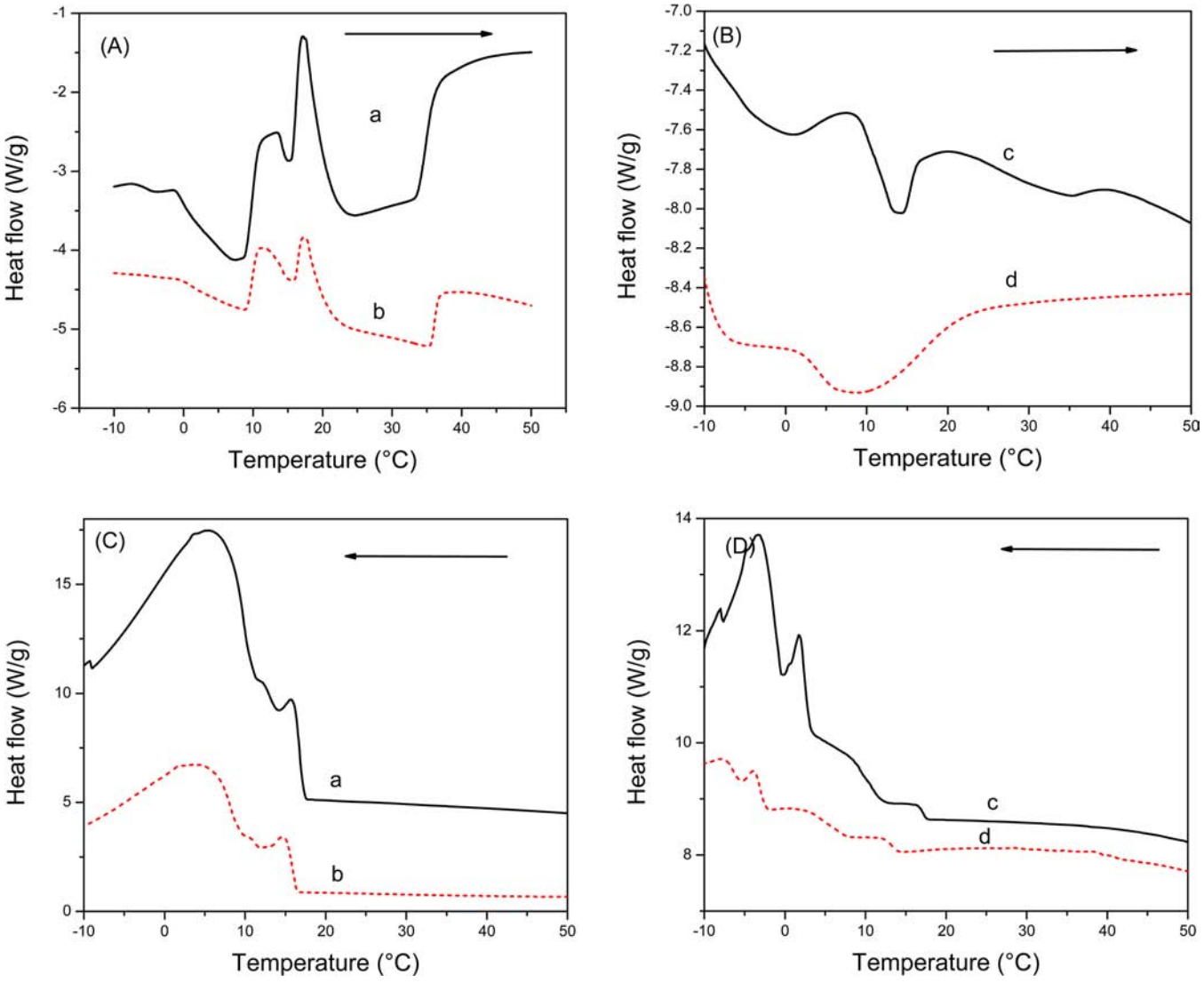

Figure 7. Thermograms of (A) AMF, (B) AMF dissolved with β-carotene, (C) NLCs, and (D) NLCs loaded with β-carotene during heating from −15 to 90 °C at 5 °C/min (A and B) and cooling back to −15 °C at 20 °C/min (C and D), shown from −10 to 75 °C.

and crystallization temperatures suggested in Figure 7 are due to the fact that AMF is a mixture of triglycerides containing more than 400 fatty acids, both saturated and unsaturated, including oleic, palmitic, myristic, and stearic acids.[47]

The heating curve of NLCs without β-carotene showed strong endothermic peaks at 15.3 and 1.3 °C and a minor endothermic peak at about 35.4 °C (Figure 7B). In the subsequent cooling step (Figure 7D), the NLCs showed an initial crystal formation temperature similar to that of bulk AMF (Figure 7C). The major exothermic peak of NLCs occurred at around −3.2 °C, much lower than that of bulk AMF (panel C versus panel D of Figure 7). For NLCs loaded with β-carotene, the major endothermic peaks during heating occurred at 8.7 and −5.3 °C, much lower than those of NLCs without β-carotene (Figure 7B), and the minor endothermic peak at 35.4 °C observed for NLCs, however, was not present after loading with β-carotene. In the following cooling step, the initial crystal formation temperature for NLCs loaded with β-carotene, at around 14.5 °C (Figure 7D), was also lower than 17.6 °C of the NLCs without β-carotene (Figure 7C). Different from bulk AMF, loading β-carotene strongly affected the crystallization and melting properties of NLCs. The retardation of crystal formation in NLCs by β-carotene agrees with other studies after loading drugs in NLCs.[48] The broadened peak and reduced entropy of NLCs after the addition of β-carotene (Figure 7B) also indicate the less ordered arrangement of lipid structures.[49] The observation of the lower solidification temperature of NLCs than that of bulk AMF also agrees with the literature,[50] because supercooled melts can exist in NLCs.[51] The DSC (Figure 7) and XRD (Figure 6) results suggest that AMF in NLC dispersions does not have extensive ordered crystalline structures but is present as a mixture of solid- and liquid-state lipids at room temperature (21 °C) in the present study. As discussed previously, NLCs have advantages in encapsulation when compared to SLNs with highly ordered lipid crystals.[11]

Physical Storage Stability of NLCs. Figure 8 contains Abs_{600} and $d_{4,3}$ values of emulsions, stored at room temperature (21 °C) for 90 days, for NLC dispersions with and without dialysis. The initial $d_{4,3}$ and Abs_{600} of the emulsion with NaCl were 25.5 ± 3.0 nm and 0.13 ± 0.02, respectively. During the storage, there was no significant changes in the Abs_{600} and $d_{4,3}$ ($p > 0.05$), which were 25.4 ± 1.3 nm and 0.10 ± 0.00, respectively, at the end of the storage. Furthermore, dialysis did

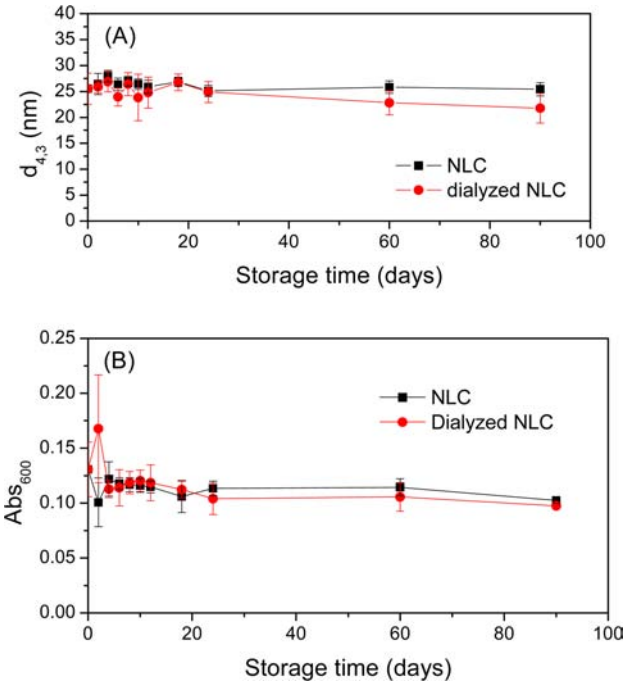

Figure 8. Changes of (A) volume-length mean diameter ($d_{4,3}$) and (B) absorbance at 600 nm (Abs_{600}) of NLC dispersions, with and without dialysis, during 90 days of storage at room temperature (21 °C). NLCs prepared by heating at 90 °C for 30 min using an emulsion prepared with 10% (w/w) AMF in an aqueous phase with 30% (w/w) Tween 80 dissolved in a 0.8 M NaCl solution. Error bars are standard deviations from duplicate samples.

not change Abs_{600} and $d_{4,3}$ of samples and their stability during storage. Therefore, the AMF-based NLC dispersions possess excellent physical stability during storage, which again can be attributed to the elimination of coalescence and Ostwald ripening because of use of solid lipids as the dispersed phase.

Stability of β-Carotene during Storage. Figure 9 shows changes in the antioxidant potential and concentration of β-carotene loaded in NLCs during 16 days of storage at room temperature (21 °C). To study the influence of physical structures of lipid droplets on the stability of loaded bioactive compounds, an emulsion control was prepared by substituting AMF with soybean oil that has a similar average fatty acid chain length as AMF. The $d_{4,3}$ of soybean oil nanoemulsion (29.8 ± 0.0 nm) was comparable to that of NLCs and was stable after 16 days of storage (30.3 ± 1.0 nm). The net antioxidant potential of β-carotene in nanodispersions was determined by subtracting the measured potential of the corresponding nanodispersion without β-carotene at day 0. Both net antioxidant properties and concentration of β-carotene gradually decreased during storage, but β-carotene encapsulated in NLC treatment showed much slower degradation than its encapsulation with soybean oil. By the end of storage, about 94.8% loss of the β-carotene concentration and 97.4% loss of the net antioxidant potential were observed for the latter system. In contrast, the concentration and net antioxidant potential of β-carotene encapsulated in NLCs only decreased by 47.3 and 66.7%, respectively. The concentration change of encapsulated β-carotene (Figure 9B) followed the first-order kinetics, as reported previously for β-carotene in safflower seed oil.[52] The results indicate the effectiveness of NLCs in reducing the degradation of encapsulated bioactive compounds during storage.

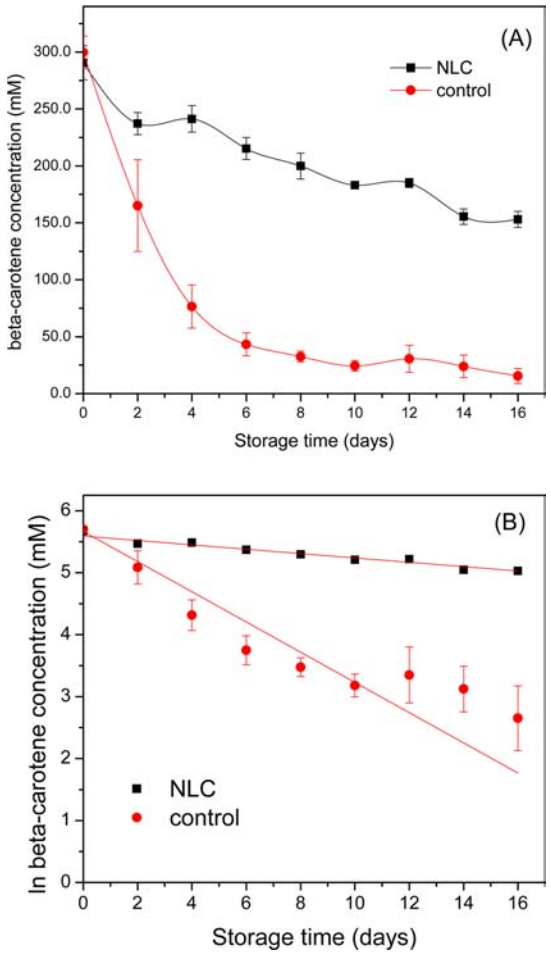

Figure 9. Changes of (A) antioxidant properties of β-carotene-loaded NLC dispersion and (B) concentration of β-carotene, in natural logarithm values, during storage at room temperature (21 °C), in comparison to a control with β-carotene encapsulated in soybean-oil-based nanoemulsion. The numbers in panel A are subtracted by the corresponding dispersion without β-carotene. Error bars are standard deviations from duplicate samples.

To prove that the reduced degradation of β-carotene in NLCs than in soybean oil nanoemulsion is due to the lowered mobility in the amorphous lipid core, 0.0125 mM β-carotene was dissolved in soybean oil or AMF and stored at 65 °C and room temperature (21 °C) for 16 days. The absorbance at 450 nm was measured during storage to monitor changes in the β-carotene content, with the corresponding lipids as a control. β-Carotene degraded much faster in melted AMF (79.0%) than in soybean oil (28.9%) at 65 °C after 16 days, suggesting soybean oil as having better antioxidant properties. At 21 °C, β-carotene did not show obvious degradation in both lipids during storage. Because soybean oil has better antioxidant properties, it suggests that the amorphous solid state of AMF reduces the mobility of molecules and free radicals to reach similar stability as β-carotene in soybean oil. The degradation tests in bulk lipids further support the conclusion that the enhanced stability of β-carotene in NLCs is due to the amorphous lipid structures of the AMF core.

Advantages of the PIT Method for Preparation of Transparent NLC Dispersions. The PIT method does not require specialized equipment, such as high-pressure homogenizers, which reduces capital, maintenance, and operation

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costs. Because heating is also needed in HPH to melt solid lipids, the PIT method may be more cost-effective than HPH to prepare SLNs and NLCs. The SLNs prepared by HPH are typically bigger than 100 nm, and dispersions may not be transparent.[53−55] The catastrophic phase-inversion method is another low-energy method used to prepare nanoemulsions with droplet diameters of 100−200 nm[56] and may be studied for the possibility of preparing SLNs and NLCs. As discussed previously, the use of NaCl in the present work replaces propylene glycol that was used to prepare transparent emulsions of lemon oil using the PIT method.[14] Because the NLCs are dialysis-stable (Figure 8), NaCl in transparent dispersions can be removed by diafiltration, an industrial process that is used in whey protein purification, and the permeate with NaCl and possibly free surfactants can be reused to prepare NLCs to reduce the production cost.

In summary, the present study demonstrated the feasibility to prepare transparent dispersions of NLCs composed of AMF as the solid lipid dispersed phase and Tween 80 as a non-ionic surfactant using the PIT method. Thermal treatment conditions, salinity, and surfactant/oil ratio all played important roles in the effectiveness of transforming turbid emulsions into transparent emulsions after heating. An increase of salinity lowered the PIT, thus favoring the formation of transparent NLC dispersions. A higher surfactant concentration enabled the incorporation of a higher amount of AMF in transparent NLC dispersions. NLC dispersions remained stable upon dilution and showed practically no changes in particle size and turbidity during storage for 90 days at 21 °C. β-Carotene was successfully encapsulated in NLCs using the PIT method and had much better stability in NLCs than in soybean-oil-based nanoemulsions. Therefore, the simple PIT method and AMF can be used to form stable and transparent NLC dispersions for incorporation of a variety of lipophilic bioactive compounds in functional beverages.

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Notes

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