Transparent Dispersions of Milk Fat-Based Solid Lipid Nanoparticles for Delivery of beta-Carotene

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Linhan Zhang

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ABSTRACT

Solid lipid nanoparticles (SLNs) are a category of delivery systems applicable to various bioactive compounds in the food industry. Compared to conventional emulsions that have a fluidic oil phase, the mobility and release of bioactive compounds can be controlled by encapsulation in the solid lipid matrix with appropriate properties. Common approaches of preparing SLNs are high energy methods and solvent evaporation methods, which have can lead to degradation of compounds during processing and residues of organic solvent, respectively. In this thesis, a low energy approach based on the phase inversion temperature method has been used to prepare SLNs based on anhydrous milk fat (AMF). Food grade surfactant Tween 80 was used as a surfactant, and beta-carotene was used as a model lipophilic bioactive compound. AMF and surfactant solution with 0-1.0 M NaCl were mixed to form coarse emulsions that were heated at 80-95 °C [Celsius degree] for 30 min to induce phase inversion, followed by a fast cooling process in ice bath. The phase inversion temperature decreased from >95 °C to 73°C when NaCl increased from 0 to 1.0 M in the aqueous phase. Up to 10% w/w of AMF can be encapsulated in the system as transparent dispersions, with particle mean diameter smaller than 25 nm. The SLN dispersions were dilution and dialysis stable, and the particle size and turbidity maintained unchanged during the 90-day storage at room temperature. Compared with beta-carotene encapsulated in soybean oil-based nanoemulsion, degradation of beta-carotene in SLNs was much reduced. The studied SLNs may find unique applications in incorporating lipophilic bioactive compounds in transparent beverages.

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CHAPTER I

Introduction and Literature Review

ABSTRACT

SLNs are nanoparticles that contain solid lipid cores. Like nanoemulsions, SLNs have a lot of advantages, for example, more stable, have better bioavailability, and have a relatively transparent appearance. Compared to nanoemulsions that have a fluidic oil phase, the mobility and release of bioactive compounds in SLNs can be controlled by encapsulation in the solid lipid matrix with appropriate properties. Approaches of preparing SLNs, high energy methods and low energy methods, and microemulsion based SLNs, have been compared and discussed in this review. Many factors may affect the stability of SLNs. Parameters used to characterize the stability of SLNs including particle size, zeta-potential, thermal and crystallization of lipids, and so on. In this literature review, bioactivity and bioavailability of bioactives incorporated in SLNs were also discussed. The studied SLNs may find unique applications in incorporating lipophilic bioactive compounds in transparent beverages.

1. Introduction

Various delivery systems have been studied in recent years to incorporate lipophilic bioactive compounds for improved distribution, increased stability during processing and storage, and enhanced bioactivity or bioavailability [1]. Oil-in-water (O/W) emulsions are commonly studied because the lipid core can serve as a carrier of lipophilic bioactives. Nanoemulsions have received extensive attention because, when compared with conventional emulsions that have a mean diameter in the range from 200 nm to 100 μm, the much smaller diameters (about 10-200 nm) of nanoemulsions offer several advantages [2]. When particles are sufficiently small, thermal energy becomes more significant than gravitational energy. Therefore brownian motion is the main underlying force that cause the sedimentation due to density difference between the dispersed and continuous phases, which extends the shelf-life [3]. Unlike the turbid appearance of conventional emulsions, nanoemulsions are usually translucent or transparent, due to the much reduced ability of nanoparticles to scatter visible light. As delivery systems, studies have shown the enhanced stability and bioavailability of bioactive compounds like beta-carotene, polymethoxyflavones, resveratrol, curcumin, and dibenzoylmethane after encapsulation in nanoemulsions [4].

However, nanoemulsions have several limitations. For example, they are often stable only under a particular set of conditions and may be disintegrated upon dilution [5]. Like conventional emulsions, coalescence and Ostwald ripening can result in the destabilization of nanoemulsions. When used to encapsulate compounds that can be degraded by environmental factors such as oxygen and light, the liquid state of the lipid core allows the diffusion of compounds to oil droplet surfaces where many degradation reactions occur, which is more

problematic for nanoemulsions because the small dimension of nanodroplets reduces the time scale to diffuse to droplet surface and provides a large surface area for reactions [6].

Solid lipid nanoparticles (SLNs), possessing a solid lipid core, offer unique features overcoming some limitations but still maintain the advantages of nanoemulsions with a liquid core. Bioactive compounds in the solid lipid core have limited mobility and therefore lowered rate of diffusion to the particle surface [7]. This can reduce the degradation of bioactive compounds by reactions such as exchange oxidation and therefore increase the chemical stability (Figure 1.1.). The protective properties of the incorporated active compounds of SLNs have been demonstrated for co-Q10 [8]. Finally, the solid state of lipid core can limit coalescence to improve physical stability during shelf-life storage. In this chapter, methods of preparing SLNs are discussed, followed by typical parameters used to characterize SLNs, before advancing to reviews about incorporation and release properties of bioactive compounds. Factors affecting the stability of SLNs, including physicochemistry of lipids and surfactants and preparation condition, are also discussed.

2. Preparation of SLNs

Various methods have been developed to prepare SLNs. Most techniques established to prepare nanoemulsions, high energy methods and low energy methods, can be used to prepare SLNs. In addition, SLNs can be manufactured by forming thermodynamically stable water-in-oil microemulsions at a temperature corresponding to the liquid state of lipids, followed by cooling to solidify the lipid core [2]. The choice of a preparation method depends on the properties of lipids and surfactants to be used and the applicability to bioactive compounds to be encapsulated.

2.1. High energy emulsification methods

The most common methods used to prepare SLNs are high energy methods, similar to those employed to prepare conventional nanoemulsions [9]. Coarse emulsions are initially prepared and a mechanical device is then used to generate intense forces to disrupt the large oil droplets to fine ones. The established high-energy devices include high pressure homogenizers, microfluidizers, and high power sonication systems. With respect to the melting temperature of lipids, high energy methods can be classified as being either melt (hot) and cold ones.

2.1.1. High pressure homogenization (HPH) method

HPH is a suitable method for preparation of SLNs. This method generates a disrupt force through a high pressure, and then the very high shear stress and cavitation forces disrupt the particles to very small size [10]. Steps of preparing SLNs using melt and cold homogenization methods are summarized in Figure 1.2. [9].

In melt, or hot, homogenization, bioactive compounds are dissolved or dispersed in melt lipid(s) first, and the hot lipid phase is mixed with a hot aqueous surfactant solution and stirred to form a coarse emulsion. The quality of coarse emulsion can significantly affect the quality of a final product. The coarse emulsions are then fed to a high pressure homogenizer and processed to fine nanoemulsions at temperatures above the melting point of lipids. SLNs are finally produced by cooling the nanoemulsions under controlled conditions [11]. The diameter of SLNs processed using this method is about 80-300 nm, depending on chemistry and concentration of surfactants and lipid phase, temperature, and the pressure and number of passes during homogenization [12]. The average size of SLNs may be increased by lipids by using higher melting temperatures and higher lipid concentration [13]. In general, the higher the temperature used in HPH, the smaller the

particles size can be obtained [14]. This occurs because high temperatures decrease the viscosity of the dispersed phase [15].

The hot HPH method is an effective approach to decrease particle size when preparing SLNs. However, its high temperature and shear stress have been assumed to be the major cause of free radical formation, which can cause the degradation of incorporated drug [15]. When a bioactive compound is extremely temperature-sensitive, cold homogenization technique may become a more viable option [12b]. To support, there are also studies reporting the activity of peptides did not change after cold homogenization [16]. In this approach, lipids are heated and then mixed with bioactive compounds, followed by cryo-processing in liquid nitrogen or dry ice. The frozen mixture is then milled to reduce dimension, followed by its dispersal in a cold surfactant solution and subsequent HPH treatment to form SLNs [17]. Besides reducing the degradation of encapsulated compounds, cold homogenization has two addition advantages. First, it can prevent the formation of super-cooled melts that are liquid lipids at temperatures below the crystallization point. Super-cooled melts formed due to the small particle dimension and the effect of surfactant(s). This is because lipids are precooled to form solids before HPH. Second, cryo-processing solidifies the melt rapidly, which results in a homogenous distribution of bioactive compounds in the lipid phase and avoids the expulsion from the lipid core as observed in melt homogenization [18]. Effects of HPH on the structure of SLNs and encapsulated compounds are discussed in greater details below.

2.1.2. Combination of high shear homogenization and ultrasonication methods

High shear homogenization and ultrasound methods are other high energy methods that can be combined to prepare SLNs at a temperature above the melting point of lipids. Both

methods are well developed and easy to handle. However, metal contamination due to ultra-sonication is a potential problem [19]. Typically, a coarse emulsion is first prepared with an ultrasonic instrument, and high speed stirring is used to further reduce particle size [20]. It was also reported that a higher shear rate during homogenization did not significantly change particle size but decreased the polydispersity index of emulsions [21]. In another study, Sliva et al. [22] compared risperidone SLNs prepared by hot HPH and ultrasound technique. The authors did not observe significant difference in the dimension and shape of particles prepared by the two methods. SLNs produced by HPH however demonstrated better physical stability and a higher drug-loading capacity.

2.1.3. Supercritical fluid extraction methods

A novel supercritical fluid extraction of emulsions (SFEE) method has also been developed to produce SLNs to encapsulate drugs [23]. This method is based on the principle that supercritical fluids can extract an organic solvent from a pre-formed lipid dispersion [24]. A carrier lipid and a lipophilic drug are first dissolved in chloroform, which is then mixed with an aqueous phase with surfactants. The coarse emulsion can be processed to fine oil droplets by HPH. The fine emulsion is then sprayed into a continuous extraction chamber for extraction by a counter-flow supercritical carbon dioxide (Figure 1.3.). SLNs with a narrow size distribution and a mean diameter below 30 nm were reported, and the residual solvent content in the final dispersion was consistently low (below 20 ppm). For food applications, the organic solvent may be adopted for generally-recognized as safe ethanol and others that can be used as processing aides.

2.2. Low energy emulsification methods

In low-energy approaches, the formation of tiny oil droplets within oil-water-emulsifier mixture systems is spontaneous when the composition or environmental conditions are altered [2]. In the solvent evaporation method, lipids and the compound to be encapsulated are first dissolved in an organic solvent such as ethanol and acetone. After formation of a coarse emulsion, the organic solvent is evaporated, e.g., at a temperature above the melting point of lipids, to reduce the particle size, followed by cooling to form SLNs [25]. The approach has also been used to prepare W/O/W SLNs [26]. A problem of this method is the low concentration of lipids in preparation, because a limited concentration of lipids can be dissolved in the organic solvent. The organic solvent residue is another limitation for food applications.

Theoretically, all low energy approaches used to prepare nanoemulsions can be used to prepare SLNs by adopting a cooling step. For example, the phase inversion temperature method, which involves a heating step and another cooling process, may be adopted to prepare SLNs. However, these low energy methods are not well studied for the preparation of SLNs.

2.3. Microemulsion-based SLNs

Besides adopting techniques used to prepare nanoemulsions, SLNs can be developed from microemulsions. Principally, warm microemulsions are first prepared with a melt lipid core, followed by dilution into cold water under stirring to solidify the lipid core in order to form SLNs. The diluted emulsion can be concentrated by ultrafiltration or freeze drying [27]. Microemulsion-based SLNs preparations do not involve high energy to reduce the particle size, which is adventages to sensitive bioactive compounds. However, a high surfactant concentration

is required to prepare microemulsions, and the final process of concentrating SLNs may promote the aggregation of SLNs and thus affect their dimension [28].

3. Factors affect stability of SLNs and their measurements

Stability of SLNs system is affected by many factors, including chemical and thermal properties of lipid phase, type and amount of surfactant, and preparation and storage conditions, which will described below. To characterize the effects of these factors, particle size, zeta potential and other measurements are used. The adequate characterization of these parameters of SLNs is also significant to control the quality of product.

3.1. Particle size

Particle size affects both the stability and appearance of colloidal systems. Typically, colloidal systems with small particle size are more stable and have clearer appearance [29]. For example, delivery systems with particle diameters less than 60-80 nm are transparent but become turbid or opaque at larger particle size [30]. Thus, optical clarity can be used to characterize the stability of systems with small particles [5]. Many factors affect the dimension of SLNs. The choice of lipid(s) is one of the key parameters in controlling the properties and structure of SLNs. For example, the average size of SLNs can be decreased by lipids with higher melting temperatures [13a]. This is due to the higher viscosity of lipid core during hot homogenization. Increasing the lipid content also increases particle size and broadens the particle size distribution [13b].

In emulsion systems, surfactant type and concentration also have a great effect on particle size and storage stability. Surfactants decrease interfacial tension and provide sufficient repulsive interaction forces to prevent flocculation and coalescence. For SLNs, especially thosed formed

by melt methods, the effect of emulsifier is of the same importance to properties of SLNs as to nanoemulsions. For tripalmitin SLNs prepared using melt HPH method [31], when stored at a temperature lower than 20 °C, gelation of SLNs stabilized with modified starch was retarded compared with those coated with Tween 20. This is due to the formation of a thick polymeric layer around the lipid particles that inhibited partices from aggregating. But the gelation would still occur eventually for SLNs coated with modified starch. While when stored at 37 °C, SLNs coated with Tween 20 showed a longer stability against aggregation and gelation, which may due to the not fully crystallized lipid core compared with the fully crystallized lipid core of modified starch coted SLNs.

Other preparation conditions may also affect the physical stability of SLNs. SLNs with Compritol 888 ATO as lipid carrier were prepared using hot HPH method [32]. The energy introduced to this system (temperature, light) led to particle size growth and gelation, also to an increase in zeta potential. On the other hand, packing material (siliconized vials of glass quality) did not show pronounced effect on the stability of SLNs.

Dynamic light scattering (DLS) is the most commonly used method to measure particle size of colloidal systems. In DLS, the fluctuation of the intensity of the scattered light, due to the Brownian motion of particles, is measured [33], and the hydrodynamic radius of individual particles can be determined via the Stokes-Einstein equation [34]. The hydrodynamic radius of a dispersion can be obtained as the average of individual hydrodynamic radii after weighing their scattered light intensities [35]. This method can be used to measure particles with a dimension from several nanometers to about 3 micrometers [9] but is inappropriate for larger particles.

However, it should be noted that DLS does not measure particle size directly and the assumption of "infinite dilution" [33] is difficult to fulfill. Therefore, additional techniques are recommended to complement DLS studies, for example, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM). The advanced microscopy techniques provide not only particle dimensions but also additional information such as particle morphology.

3.2. Surface charge

Many polymers used to prepare emulsions, for example, proteins and polysaccharides, are charged. Information of the electrical charge of particles in a delivery system is of great importance. Particle charge affects interpartilce against aggregation, binding with biological surfaces, and interactions with other ingredients in a food matrix [29b].

Surface charge properties of SLNs are important to the stability because of their significance on the long-range electrostatic repulsion between SLNs. Surface charge of a colloidal system is usually measured for zeta potential. Generally, dispersions with a zeta-potential magnitude higher than 30 mV are physically stabilized by electrostatic repulsion alone [36]. A zeta-potential as low as 8-9 mV in combination with a steric stabilization was also reported to stabilize SLNs in artificial gastrointestinal media [37].

3.3. Crystallization and thermal properties

Lipids used to prepare SLNs include triglycerides (e.g. tristearin), partial glycerides (e.g. Imwitor), fatty acids (e.g. stearic acid), steroids (e.g. cholesterol), and waxes (e.g. cetyl palmitate) [38]. Thermal properties and crystalline characteristics of bulk lipid and prepared SLNs are of great importance, because these parameters are strongly correlated with the incorporation

and release rates of bioactive compounds [39]. The melting point of core lipid also influences the preparation temperature, the cooling temperature, and cooling rate in the melt HPH method. The lipid composition may also affect loading parameters [7, 13b].

Crystallization behavior of lipids in SLNs is different from bulk lipids. The solidification temperature of lipid core in SLNs may be lower than that of the bulk lipids [9]. Nanoparticles prepared from bulk lipids that are solid at room temperature can exist as super-cooled melts at room and even refrigeration temperatures over several months [40]. Thus, some nanoparticles prepared from lipids that are solid at room temperature may not be considered as SLNs due to the present of super-cooled melts at room and refrigerator temperature.

The main reason of formation of supercooled melts is the nano-scale size of SLNs. The presence of nuclei is a prerequisite of crystallization [41], and the formation of a sufficient number of nuclei is less possible in nanoparticles. Besides the small size of nanoparticles, surfactants play an additional role in controlling crystallization process because the number of lipid molecules interacting with the hydrophobic emulsifier tail groups can be large enough to modulate the crystallization properties. Thus, surfactant chemistry can affect both melting point and melting enthalpy of lipids [42]. Saturated phospholipids can increase the crystallization temperature, thus promote crystallization of SLNs at a higher temperature during cooling process[42]. Egg lecithin was also reported to induce crystallization at higher temperatures than soybean lecithin [43].

The lipid composition also determines polymorphisms of lipid crystals (Figure 1.4). The β-form crystals are thermodynamically more stable than the α-form crystals and have the highest melting point [13b]. Lipids tend to transform from α to β polymorphic form during storage. This

transformation of the lipid crystal leads to an overall morphological change of the lipid particles from a spherical to a needle-like shape, which increases the total surface area. During the transformation, non-polar lipid surfaces are exposed to water, which, if surfactant present is insufficient, can causes aggregation of lipid particles to reduce the unfavorable contact between oil and water [44]. As described above, this process can cause precipitation or gelation after certain storage period. To prove this mechanism, additional surfactant was added to the aqueous phase prior to crystallization of the lipid phase but after the homogenization [45]. In this way, droplet size was maintained because additional surfactant can interact with the newly formed surfaces during transformation of crystal polymorphisms, and the SLNs dispersions remained fluid-like, contrasting with the gel formation of the control group.

Chemical properties of surfactant, lipid phase, and preparation conditions all have an effect on the polymorphic transition behavior of lipids. Mixing tripalmitin with medium chain triglycerides or short chain lipid (orange oil) can slow the transition process and improve the stability of SLNs against particle aggregation and gelation [31]. The lipid transition would also related to surfactant type used. SLNs prepared form high melting surfactants (high melting lecithin and Tween 60) had a higher α crystal fraction than SLNs prepared form low melting surfactants (low melting lecithin and Tween 80) [46]. The polymorphic transitions of triglycerides can be slowed by the presence of saturated lecithin as emulsifier [43]. When preparing SLNs with hot HPP approaches, cooling and heating conditions are important parameters to control crystal structure. Figure 1.5 shows the effect of cooling conditions on crystal structure. The α-form crystals are formed around 43 ℃ while β-crystals are usually formed around 61 ℃ [34]. When cooling speed changes, the crystal type formed can also change. A fast cooling rate favors the formation of α crystals and helps to maintain the spherical structure of SLNs and thus is

preferred for the stability of SLNs. Heating and cooling rates also affect the transition of SLNs from α to β polymorphic forms, which is related to particle aggregation and gelation. A fast cooling rate decreases the gelation temperature during cooling, while a fast heating rate increases the gelation temperature during heating. Fast cooling and heating rates also decrease coalescence enthalpy, but favor the formation of supercooled melt. Thus, careful selections of cooling and heating rates are required to form stable SLNs suspensions [47].

Differential scanning calorimetry (DSC) and X-ray diffraction (XRD) are widely used to investigate the structure of lipids. In DSC, thermal properties are measured based on the fact that lipid structures formed at various conditions possess different melting points and melting enthalpies. Conversely, XRD can used to study the existence and type of crystalline structures [48]. These two methods are usually combined to determine the melting point, melting enthalpy, crystal type, crystal transformation and solid state of lipids in SLNs [49].

4. Release and bioavailability of bioactives incorporated in SLNs

Many SLNs systems have been developed for topical applications, oral administration and parenteral administration of bioactives, listed in Table 1. SLNs systems developed for food applications are rare compared with SLNs for pharmaceutical and cosmetic applications. However, many drugs are loaded in SLNs using food grade lipids and emulsifiers (lecithin, Tween, and so on) [12c], which can be used to guide studies targeted for food applications. Physical stability of SLNs and stability, incorporation, release, and bioavailability of drugs loaded in SLNs are significant parameters to be considered to study these systems.

Three types of SLNs have been developed to incorporate active ingredients: homogenous matrix model, drug-enriched shell model, and drug-enriched core model (Figure 1.6.) [50]. These

models are due to the partitioning of drug between lipid phase and aqueous surfactant phase during particle formation. The homogenous matrix model is thought to be formed when applying a cold homogenization method or when incorporating very lipophilic drugs, while the other two models are obtained due to phase separation and fast precipitation of drugs during the cooling process, respectively [18]. When producing SLNs by hot methods, drugs are first loaded in melt lipids and tend to partition to the aqueous phase. When temperature rises or when surfactant concentration increases, the solubility of drugs in the aqueous phase increases, resulting in the increased drug partitioning to the aqueous phase. During cooling, the solubility of drugs in the aqueous phase decreases and drugs start to repartition to the lipid phase. When temperature reaches the recrystallization temperature, a solid lipid core starts to form, incorporating drugs with an amount depending on the solubility of drugs. Upon further cooling, drugs can continue to partition to the lipid phase but the already-solidified lipid core cannot incorporate anymore drugs, forming the outer shell rich in drugs. Lastly, SLNs with a drug-enriched core are formed when the drug precipitates before lipids. This can occur in the case when dissolving a drug in lipid melts close to its saturation solubility. If drugs supersaturate in the lipid phase during cooling and crystallize before the lipids recrystallize. Further cooling leads to further crystallization of drugs until reaching the recrystallization temperature of lipids. In the final stage, a solid lipid shell forms around the solid core, resulting in SLNs with drug-enriched core [12b].

The properties of drugs being released from SLNs are a function of SLNs structures. When drugs are mostly in the shell (Figure 1.6), burst release is commonly observed [19]; [51]. In contrast, a prolonged release can also be achieved by forming SLNs with drug-rich cores or a homogenous structure [11a, 52].

Particle size (surface area) of SLNs greatly affects the extent of burst release [19, 50]. Reduced burst release was observed with an increase in particle size, and a prolonged release can be obtained when the particle size is sufficiently large [17]. In the drug-enriched shell model (Figure 1.6.), the drug in the outer shell has a relatively short distance of diffusion, resulting in a burst release. This was demonstrated for nisin encapsulated in SLNs, showing a high percentage of release in the first day but a prolonged release through the 25 day period [53]. This enabled the antibacterial activity against *Listeria monocytogenes* and *Lactobacillus plantaru*m for up to 20 and 15 days, respectively, which was much longer than only the one and three-day activity for free nisin [53].

During storage, α and β' type crystals can transform to more stable, ordered β type crystals [13b]. The formation of highly crystallized lipid with perfect lattices can lead to drug expulsion and formation of drug crystals in the aqueous SLNs dispersions, resulting in increased drug degradation [58]. To avoid drug exclusion, increase drug incorporation, and prolong drug release, lipid cores with multiple components can be adopted to increase the distance between fatty acid chains of the glycerides and decreases the perfectness of crystals. Hard fat is a mixture of partial glycerides and liquid fractions. SLNs based on hard fat tend to form the less structured lipid core that increases drug inclusion and avoids drug expulsion [54]. This occurs because the impurities of lipids can retard the recrystallization process. A mixture of solid and liquid lipids can lead to more imperfections in the crystal, and thus can increase drug loading [55]. Some researcher also call this kind of delivery systems nanostructured lipid matrices (NLC) [12b], which are solid but not crystalline. In general, the solubility of drugs in the lipid melt is higher than that in the final solid lipids. A high concentration of drugs leads to immediate drug expulsion during cooling process. Thus, a new SLNs type with drug-loaded liquid lipid dispersed in the solid lipid

core (O/F/W type) has been developed [12b, 56]. An increased drug loading was observed in this delivery systems because of the high solubility of drug in the liquid lipid. At the same time, the solid lipid around the liquid core can still protect drugs from degradation, as in other SLNs systems.

Other factors affecting drug release include drug chemistry, emulsifier type and concentration, the nature of lipid matrix, and preparation methods [51, 57]. SLNs stabilized by high-melting surfactants were observed to protect the encapsulated beta-carotene against chemical degradation better than those with lower melting temperatures [58]. This occured because high-melting surfactants can affect the polymorphic transition behavior of lipids to form more α-crystals that not only improve the chemical stability but also accommodate a higher level of bioactive compounds [46, 58]. The effects of drug chemistry were demonstrated in a study showing burst release of tetracaine and etomidate but prolonged release of prednisolone when these three compounds were incorporated in SLNs using the same production method [11a].

The high temperature used in hot HPH method and high surfactant concentration can increase the burst release, which may be prevented by adopting the cold homogenization method [18]. The burst release can also be reduced by adopting a surfactant that does not dissolve drugs or possibly excluding surfactant in SLNs preparation. Exceptions have also been observed. For example, proteins loaded in W/O/W SLNs prepared with the solvent evaporation method showed the reduced burst release at a higher surfactant concentration [59]. This may have been caused by differences in SLNs structures, W/O/W in this study vs. no aqueous phase in regular SLNs. As for the lipid matrix, the structure can be engineered by blending medium chain triglycerides (liquid oils) and long chain ones (solid fat), also known as NLC [60]. The controlled release can

thus be obtained by a built-in trigger mechanism to initiate the transformation from α to β form crystals [55a, 56].

The controlled release of drugs from SLNs has been studied *in vitro* and *in vivo*. For rifampicin, isoniazid, and pyrazinamide incorporated in SLNs using an "emulsion solvent diffusion" method, drug concentrations were maintained in the plasma for 8 days and in the organs for 10 days following the oral administration to mice. This contrasted with free drugs that were cleared in 1-2 days [25a]. When tested for mice infected by *M. tuberculosis* $H_{37}Rv$, delivery of drugs using SLNs reduced the dosing frequency [25a]. A SLNs formulation of compritol for intravenous injection also showed reduced phagocytic uptake *in vitro* and *in vivo*, corresponding to the prolonged circulation time [61]. Small interfering RNA (siRNA) loaded in SLNs also showed the prolonged release both *in vivo* and *in vitro*, and the *in vitro* cell studies showed that the released siRNA maintained its activity [62]. Idarubicin loaded in SLNs and in the solution form was administered to rats by the duodenal route or intravenously [63]. A prolonged release and higher bioavailability were observed for idarubicin loaded in SLNs when compared to the solution control. For the SLNs treatment, the drug and its metabolite were observed in the brain, which was not observed for the solution treatment. The ability of SLNs passing the blood-brain barrier was also reported in others studies, which suggests that the prolonged circulation time of SLNs causes the accumulation in the brain but not in other organs[11b, 64]. This characteristic, if carefully controlled, can be used for the targeted delivery of drugs using SLNs.

5. Conclusions

SLNs are a group of delivery systems with potential for application in the food industry. SLNs can be prepared using many approaches that are used for conventional nanoemulsions,

with modifications. SLNs can also be prepared from self-assembled microemulsions. The physical stability of SLNs can be controlled for particle dimension and surface charge, as well as the structures of lipid core including the composition and type of crystals. When compared to conventional emulsions, the mobility and release of bioactive compounds can be controlled by the properties of solid matrix and thus the degradation of bioactive compounds due to exchange can be reduced. The release properties of the encapsulated compounds, however, are dependent on the exact structure of SLNs, with burst release commonly observed for the presence of the encapsulated compound mostly in the outer shell and prolonged release corresponding to compounds rich in the lipid core or distributed evenly in the lipid matrix. There are several problems related to the stability and controlled drug release, including lipid crystal transformation, super-cooled melts, drug expulsion and degradation of incorporated drug during processing. These problems can usually be overcome by adopting appropriate lipid compositions, surfactant type and concentration, and preparation conditions.

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Appendix

Table 1.1. Examples of compounds incorporated in SLNs and the surfactants and methods used in preparation.

Incorporated drug	**Emulsifier(s)**	**Lipid(s)**	**Preparation Method**	**Reference**
Tetracaine, etomidate, prednisolone	Poloxamer 188*	Compritol 888 ATO*	High pressure homogenization	[15]
Camptothecin	Soybean lecithin, Poloxamer 188*	Stearic acid	High pressure homogenization	[16]
Camptothecin	Soybean lecithin, Poloxamer 188*	Stearic acid	High pressure homogenization	[67]
Doxorubicin	Epikuron 200*	Stearic acid	Microemulsion	[82]
Retinol	Xanthan gum,	Campritol 888 ATO*, Miglyol 812*	High pressure homogenization	[72], [39], [37]
Retinoic acid	Soy lecithin, Tween 80*	Campritol 888*	High pressure homogenization	[36]
Idarubicin	Epikuron 200*	Steric acid	Microemulsion	[79]

Table 1.1. Continued.

Incorporated drug	Emulsifier(s)	Lipid(s)	Preparation Method	Reference
Retinyl acetate, progesterone, sodium cromoglycate	Gelatin, Polyvinyl alcohol (PVA)	Cetyl alcohol, cholesterol, tristearin, monostearate	Melt dispersion - solvent evaporation	[73]
Cyclic undecapeptide cyclosporin A (CyA)	Epikuron 200*	Stearic acid,	Microemulsion	[83]
rifampicin, isoniazid, and pyrazinamide	PVA	Steric acid	Solvent diffusion	[34]
Bovine serum albumin, Lysozyme, Insulin	Poly (lactic-co-glycolic acid)	Hydrogenated castor oil	Solvent evaporation	[79]
Small interfering RNA (siRNA)	Lecithin, DSPE-Polyethylene glycol	Tristearin, DOTAP(1,2-dioleoyl-3-trimethylammonium-propane	Solvent evaporation	[78]

Table 1.1. Continued.

Incorporated drug	**Emulsifier(s)**	**Lipid(s)**	**Preparation Method**	**Reference**
Beta-carotene	High-melting lecithin, low-melting lecithin, Tween 60*, Tween 80*	Tripalmitin, medium chain triglycerides	High pressure homogenization	[74]
Hesperetin	Tween 80*	Glycerol monostearate, stearic acid	Sonication	[84]
Nisin	Poloxamer 188*, sodium deoxycholate	Cetylpalmitate, Softisan 378*, Softisan 154*, Imwitor 900*	High pressure homogenization	[68]
Beta-carotene	Stearic acid	Lecithin, sodium taurocholate	High shear homogenization	[85]
Retinyl palmitate	Compritol 888 ATO*, palmitic acid	Phosphatidylcholine, cetearyl alcohol and cetearyl glucoside,	Microemulsion based, HPH	[86]
	Compritol 888 ATO*, palmitic acid	Phosphatidylcholine, cetearyl alcohol and cetearyl glucoside,	Microemulsion based, HPH	[87]

Table 1.1. Continued.

*Chemical structures of corresponding commercial names.

Poloxamer 188: poly (ethylene glycol)-*block*-poly (propylene glycol)-*block*-poly(ethylene glycol).

Campritol 888 ATO: Cetyl palmitate, glyceryl behenate.

Epikuron 200: soya phosphatidylcholine.

Miglyol 812: caprylic/ capric Triglyceride.

Tween 80: polyoxyethylene (20) sorbitan monooleate.

Tween 60: polyoxyethylene (20) sorbitan monostearate.

Softisan 378: a blend of triglycerides based on saturated even-numbered, unbranched natural fatty acids of vegetable origin with a chain length of $C_8 – C_{18}$.

Softisan 154: a blend of triglycerides based on saturated even-numbered, unbranched natural fatty acids of vegetable origin with a chain length of $C_{14} – C_{18}$.

Imwitor 900: glycerylmonostearate, 40-55 %.

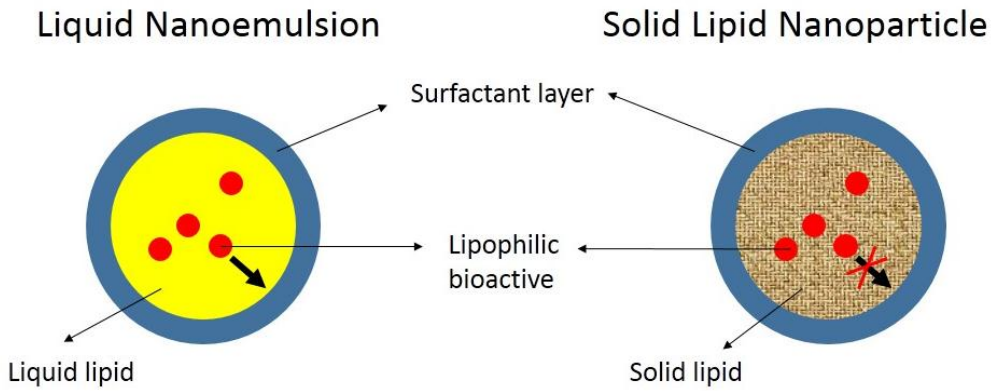

Figure 1.1. Comparison of a liquid nanoemulsion (left) and solid lipid nanoparticles (right) with respect to the stabilization of encapsulated lipophilic bioactive compounds. Redrawn based on [7].

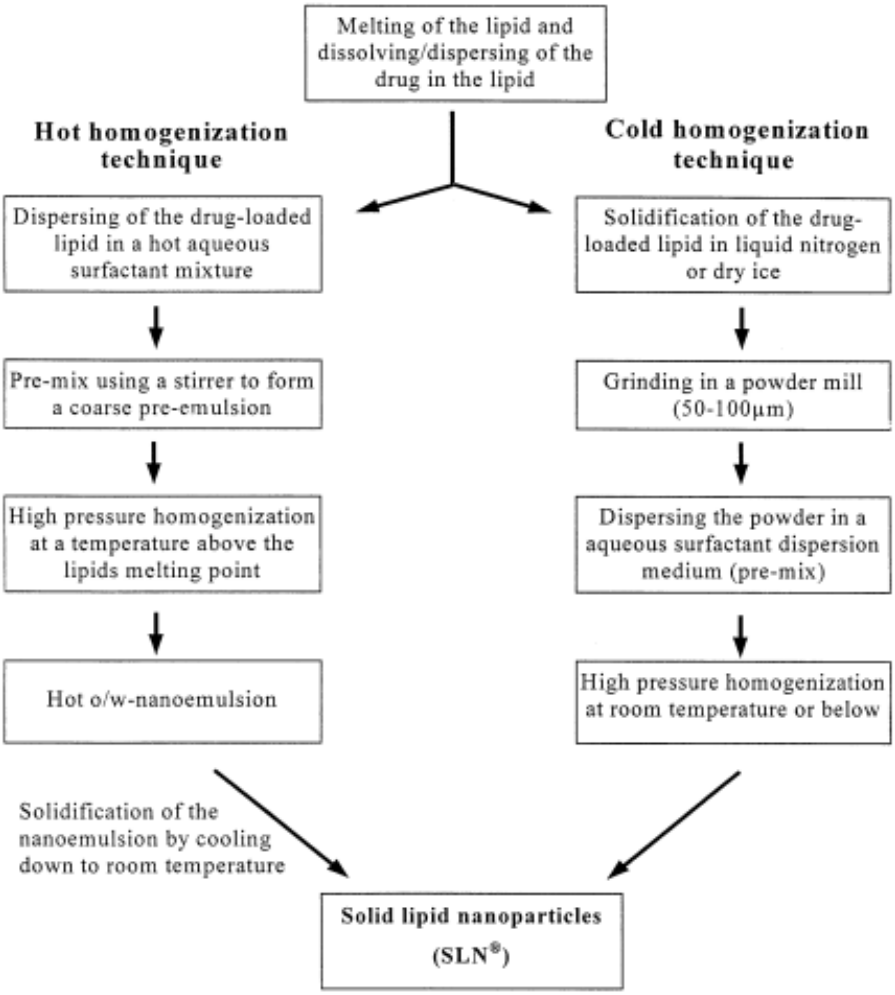

Figure 1.2. Common procedures in melt and cold homogenization approaches used to prepare SLNs. Adapted from Mehert et al. (2001).

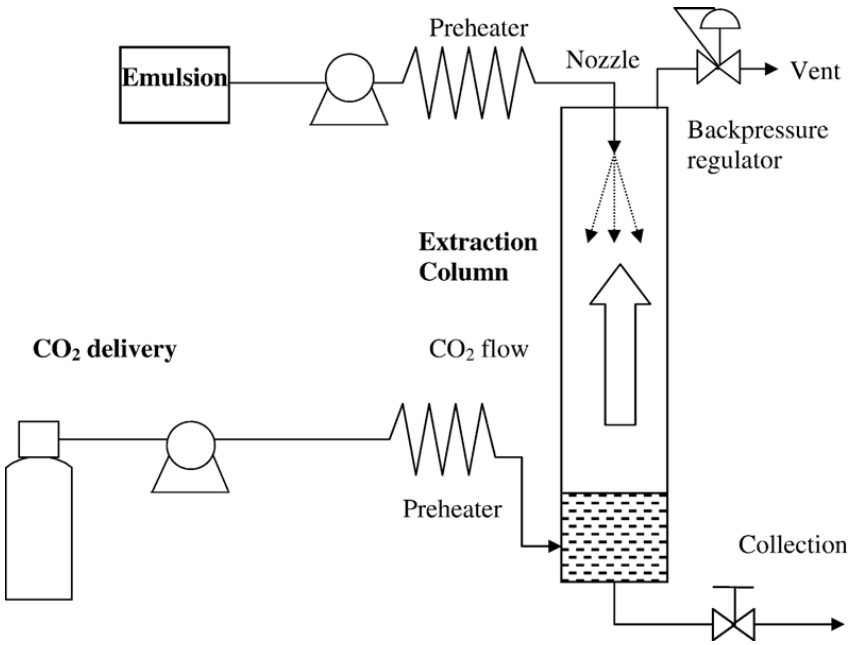

Figure 1.3. Processes used to prepare SLNs by supercritical extraction of emulsion. Adapted from Chattopadhyay, et al., (2007).

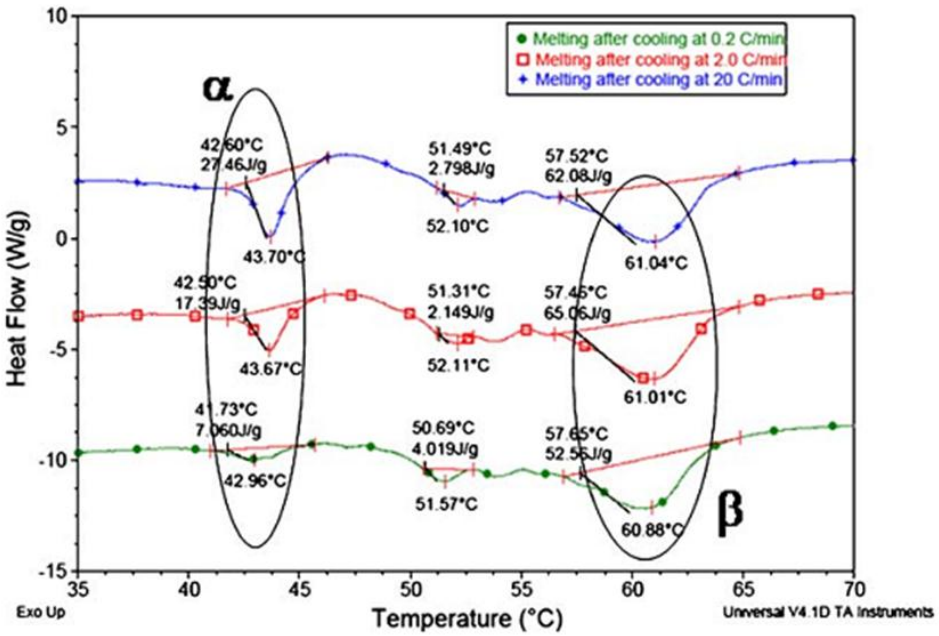

Figure 1.4. Comparison of differential scanning calorimetry patterns of bulk triglycerides and the corresonding nanodispersion during cooling at various rates. Green, red and blue curves represented cooling at 0.2 °C/min, 2.0 °C/min, and 20 °C/min respectively. The figure is adapted from Westesen, Bunjes, & Koch, (1997).

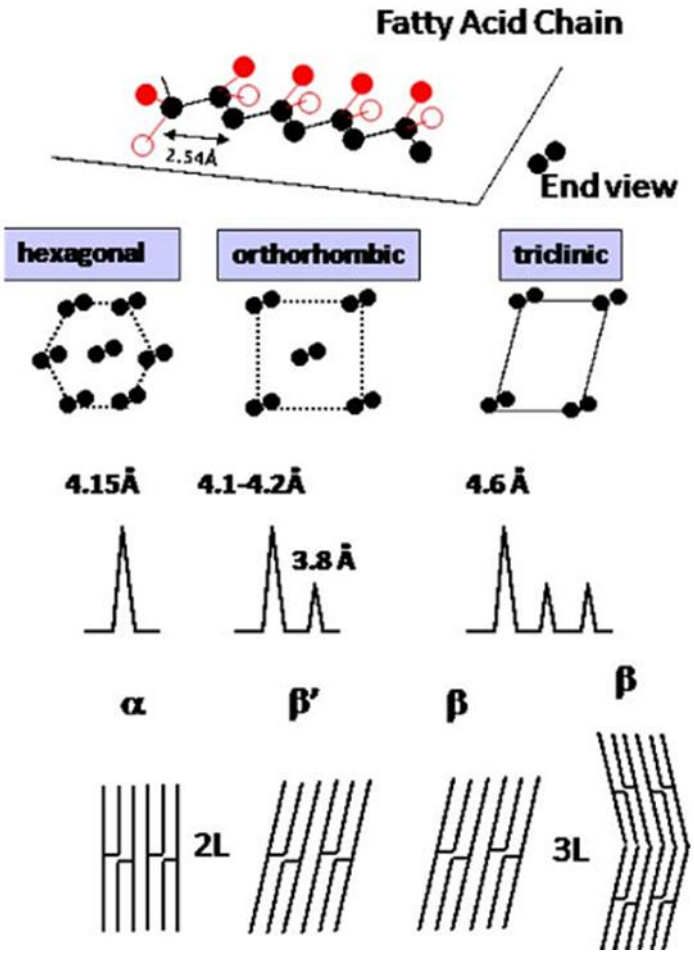

Figure 1.5. Correlations between hexagonal, cubic, and orthogonal arranges of triaglycerides, x-ray scattering patterns, and α, β′, and β-crystal polymorphisms. Adapted from Westesen, Bunjes, & Koch, (1997).

Figure 1.6. Three models showing the distribution of active compounds in SLNs: Homogeneous matrix (left), drug-enriched shell (middle), and drug-enriched core (right). Redrawn based on [12b].

CHAPTER II

Transparent Dispersions of Milk Fat-Based Solid Lipid Nanoparticles for Delivery of Beta-Carotene

This chapter is a lightly revised version of a paper by the same title submitted to the *Journal of Agricultural and Food Chemistry* by Linhan Zhang, Douglas G. Hayes, Guoxun Chen and Qixin Zhong. The use of "our" in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the preparation of sample, (2) the data collection and analysis, (3) the gathersing and interpretation of literature, and (4) the writing.

Abstract

Solid lipid nanoparticles (SLNs) are possible vehicles to incorporate lipophilic bioactive compounds in transparent functional beverages. In this work, AMF and Tween 80 were used to prepare SLNs by using a phase inversion temperature method, and beta-carotene was used as a model lipophilic bioactive compound. The phase inversion temperature decreased from >95 °C to 73 °C, when NaCl was 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 SLNs dispersions were observed at AMF level higher than 10% w/w, corresponding to particles smaller than ~25 nm. The SLNs dispersions were dilution and dialysis stable and maintained turbidity level and particle size during 90-day storage at room temperature. The degradation of beta-carotene encapsulated in SLNs was much reduced when compared with its encapsulation in the soybean oil-based nanoemulsion.

Keywords: Solid lipid nanoparticles, phase inversion temperature method, anhydrous milk fat, transparent dispersion, beta-carotene, storage stability

1. 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, either in 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 like beta-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 of degradation occurs [6]. A variety of SLN systems have been developed as effective delivery agents [7-9]. But much work is needed to fabricate transparent SLN dispersions using low-cost and scalable processes.

SLNs are usually formed by high energy methods such as high pressure homogenization above or below the melting temperature of lipids [8, 10-11]. 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 due to 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 [12]. Since heating lipids above the melting temperature is needed to dissolve lipophilic compounds before preparing SLNs, it is logical to form transparent SLN dispersions using the PIT method, which has not been studied.

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 nonionic surfactants) [13].

$$\mathrm{HLD} = \alpha - EON - \mathrm{k}\, ACN + \mathrm{b}\, S + \Phi(A) + \mathrm{C_T}\,(T - T_{\mathrm{ref}}) \qquad (1)$$

Negative and positive HLD values indicate the possibility of forming O/W and W/O type emulsions, respectively, while a bicontinuous phase behavior corresponds to a HLD value of 0 [14]. For an emulsion with a fixed composition, a fine emulsion with small particle size can be formed during the process of inversion between W/O and O/W emulsions due to changes in temperature [15-16]. Partial inversion was observed for transformation of turbid coarse O/W emulsions of lemon oil emulsified by Tween 80 (polyoxyethylene (20) sorbitan monooleate) to transparent nanoemulsions after heating at 90 °C for 30 min [12].

where α is a characteristic parameter of the lipophilic 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 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 wt% of NaCl or equivalent salt; T is the temperature (°C); T_{ref} is generally taken at 25 °C; and k, b, and $\mathrm{C_T}$ are constants characteristic of the surfactant type and electrolyte [13]. The value of temperature coefficient $\mathrm{C_T}$ is typically larger for an ethoxylated nonionic surfactant than an ionic one [17].

The first objective of this work was to study the formation of transparent dispersions of SLNs using the PIT method. AMF was chosen as the solid lipid because it is a commercially available dairy ingredient. Variables in SLN formation were studied for thermal treatment conditions, salinity, and composition. The second objective was to study the prevention of degradation for beta-carotene, a model lipophilic bioactive compound, when encapsulated in SLNs.

2. Materials and Methods

2.1. Materials

AMF was kindly donated by Land O'Lakes, Inc. (St. Paul, MN). Polyoxyethylene (20) sorbitan monooleate (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). Beta-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).

2.2. Preparation of SLNs dispersions

2.2.1. 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 ℃ before use.

2.2.2. Preparation of coarse emulsions

Preparation of coarse emulsions and SLN nanodispersions was investigated based on a PIT method detailed previously [12], with some modifications. To form a coarse emulsion, AMF was heated to 60 ℃ 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, beta-carotene was dissolved in warm AMF at a concentration of 6.25 mM before emulsification. To compare impacts of the structure of lipid body (solid fat vs. liquid oil) on the stability of encapsulated beta-carotene, a control nanoemulsion was prepared using soybean oil at conditions comparable to the treatment using AMF.

2.2.3. Thermal treatment for preparation of SLNs

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 ℃) without stirring. After thermal treatment, these samples were first cooled at ambient conditions with hand shaking till a homogenous appearance, followed by quenching in an ice bath under static conditions.

2.3. Turbidity measurement

Turbidity of samples was measured for absorbance at 600 nm (Abs_{600}) using a UV/vis spectrophotometer (Unicam, Cambridge, UK). Tween 80 solutions at corresponding concentrations were used as controls.

2.4. Particle size determination

The particle size of samples was measured by using a Delas™ Nano particle analyzer (Beckman Coulter, Fullerton, CA). In order 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 following:

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

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

2.5. 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 = 28 mm and cup inner diameter = 30 mm). After loading the sample, heating to and equilibrated at 95 ℃ for 2 min, a temperature sweep with a fixed shear rate of 100 s^{-1} [13] was applied from 95 to 25 ℃ at 3 ℃/min. A thin layer of soybean oil was applied on the top of sample to minimize water evaporation.

2.6. Differential scanning calorimetry (DSC)

DSC (model Q2000, TA Instruments, New Castle, DE) was used to study melting and crystallization properties of AMF and SLNs during heating and cooling. The transparent SLN dispersion prepared with 15% AMF and 45% Tween 80 in 0.8 M NaCl as the aqueous phase was used to generate detectable endothermic and exothermic peaks. A sample corresponding to 5-8 mg AMF or 15-20 mg SLN dispersion was placed in an aluminum pan and hermetically sealed. An empty pan was used as a reference. The thermal profile was studied by two cycles, with each cycle following the steps of holding at -15 °C for 5 min, heating from -15 to 90 ℃ at 5 ℃/min and cooling from 90 to -15 ℃ at 20 ℃/min. Heat flow of the samples was recorded using the instrument software.

2.7. Atomic force microscopy (AFM)

The shape and dimension of particles were characterized using a NanoScope IIIA Multimode AFM (Veeco Instruments Inc., Santa Barbara, CA). The SLN dispersion was first diluted to 10 ppm of AMF using deionized water. Two 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.

2.8. X-ray diffraction (XRD)

Crystallinity of pristine beta-carotene, AMF and beta-carotene-loaded SLNs was studied by XRD (Panalyticla, Westborough, MA). SLN 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 KR radiation (λ = 1.54 Å) was generated at 30 kV and 10 mA.

2.9. Antioxidant properties

The antioxidant properties of the emulsions were estimated using an ABTS method [18-19]. 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. Two 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

concentration of Trolox and emulsion. The antioxidant properties were obtained by referring to a standard curve established from Trolox and expressed as mM Trolox equivalents per mL.

2.10. Quantification of beta-carotene concentration

Beta-carotene concentration in the emulsion was determined using absorbance [20]. Twenty µL of an emulsion sample was extracted by using a mixture composed of 2 mL ethanol and 3 mL 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-Visible spectrophotometer (Unicam, Cambridge, UK). The concentrations of beta-carotene in emulsions were obtained by referring to a standard curve established from standard solutions with different amounts of beta-carotene in hexane.

2.11. Storage stability of SLNs and encapsulated beta-carotene

Physical stability of SLNs

Selected SLN dispersions without beta-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 cut-off 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. Two mL dispersions were 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 beta-carotene

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

2.12. 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 by using Statistical Analysis Software (V9.2, SAS Institute, Cary, NC).

3. Results and Discussion

3.1. Influence of salinity and temperature on SLNs formation

The first group of experiments was used to study temperature and NaCl concentration that are important parameters of HLD (eq 1). Furthermore, based on our preliminary trials, emulsions with high surfactant concentrations can form gels at high NaCl concentrations during thermal treatments, while a low surfactant-to-oil ratio (SOR) 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 2.1. Emulsions with 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 2.1 also indicates that a lower NaCl concentration was required to transform turbid emulsions to transparent ones when heated at 95 °C than at 90 °C.

Figure 2.1 suggests that 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 [21-22]. Figure 2.2 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 ℃, the viscosity increased quickly and reached a maximum value at 65 °C. Upon further cooling, viscosity decreased until reaching about 55 ℃, followed by an increase with a further decrease in 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 [21]. For systems with a large water fraction (fw) as the case of the present study, the viscosity maximum above the PIT is usually not observed [13], possibly due to the formation of multiple W/O/W emulsions instead of W/O ones. The PIT of systems with high water content can be approximated by the temperature at which the viscosity starts to increase during cooling, e.g., 76 ℃ for the system containing 0.8 M NaCl. As presented in Figure 2.2, 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 an HLD value of zero, i.e. phase inversion. The remaining experiments to form SLNs were conducted at NaCl concentration of 0.8 M and thermal treatment of 90 °C for 30 min.

3.2. Influence of surfactant: oil ratio on SLNs formation

The impact of composition on SLN 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 microemulsions. 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 2.3A 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 Figure 2.4A and 2.4B, 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 SLNs 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 SLNs are summarized in Figure 3B for this group of treatments, in the form of a phase diagram.

Our results generally agreed with those of Rao and McClements [12]. 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 SLN dispersions in our preliminary studies. This may be due to the lower solubility and higher melting temperature of AMF than those of lemon oil.

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 [12], 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 SLNs are presented in 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.

3.3. Structure of SLNs studied by AFM and XRD

3.3.1 AFM

Figure 2.5A displays an AFM image of SLNs in a transparent dispersion. Particles were discrete and mostly spherical. When compared to particle size distribution (Figure 2.5C), particle

heights measured by AFM (<5 nm, Figure 2.5B) were much smaller, as reported in other studies [23-25]. The difference may be due to the underlying theory for the two methods. An assumption used in dynamic light scattering (DLS), "infinite dilution" [26], 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 particle size obtained by the Stokes-Einstein Equation may be overestimated. Additionally, the dimension occupied by particles may decrease after drying the SLN dispersion for AFM.

3.3.2 XRD

XRD is a convenient technique not only for characterizing SLNs [27-28] but also for confirming the encapsulation of lipophilic compounds [29], especially beta-carotene, which is highly crystalline [30-31]. AMF also has crystalline structures below its melting temperature [32]. Because AMF is composed of a mixture of lipids, its crystalline structures are dependent on temperature and cooling rate [33-34]. Figure 2.6 shows the XRD spectra of beta-carotene, AMF, and beta-carotene-loaded SLNs. Beta-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 crystalline structure is also present in AMF at room temperature [35]. For SLNs loaded with beta-carotene, because Tween 80 is a non-crystalline compound at ~20 °C[36-38], the smooth XRD curve of the beta-carotene-loaded SLNs and its similarity to the spectrum of AMF suggest that beta-carotene was embedded in SLNs [39]. Detailed structures of AMF crystals however were not studied in the present work, as it requires temperature-controlled small angle XRD [40-42].

3.3.3 DSC

Thermograms of AMF and SLNs during heating and cooling cycles are shown in Figure 2.7. 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. During the subsequent cooling process, the crystal formation started at around 17.6 ℃ and the crystalline process of mostly oil happened around 5.0 °C. The two cycles of heating-cooling process do not showed any differences, indicating bulk recrystallization of AMF would not affect its crystal type and melting temperature. The DSC results of AMF agree with previous studies [43-44]. Multiple melting temperatures suggested in Figure 2.7 are due to the fact that AMF is a mixture of triglycerides containing more than 400 fatty acids, both saturated and unsaturated, including oleic acid, palmitic acid, myristic acid, and stearic acid [45]. First cycle of heating curves of SLN showed a strong endothermic peak at 15.3 °C. While the other two melting peak showed in the heating curved of bulk AMF do not present as obvious as in the heating curve of SLN. In the subsequent cooling cycle of SLN, initial crystal formation temperature is the same as bulk AMF. But the main exothermic peak happened around -3.2 °C, much lower than bulk AMF. The differences of crystallization behavior of lipids in SLN agrees with previous study that the solidification temperature of lipid core in SLN may be lower than that of bulk lipids[46]. Lipids may exist as super-cooled melts in SLN[47]. The differences between first and second cycle of SLN may due to particle coalescence or free oil in the SLN[48-49]. Thus, AMF in SLN dispersion is present as a mixture of solid state and liquid state at room temperature (21 °C) in the present study. The DSC (Figure 2.7) and XRD (Figure 2.6) results suggest that AMF in SLNs does not have extensive ordered crystalline structures but is present at the amorphous solid state, corresponding to

nanostructured lipid carriers that are advantageous in encapsulation when compared to SLNs with highly ordered crystals. [11]

3.4. Physical storage stability of SLNs

Figure 2.8 contains Abs_{600} and $d_{4,3}$ values of emulsions, stored at room temperature (21 °C) for 90 days, for SLN 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 not change Abs_{600} and $d_{4,3}$ of samples and their stability during storage. Therefore, the AMF-based SLN dispersions possess excellent physical stability during storage, which again can be attributed to the elimination of coalescence and Ostwald ripening due to use of solid phase lipids as the dispersed phase.

3.5. Stability of beta-carotene during storage

Figure 2.9 shows changes in antioxidant potential and concentration of beta-carotene loaded in SLNs during 16-day 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 with that of SLNs and was stable after 16 day-storage (30.3 ± 1.0 nm). The net antioxidant potential of beta-carotene in nanoemulsions was determined by subtracting the measured potential of the corresponding nanoemulsion without beta-carotene. Both net antioxidant properties and concentration of beta-carotene gradually decreased during storage, but the beta-

carotene encapsulated in SLN treatment showed much slower degradation than its encapsulation with soybean oil. By the end of storage, about 94.8% loss of the beta-carotene concentration and 97.4% loss of net antioxidant potential were observed for the latter system. In contrast, the concentration and net antioxidant potential of beta-carotene encapsulated in SLNs only decreased by 47.3% and 66.7%, respectively. The concentration change of encapsulated beta-carotene (Figure 2.9B) followed the first order kinetics, as reported previously for beta-carotene in safflower seed oil [50]. The results indicate the effectiveness of SLNs in reducing the degradation of encapsulated bioactive compounds during storage.

To prove the reduced degradation in SLNs is due to the lowered mobility in the solid lipid phase, 0.0125 mM beta-carotene was dissolved in soybean oil or AMF and stored at 65 °C and room temperature (21 °C) for 16 days, and the absorbance at 450 nm was measured to monitor changes in beta-carotene content, with the corresponding lipids as a control. Beta-carotene degraded much faster in melted AMF (79.0 %) than in soybean oil (28.9%) at 65 °C, suggesting soybean oil having better antioxidant properties. At 21 °C, beta-carotene did not show obvious degradation in both lipids. Since 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 beta-carotene in soybean oil. The degradation tests in bulk lipids further support the conclusion that the enhanced stability of beta-carotene in SLNs is due to the amorphous solid state of AMF.

In summary, the present study demonstrated the feasibility to prepare transparent dispersions of SLNs composed of AMF as the solid lipid dispersed phase and Tween 80 as a nonionic surfactant by using the PIT method. Thermal treatment conditions, salinity, and surfactant-to-oil ratio all played important roles in the effectiveness of transforming turbid

emulsions into transparent ones after heating. An increase of salinity lowered the PIT, thus favoring the formation of transparent SLN dispersions. A higher surfactant concentration enabled the incorporation of a higher amount of AMF in transparent SLN dispersions. SLN dispersions remained stable upon dilution and showed practically no changes in particle size and turbidity during storage for 90 days at 21 °C. Beta-carotene was successfully encapsulated in SLNs using the PIT method and had much better stability in SLNs than in soybean oil-based nanoemulsions. Therefore, the simple PIT method and AMF can be used to form stable and transparent SLN dispersions for incorporation of a variety of lipophilic bioactive compounds in functional beverages.

4. Acknowledgements

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Appendix

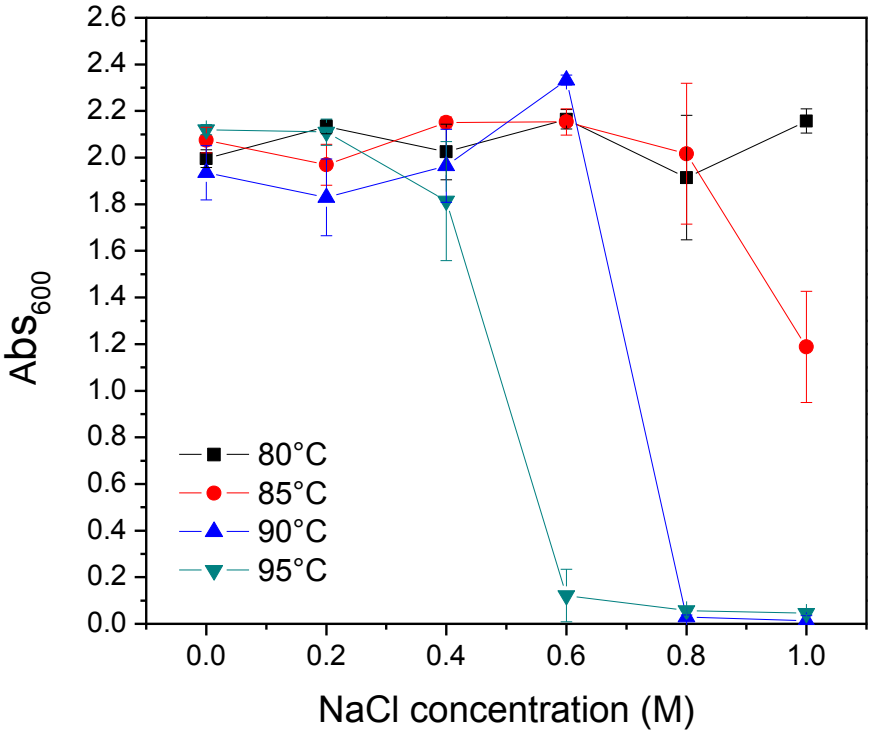


Figure 2.1. Absorbance at 600 nm (Abs_{600}) of dispersions containing 1% w/w AMF and aqueous phase with 15% w/w Tween 80 dissolved in 0-1.0 M NaCl solutions after heating at a temperature between 80 and 95 °C for 30 min and cooling to room temperature. Error bars are standard deviations from duplicate samples.

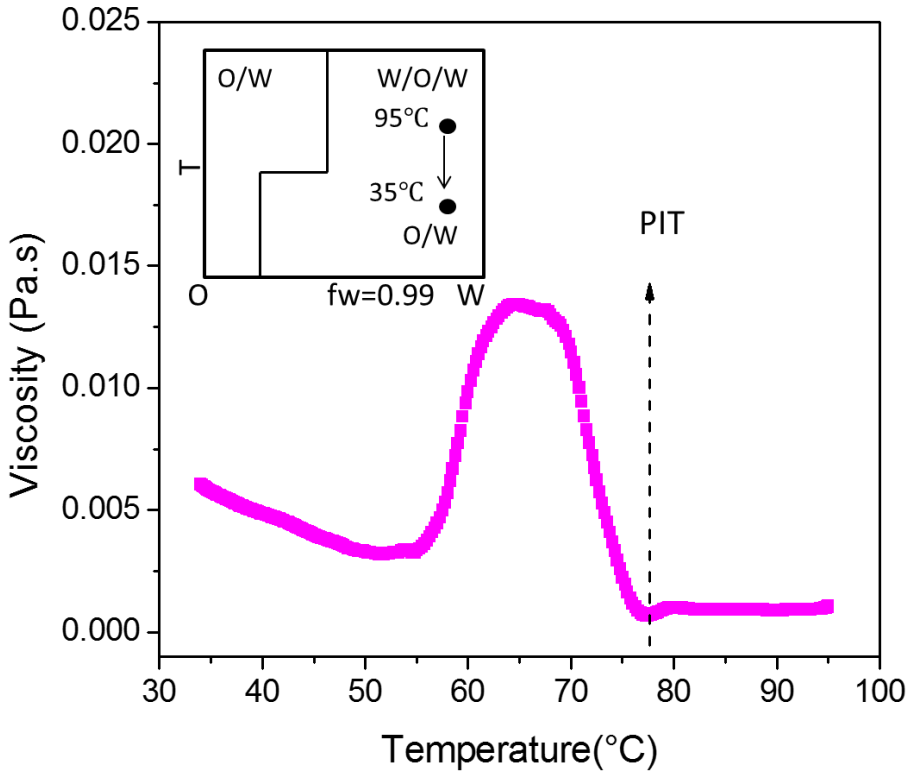


Figure 2.2A. The change of viscosity during cooling from 95 to 35 ℃ for an emulsion. The phase inversion temperature (PIT) was determined from the local minimum, as depicted. As shown in the inset, cooling results in the conversion of a W/O/W emulsion to an O/W emulsion. The emulsion contained 1% w/w anhydrous milk fat (water fraction fw =0.99) and an aqueous phase composed of 15% w/w Tween 80 dissolved in a 0.8 M NaCl solution.

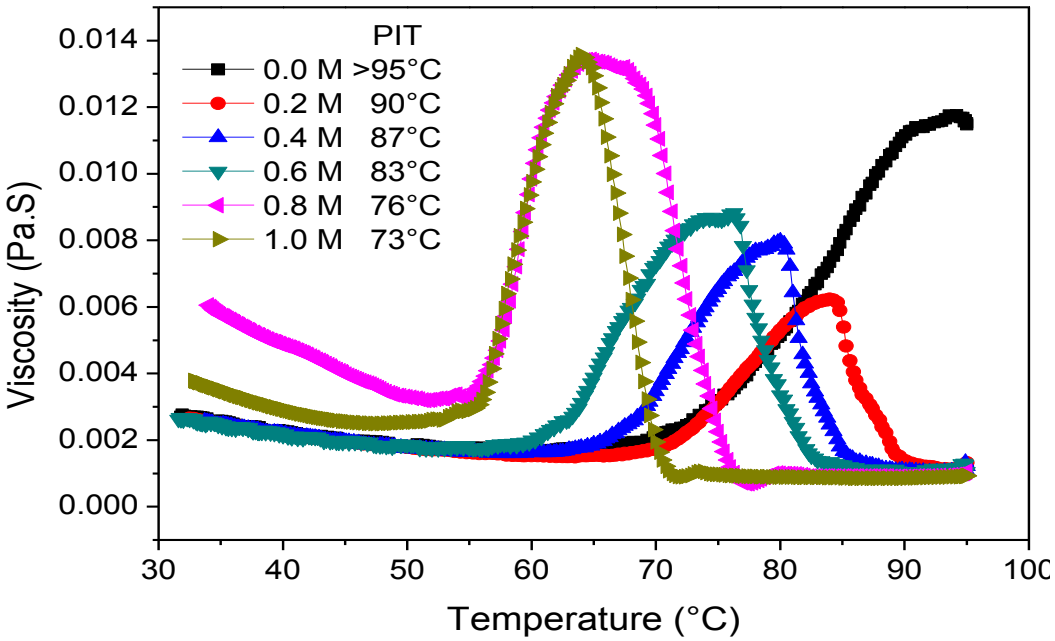


Figure 2.2B. Effects of NaCl concentration on the viscosity of emulsions during cooling from 95 to 35 ℃. Emulsions contained 1% w/w AMF and an aqueous phase with 15% w/w Tween 80 dissolved in 0-1.0 M NaCl solutions. Values of the phase inversion temperature (PIT), obtained as described in Figure 2.2A, were listed in the legend.

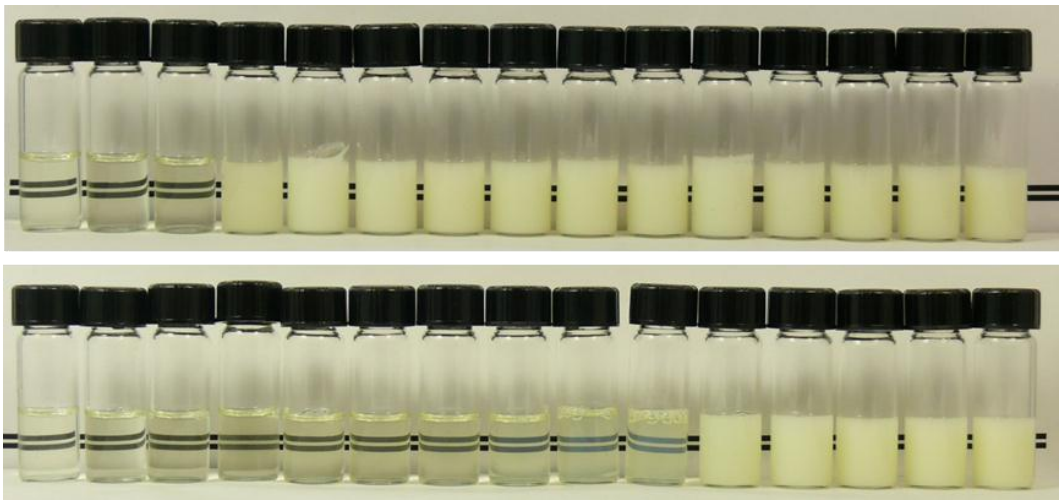


Figure 2.3. Appearance of emulsions before (top) and after (bottom) heating at 90 °C for 30 min. Emulsions before heating were prepared with 1.0-15.0% w/w AMF and an aqueous phase with 30% w/w Tween 80 dissolved in a 0.8 M NaCl solution. AMF concentration increased by 1% w/w successively for samples ordered from left to right.

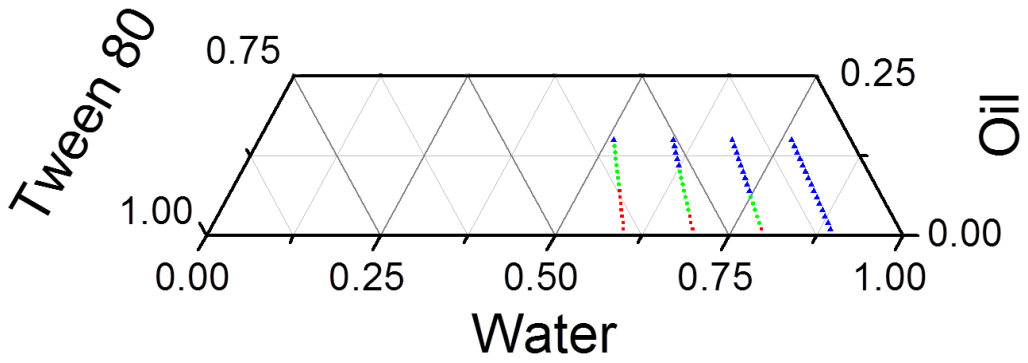


Figure 2.4. Partial phase diagram showing conditions (×100%w/w for each constituent) of forming microemulsions (red squares), transparent SLNs dispersions (green circles) and turbid or phase-separated emulsions (blue triangles) at 25 °C formed by the phase inversion temperature method. Coarse emulsions were prepared by mixing 1-15% w/w AMF with an aqueous phase with 10-40% w/w Tween 80 dissolved in a 0.8 M NaCl solution and were heated at 90 °C for 30 min.

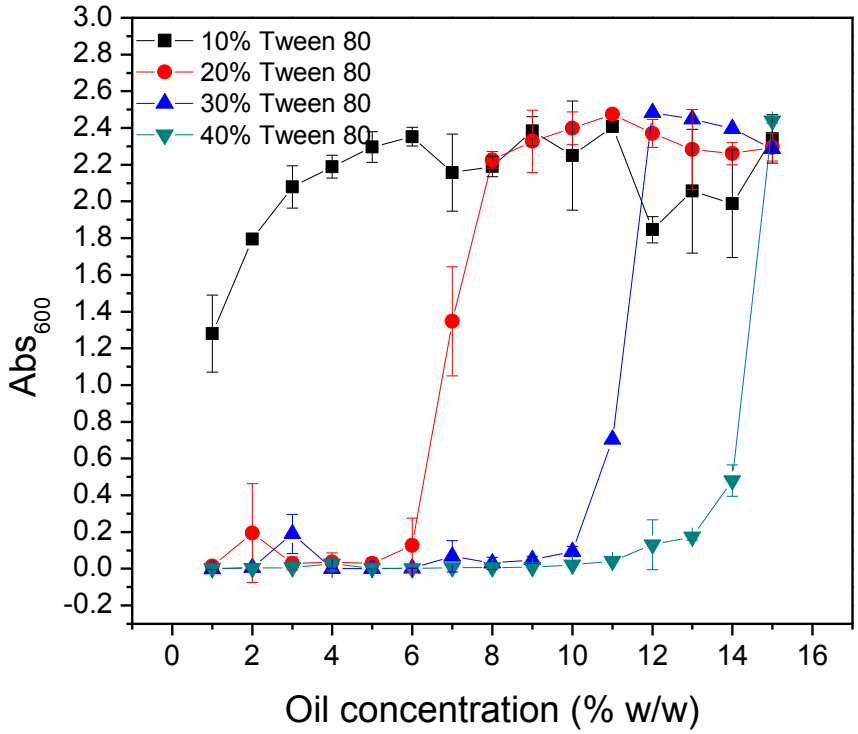


Figure 2.5. Absorbance at 600 nm (Abs_{600}) of emulsions after heating at 90 °C for 30 min and cooling to room temperature. Emulsions were prepared with 1.0-15.0% w/w AMF and an aqueous phase with 10-40% w/w Tween 80 dissolved in a 0.8 M NaCl solution. Error bars are standard deviations from duplicate samples.

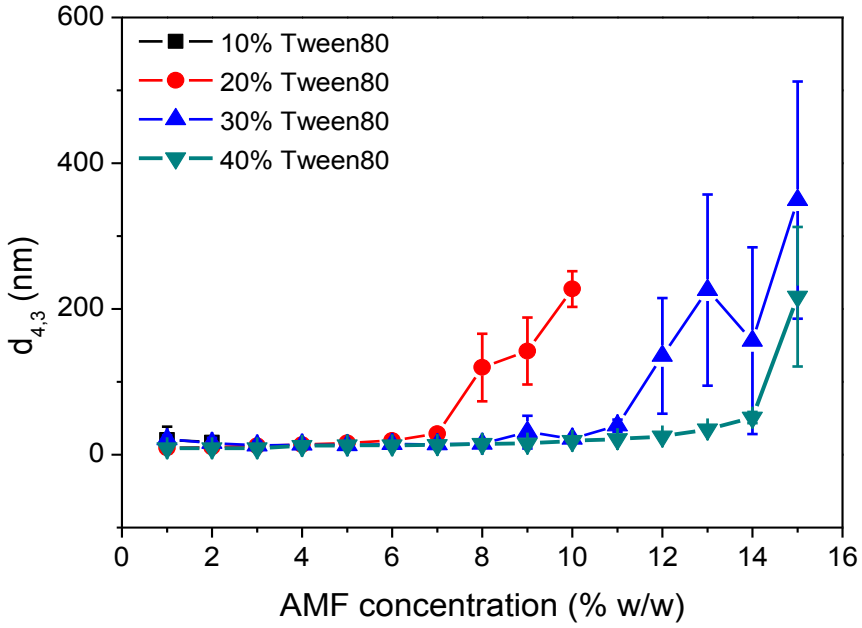
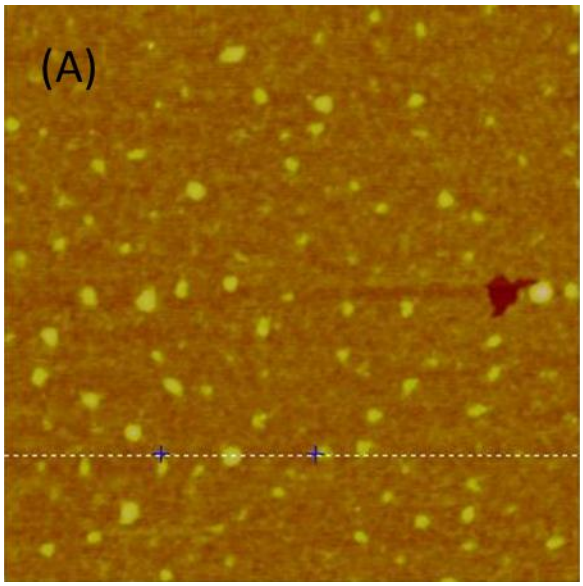
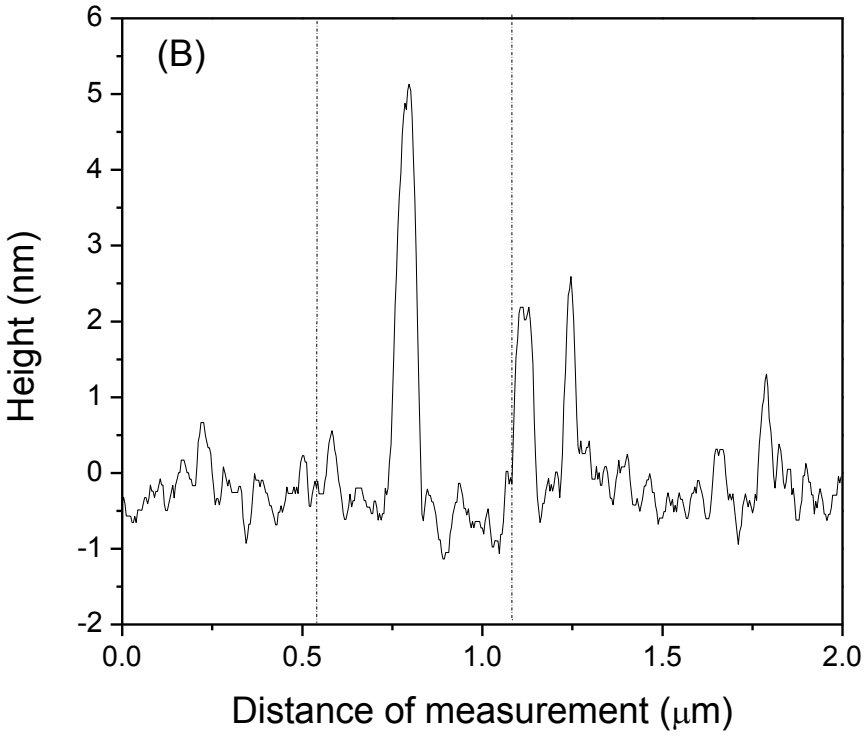
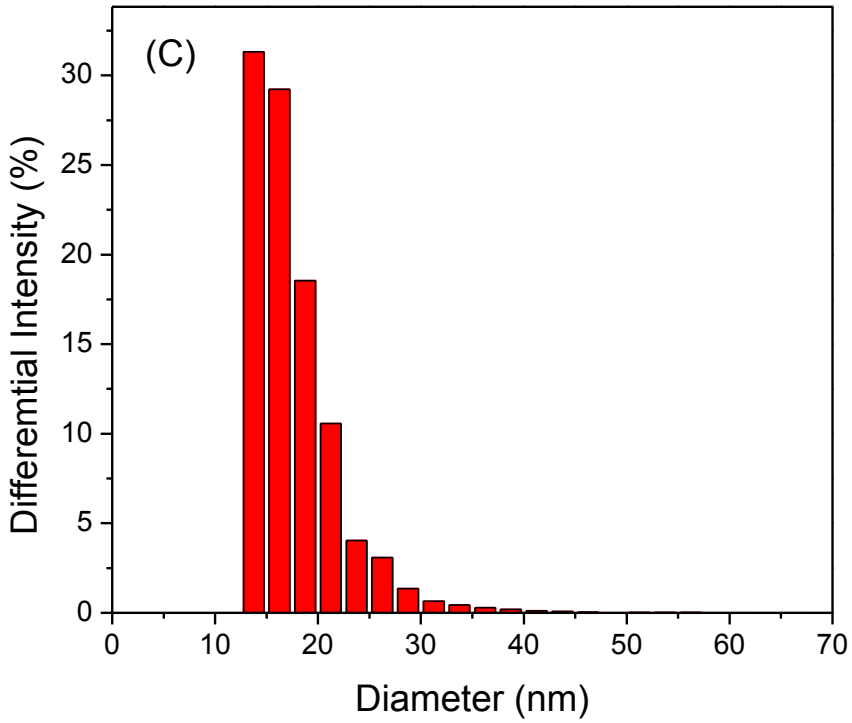


Figure 2.6. Volume-length mean diameter ($d_{4,3}$) of emulsions after heating at 90 °C for 30 min and cooling to room temperature. Emulsions were prepared with 1.0-15.0% w/w AMF and an aqueous phase with 10-40% w/w Tween 80 dissolved in a 0.8 M NaCl solution. Samples that exhibited creaming within 10 min after thermal treatment are not plotted. Error bars are standard deviations from duplicate samples.

Figure 2.7. Atomic force microscopy topographic image with a dimension of 2 × 2 μm (A) and height distribution of particles along the measurement line (B). Particle size distribution of SLNs from light scattering is plotted in (C) for comparison. The SLNs 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.


(A)


(B)
Height (nm)
6
5
4
3
2
1
0
-1
-2
0.0
0.5
1.0
1.5
2.0
Distance of measurement (μm)


(C)
Differemtial Intensity (%)
0
5
10
15
20
25
30
0
10
20
30
40
50
60
70
Diameter (nm)

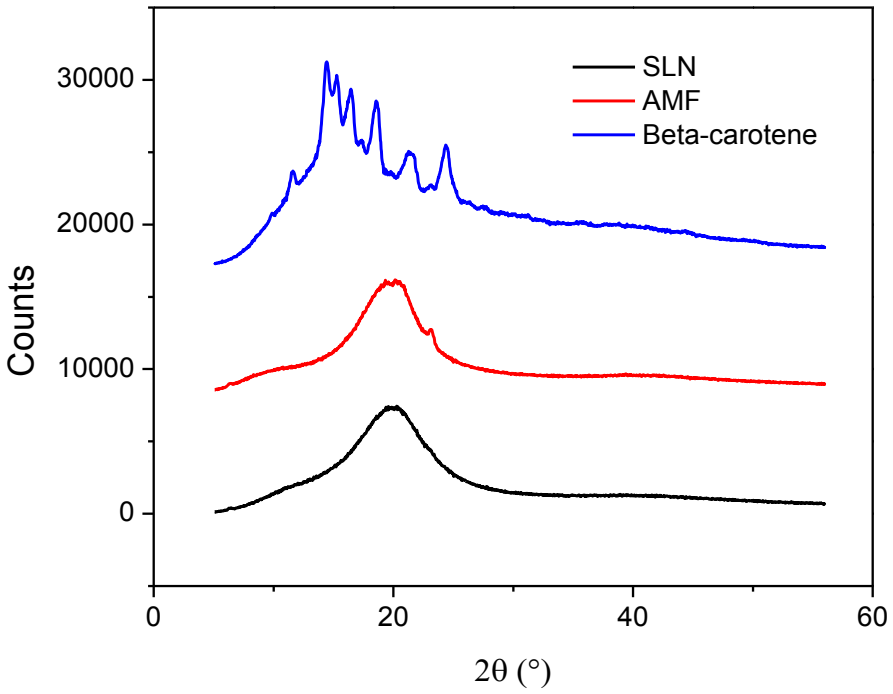


Figure 2.8. XRD patterns of pristine beta-carotene (blue), AMF (red) and beta-carotene-loaded SLNs (black). SLNs were prepared by heating at 90 °C for 30 min using an emulsion prepared with 10% w/w AMF in an aqueous phase with 30% w/w Tween 80 dissolved in a 0.8 M NaCl solution.

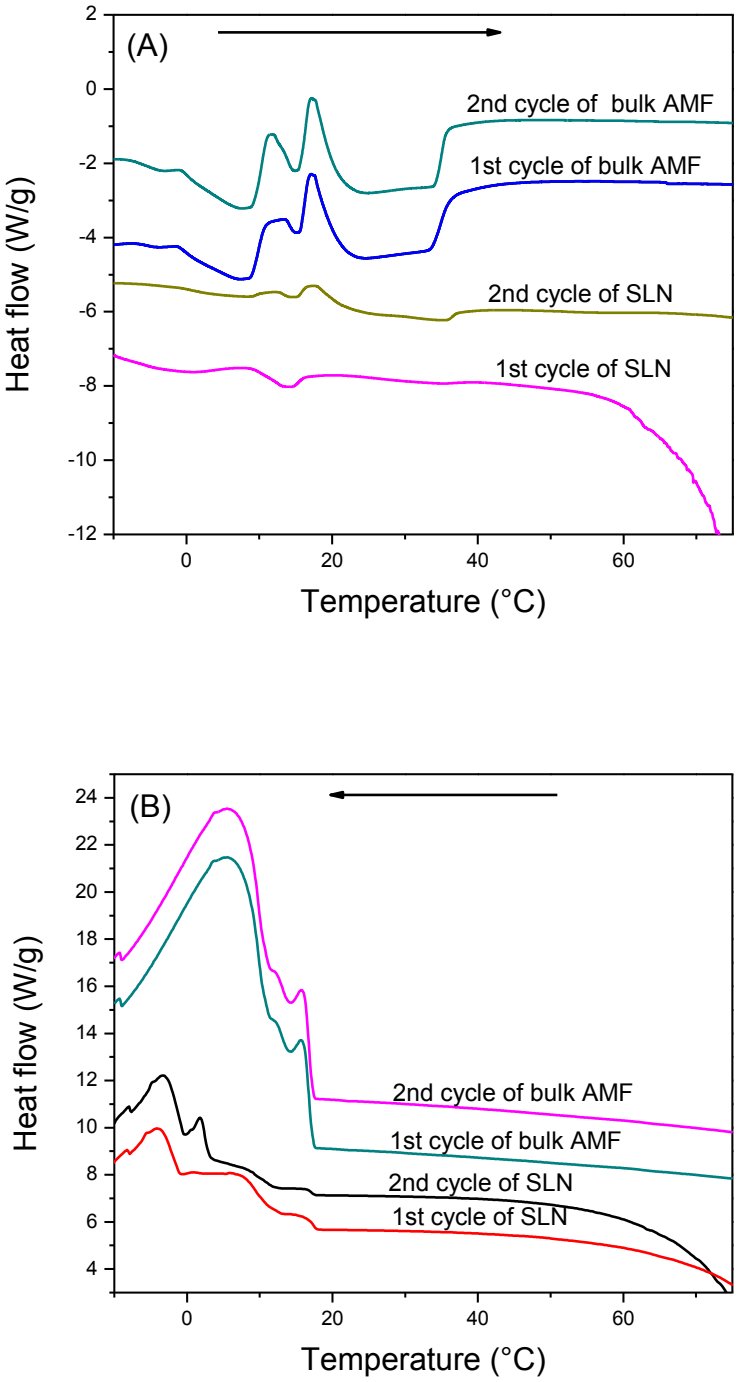


Figure 2.9. Thermograms of AMF, SLNs during two cycles of (A) heating from -15 to 90 °C at 5 °C/min and (B) cooling back to -15 °C at 20°/min , shown from -10 to 75°C.

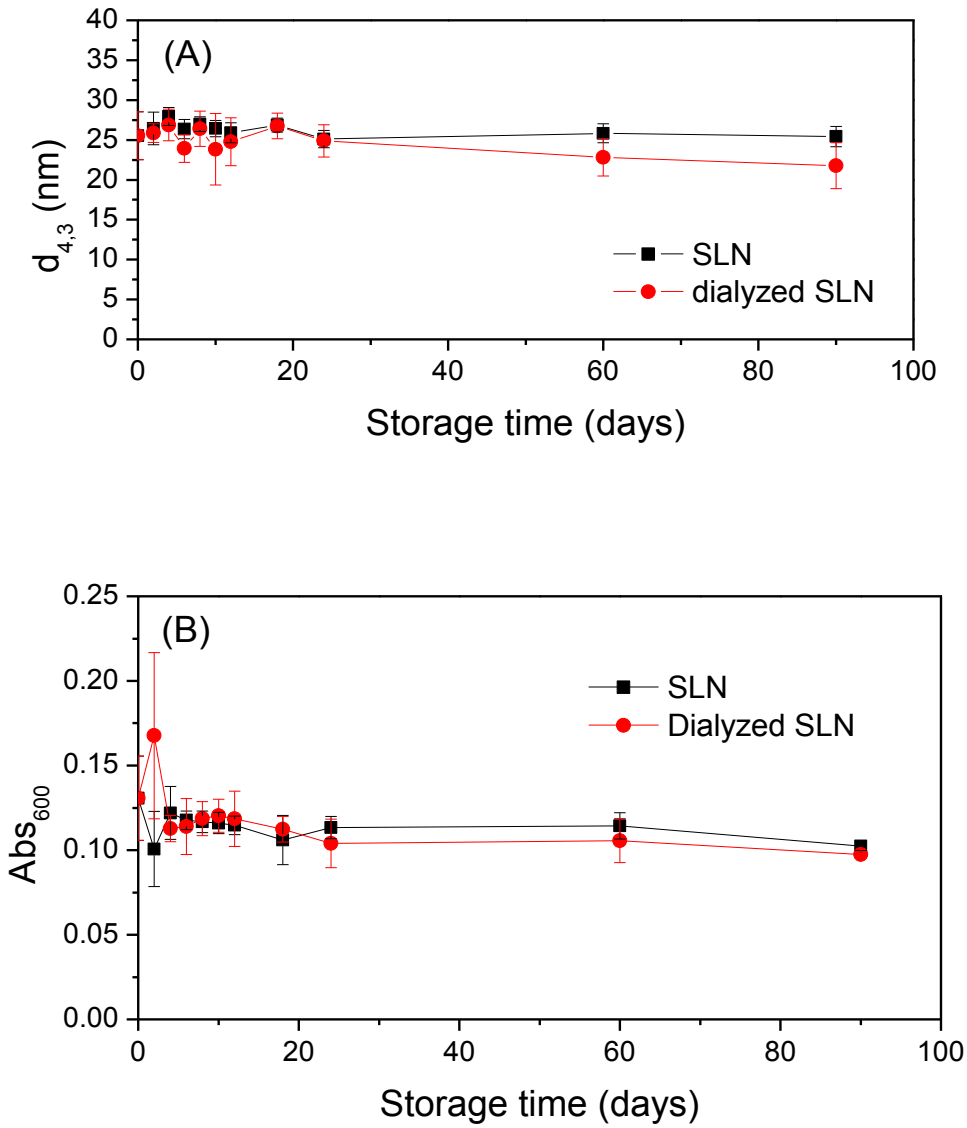


Figure 2.10. Changes of (A) volume-length mean diameter ($d_{4,3}$) and (B) absorbance at 600 nm (Abs_{600}) of SLNs dispersions, with and without dialysis, during 90-day storage at room temperature (21 °C). SLNs prepared by heating at 90 °C for 30 min using an emulsion prepared with 10% w/w AMF in an aqueous phase with 30% w/w Tween 80 dissolved in a 0.8 M NaCl solution. Error bars are standard deviations from duplicate samples.

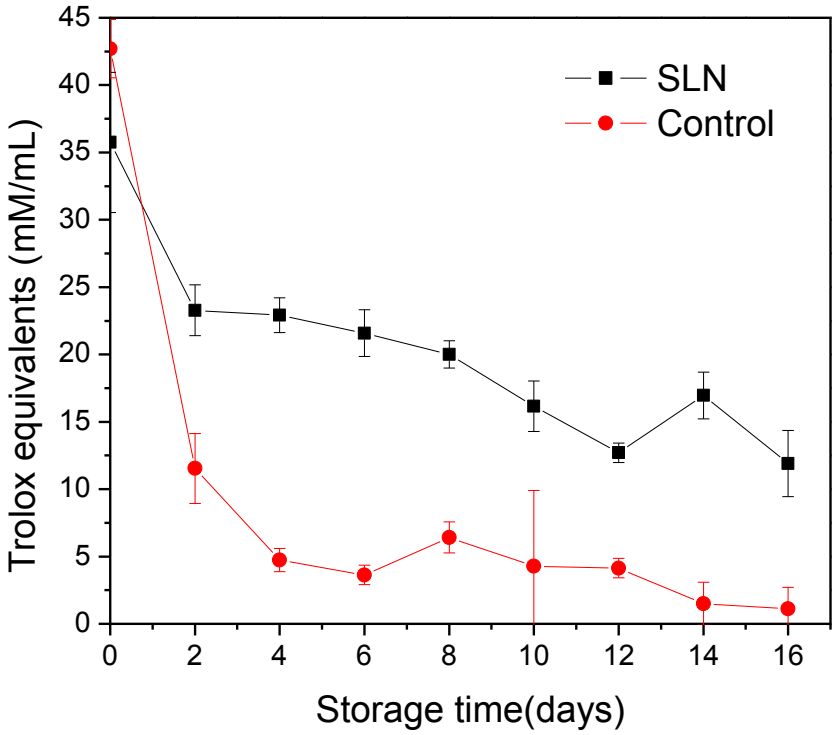


Figure 2.11. Changes of antioxidant properties of beta-carotene-loaded SLNs dispersions during storage at room temperature (21 °C), in comparison to a control with beta-carotene encapsulated in vegetable oil-based nanoemulsion. Error bars are standard deviations from duplicate samples.

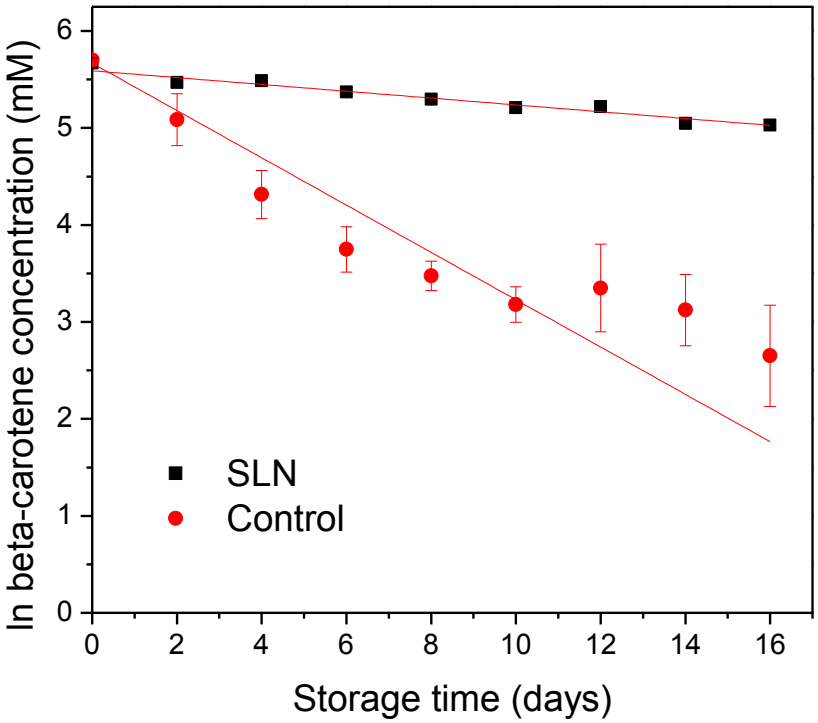


Figure 2.12. Concentration changes, in natural logarithm values, of beta-carotene loaded in SLNs dispersions, in comparison to a control of beta-carotene encapsulated in vegetable oil-based nanoemulsion. Error bars are standard deviations from duplicate samples.

Conclusions

SLNs are nanoparticles that contain a solid lipid core and have potential applications in the food industry. Many approaches used to prepare conventional nanoemulsions can be used to prepare SLNs, including high energy and low energy methods. High energy methods include hot and cold HPH methods, high shear homogenization and ultrasonication methods, while solvent evaporation is a low energy method. SLNs can also be prepared from self-assembled microemulsions. The degradation of bioactives during sample preparation and possible organic solvent residues are concerns for food applications.

The degradation and release of bioactives incorporated in SLNs mainly depend on the exact structure of SLNs with regard to the spatial distributions of the bioactives and carrier lipids. Burst release and faster degradation of bioactives are commonly observed when the encapsulated compound is mostly present in the outer shell. The prolonged release and improved stability of bioactives correspond to situations where bioactives are rich in the lipid core or distributed evenly in the SLNs.

In this thesis, a phase inversion temperature method was used to prepared milk fat-based SLNs. This method only required a thermal treatment to cause phase inversion of the system. AMF is a mixture of more than 40 kinds of lipids with different chain lengths. The impurity of lipids can benefit the formation of less perfect crystals, or even amorphous solids. This kind of structure would increase the loading of bioactives in SLNs and thus avoid the burst release. A mixture of different lipids can also increase physical stability of SLNs by preventing crystal transformation in the later storage. A food grade nonionic surfactant, Tween 80, was used to

prepare the system. NaCl was added to the system to lower the PIT and therefore facilitate phase inversion.

Thermal treatment conditions, salinity, and surfactant-to-oil ratio all played important roles in the effectiveness of transforming turbid emulsions into transparent ones after heating. An increase of salinity lowered the PIT, thus favoring the formation of transparent SLN dispersions. A higher surfactant concentration enabled the incorporation of a higher amount of AMF in transparent SLN dispersions. The prepared transparent SLNs have relatively small particle diameters (23 nm). SLN dispersions remained stable upon dilution and had consistent particle size and turbidity during 90 day storage at room temperature.

Beta-carotene was used as a model lipophilic bioactive compound. Compared with soybean oil-based nanoemulsions, beta-carotene showed a much longer storage stability in terms of residual concentrations and antioxidant properties. Therefore, the simple PIT method and AMF can be used to form stable and transparent SLN dispersions for incorporation of a variety of lipophilic bioactive compounds in functional beverages. A further study may be needed to understand the lipid core structure in SLNs, as well as their stability in model food systems.

Vita

Linhan Zhang was born in Chaoyang, Liaoning, China on March 9, 1988. After graduation from NO.1 High School in Chaoyang in 2006, she continued her education at the China Agricultural University and earned a B.S. degree majoring in Food Quality and Safety. In August 2013, she will earn an M.S degree in Food Science and Technology with a concentration in Food Chemistry from the University of Tennessee, Knoxville. Linhan will pursue a career in the food industry concentrating in biopolymers and novel product development.