Formation and Stability of Solid Lipid Nanoparticles Fabricated using Phase Inversion Temperature Method

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21 **Abstract**

22 Solid lipid nanoparticles (SLNs) are being investigated for their ability to encapsulate and protect
23 lipophilic bioactive compounds in foods, supplements, and pharmaceuticals. In this study, the phase
24 inversion temperature (PIT) method was used to fabricate SLNs using a model surfactant (Brij 30,
25 C₁₂E₄) / oil (octadecane) / water system. Surfactant/oil/water (SOW) mixtures were maintained at a
26 temperature above the PIT, and then rapidly cooled to a temperature below the lipid nanoparticle
27 crystallization point. The PIT ($\approx 40^{\circ}\text{C}$) was determined by monitoring the turbidity *versus* temperature
28 profile of the SOW system during heating. The lipid nanoparticle crystallization point, melting point,
29 and physical state were determined using differential scanning calorimetry (DSC). The stability of the
30 lipid nanoparticles after fabrication depended on the storage temperature relative to the PIT and
31 melting/crystallization points. At temperatures appreciably below their melting point ($\approx 26^{\circ}\text{C}$), the lipid
32 nanoparticles were completely solid and stable to aggregation. At temperatures around their melting
33 point, the lipid nanoparticles were partially crystalline, which led to partial coalescence and gelation.
34 At temperatures appreciably above their melting point but below their PIT, the lipid nanoparticles were
35 completely liquid and prone to coalescence and phase separation. These results have important
36 implications for optimizing the fabrication and storage conditions required to produce stable
37 nanoemulsions suitable for utilization in commercial products using low-energy methods.

38

39 **Keywords:** Solid lipid nanoparticles; Phase inversion temperature; Nanoemulsions;
40 Crystallization; Low-energy; Stability

41

1. Introduction

Bioactive lipophilic ingredients, such as oil-soluble vitamins, nutraceuticals, nutrients, antioxidants, antimicrobials, flavors, and pigments, are increasingly being utilized to fortify foods and beverages because of their potential to improve product safety, shelf-life, quality, and nutritional aspects [1, 2]. Nevertheless, the incorporation of these bioactive ingredients into foods is often challenging because of their low water-solubility, food matrix incompatibility, poor chemical stability, undesirable flavor profile, and/or low bioavailability [3, 4]. Solid lipid nanoparticles (SLNs) are a type of colloidal delivery system that has been widely studied for its potential to encapsulate, protect, and release lipophilic active ingredients in the pharmaceutical, supplement, and food industries [5-11]. SLNs consist of a suspension of crystalline lipid nanoparticles dispersed within an aqueous medium, with each nanoparticle being stabilized against aggregation by an emulsifier coating. SLNs have been claimed to have advantages over liquid lipid nanoparticles (LLNs) for certain applications [12-14]. The crystallization of the lipid phase may retard molecular diffusion within the nanoparticle interior, thereby improving the retention and chemical stability of encapsulated bioactive ingredients [5]. SLNs therefore have considerable potential for utilization within the food industry to encapsulate, protect, and release chemically unstable lipophilic food ingredients.

SLNs are typically fabricated by forming an oil-in-water nanoemulsion (NE) first, and then this system is cooled below the melting point of the dispersed lipid phase to induce crystallization [15]. In general, NEs can be produced using either high-energy or low-energy approaches [16]. High-energy approaches use specially designed mechanical devices (homogenizers) that generate intense energy fields to break up large lipid droplets into smaller ones. This type of approach is currently the most commonly used to fabricate NEs in the food industry, and the mechanical devices used include microfluidization, high-pressure homogenization, and sonication [16, 17]. High-energy approaches can be used to form nanoemulsions from a wide range of oils and emulsifiers, but they require dedicated homogenizers that must be purchased and maintained. In contrast, low-energy approaches do not need any specialized mechanical devices to form nanoemulsions. Instead, a nanoemulsion can simply be formed from certain surfactant, oil, and water mixtures by altering system composition or environmental conditions (such as temperature) in a specific way [18]. This approach mainly relies on inducing changes in surfactant properties, such as oil-water partitioning, optimum curvature, and packing characteristics, when system conditions are changed [18, 19]. A number of low-energy approaches have been developed to fabricate oil-in-water nanoemulsions, including spontaneous emulsification, phase inversion temperature (PIT), and phase inversion composition (PIC) methods

74 [11, 16, 17].

75 At present, SLNs are usually fabricated using high-energy methods such as microfluidization,
76 high-pressure homogenization, or sonication [15, 20-23]. Initially, NEs are prepared at a temperature
77 above the melting point of the lipid phase, and then they are cooled below the crystallization point of
78 the lipid phase to form SLNs [24]. In the current study, we examined the use of a low-energy method
79 (PIT) to produce SLNs that might have application for the encapsulation, retention, and release of
80 lipophilic bioactive components. The PIT method relies on changes in the properties of non-ionic
81 surfactants when temperature is changed, such as their molecular geometry, packing, and oil-water
82 partitioning [18, 25, 26]. The molecular geometry of a surfactant can be characterized by a packing
83 parameter (p) equal to the effective cross-sectional area of the hydrophobic tail (a_T) divided by that of
84 the hydrophilic head group (a_H): $p = a_H/a_T$ [27]. At relatively low temperatures ($T \ll \text{PIT}$), the head
85 groups of non-ionic surfactant molecules are highly hydrated, and so the area occupied by the head
86 group is larger than that of the tail group ($p < 1$), which favors the formation of O/W emulsions. At
87 intermediate temperatures ($T \approx \text{PIT}$), the head groups become partially dehydrated so that the head
88 group and tail group areas are approximately similar ($p \approx 1$), which favors the formation of a lamellar
89 or a bicontinuous microemulsion phase [28]. At relatively high temperatures ($T \gg \text{PIT}$), the surfactant
90 head groups become so dehydrated that their effective area is less than that of the tail group ($p > 1$),
91 which favors the formation of W/O emulsions. This change in surfactant properties with temperature
92 can be utilized to form nanoemulsions. A mixture of surfactant, oil, and water are heated to a
93 temperature around or above the PIT, and then the system is rapidly cooled with continuous stirring,
94 which induces the spontaneous formation of a nanoemulsion [29, 30]. The formation of
95 nanoemulsions with small droplets typically requires that the system is rapidly cooled with continuous
96 stirring since this promotes the breakdown of the bicontinuous microemulsion phase formed at the PIT,
97 and avoids rapid droplet coalescence just below the PIT [31, 32].

98 A number of previous studies have shown that SLNs can be formed using the PIT method. The
99 influence of lipid and surfactant type on the ability of SLNs fabricated using this approach to
100 encapsulate lipophilic pharmaceutical agents has been reported [33-38]. The ability of PIT-SLNs to
101 improve the stability of an encapsulated nutraceutical (β -carotene) against chemical degradation has
102 also been demonstrated [39]. A recent study showed that ultrafine (< 10 nm) SLNs could be produced
103 using the PIT method that were suitable for topical application of biomarkers [40]. A recent *in vitro*
104 study suggested that PIT-SLNs may be suitable colloidal delivery systems for transporting lipophilic
105 bioactive agents across the Blood Brain Barrier [41].

106 In the current study, we aimed to utilize the PIT method to fabricate SLNs, and then to
107 investigate the role of storage temperature on their physical stability. We hypothesized that the
108 temperature the lipid nanoparticles were stored at relative to their melting/crystallization points and
109 PIT would influence their long-term stability. A model surfactant-oil-water (SOW) system was used to
110 facilitate a better understanding of the underlying physicochemical mechanisms involved. Thus, n-
111 octadecane was used as a model oil and Brij 30 (C₁₂E₄) was used as a model non-ionic surfactant. The
112 approach used to form SLNs and LLNs using the PIT method is shown schematically in **Figure 1**. A
113 W/O emulsion was prepared by holding the SOW mixture at an initial temperature above the PIT with
114 continuous stirring. An O/W nanoemulsion was then formed by rapidly cooling the SOW mixture
115 below the PIT while continuing to stir. In some cases, the system was cooled to a final temperature
116 that was above the crystallization temperature of the emulsified lipid phase, which led to the formation
117 of LLNs. In other cases, the system was cooled to a final temperature that was below the
118 crystallization temperature of the emulsified phase, which led to the formation of SLNs. The resulting
119 colloidal dispersions were then stored at different temperatures to determine the influence of their
120 physical state and interfacial properties on their stability. Ultimately, the information gained from this
121 study will be useful for determining the optimum protocol for producing stable PIT-SLNs that might
122 be suitable for application in various types of commercial product.

123 **2. Materials and Methods**

124 **2.1. Materials**

125 Octadecane (99.5 % purity), C₁₂E₄ (Brij 30, Technical grade), and sodium benzoate (99 % purity)
126 were purchased from the Sigma-Aldrich Co. (St. Louis, MO). Citric acid (99 % purity) was purchased
127 from Fisher Scientific (St. Clair Shores, MI). All materials were used without further purification.
128 Double distilled water was used for the preparation of all solutions and nanoemulsions.

129 **2.2. Determination of Phase Inversion Temperature**

130 The PIT value was determined by monitoring the change in turbidity of surfactant-oil-water
131 systems during heating at a constant rate [42]. Coarse emulsions containing 20 wt% octadecane, 6 wt%
132 Brij 30, and 74 wt% water phase (pH 3.0, 0.8% citric acid, 0.08% sodium benzoate) were prepared by
133 mixing at ambient temperature using a magnetic stirrer. Turbidity *versus* temperature scans were then
134 measured for the SOW mixture using a UV/visible spectrophotometer at 600 nm (Ultra-spec 3000 pro,
135 Biochrom Ltd Cambridge, UK) with a temperature control module (Programmable Heated Cell
136 Holder, 80-2106-14, Biochrom Ltd., Cambridge, UK). Temperature control and data recording were

137 carried out using computer software (Swift TM Melting Temp V1.09, Data Capture Software V1.02,
138 Biochrom Ltd., Cambridge, UK). The samples were contained within quartz cuvettes with a path
139 length of 1.0 cm and heated from 10 °C at a rate of 1 °C min⁻¹ with continuous stirring. Double-
140 distilled water was used as a blank.

141 **2.3. Lipid Nanoparticle Preparation**

142 Initially, the PIT of the SOW system was established using turbidity-temperature measurements as
143 described previously. Weighed amounts of oil, surfactant, and water were then gently mixed together
144 in a glass beaker and heated to an initial temperature (50°C) above the PIT (≈40°C) using a water bath.
145 After this temperature was reached, the mixture was held for 10 minutes and then split into four
146 samples in four beakers (preheated to 50°C). The mixtures were continuously stirred using a
147 mechanical stirrer throughout the heating stage. The hot mixtures (50°C) were then rapidly cooled to
148 different temperatures (5°C for the experimental group and 20°C, 25°C, 32°C for the control group)
149 using ice baths with continuous magnetic stirring. The liquid lipid nanoparticles (LLNs) in the control
150 group were then stored in temperature-controlled incubators at their formation temperatures. The solid
151 lipid nanoparticles (SLNs) in the experimental group (5°C) were then split into four samples and
152 heated to different storage temperatures (5, 20, 25 and 32°C) in separate temperature-controlled
153 incubators. As a result the physical state of the lipid phase in the experimental group changed
154 depending on storage temperature.

155 **2.4. Particle Size Analysis**

156 The stability of the lipid nanoparticles was assessed by measuring changes in their mean particle
157 size and particle size distribution during storage. Particle size measurements were performed using a
158 dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK).
159 To avoid multiple scattering effects samples were diluted 100 times using buffer solutions (pH 3.0)
160 maintained at temperatures corresponding to their storage temperatures. The DLS data are reported as
161 the Z-average mean diameter. The particle size of some samples was also assessed by a static light
162 scattering (SLS) instrument (Mastersizer 2000, Malvern Instruments, Malvern, UK), which is more
163 suitable for analyzing samples containing large particles. In this case, measurements were reported as
164 the volume-weighted mean diameter, $d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$, where n_i is the number of particles of
165 diameter d_i .

166 **2.5. Differential Scanning Calorimetry**

167 A differential scanning calorimeter (DSC; Q1000, TA Instruments, New Castle, DE) was used to

168 study the thermal transformations of the emulsified lipid phases during heating-cooling cycles.
169 Emulsions (5-10 mg) were placed in hermetic aluminum pans and sealed, while sealed empty pans
170 were used as a reference. The heating and cooling rate was set at 20°C/min for all samples. Different
171 heat-cool cycles were used to study the crystallization and melting temperature of the emulsified lipid
172 phase and to establish the physical state of lipid nanoparticles stored at 20°C:

- 173 - *Crystallization and melting point determination:* Lipid nanoparticles were prepared using the PIT
174 method described above and then cooled to ambient temperature. Then the samples were loaded
175 into a DSC pan that was placed in the instrument and cooled to 5°C. After cooling, the samples
176 were heated to 60°C, and then cooled to 5°C again.
- 177 - *Physical state verification:* Samples were prepared by cooling from 50°C to 5°C and then heated
178 to 20°C (SLNs, experimental group) or cooling from 50°C to 20°C (LLNs, control group). The
179 resulting lipid nanoparticles were then heated from 20°C to 60°C in the DSC instrument.

180 **2.6. Microstructure Analysis**

181 The microstructure of the samples was observed using optical microscopy (Nikon Eclipse 80i,
182 Nikon Instrument Inc., Melville, NY) with the aid of a temperature-controlled stage (LTS 120) and a
183 temperature controller (PE 94, Linkam Scientific Instruments Ltd., Tadworth, UK). Samples were
184 pipetted onto a microscope slide, covered with a cover slip, and then placed on the temperature-
185 controlled stage.

186 For determination of the microstructures of the surfactant-oil-water systems at temperatures (50,
187 40 and 20 °C) around the phase inversion temperature, samples were initially prepared by heating the
188 mixtures to 50 °C with stirring. Images of the mixtures were then recorded at 50 °C, and after they
189 were cooled from 50 to 40°C at a rate of 10°C min⁻¹. The image of the mixture at 20 °C was obtained
190 by cooling the mixture from 50 to 20 °C in a test tube with constant stirring (since this procedure is
191 needed to form nanoemulsions).

192 For determination of the microstructures of the samples at different storage temperatures, images
193 were obtained by placing an aliquot of the samples on microscope slides placed on the temperature-
194 controlled stage set to the appropriate temperature.

195 **2.7. Statistical Analysis**

196 All experiments were carried out in triplicate and the results are reported as means and standard
197 deviations. Statistical differences were tested using statistical analysis software (Minitab 17, Minitab
198 Inc., State College, PA). A student's t-test was used to determine the statistical differences between
199 two groups. Tukey's test was used in an analysis of variance (ANOVA) mode for the comparison of

200 differences amongst more than two groups. In all cases, $P < 0.05$ is considered a significant effect.

201 3. Results and Discussions

202 3.1. Identification of Phase Inversion Temperature

203 The PIT of the surfactant-oil-water (SOW) system used in this study was determined by
204 measuring the change in turbidity with temperature. Initially, a coarse O/W emulsion was prepared by
205 stirring surfactant, oil, and water at ambient temperature, and then the turbidity was measured as the
206 temperature of this mixture was increased from 10 to 50 °C at a controlled rate. The turbidity remained
207 relatively high and constant from 10 to 38 °C and the system had an opaque appearance, which can be
208 attributed to strong light scattering by the relatively large droplets in the coarse O/W emulsion (**Figure**
209 **2a**). The formation of an O/W emulsion is preferred at lower temperatures because the surfactant
210 head groups are highly hydrated, and so that their cross-sectional areas are appreciably larger than
211 those of their tail groups [43]. From 39 to 40 °C, the turbidity fell steeply and the samples had a
212 translucent appearance (**Figure 2a**), which is indicative of the formation of a bicontinuous
213 microemulsion. In this temperature range, taken to correspond to the PIT, the cross-sectional areas of
214 the surfactant head and tail groups are nearly balanced, which favors a surfactant monolayer curvature
215 close to zero thereby facilitating bicontinuous microemulsion formation [28]. Upon further heating,
216 the samples became highly turbid with an opaque appearance (**Figure 2a**). At these higher
217 temperatures, the surfactant head groups become highly dehydrated so that their cross-sectional areas
218 were less than that of their tail groups, which favors the formation of W/O emulsions [27]. During
219 cooling, a steep decrease in turbidity was observed when the temperature fell below 40 °C and the
220 samples became translucent (**Figure 2a**), which was again attributed to the formation of a bicontinuous
221 microemulsion [32]. Upon further cooling, there was a steep increase in turbidity and the samples
222 became opaque (**Figure 2a**), which can be attributed to rapid droplet coalescence just below the PIT
223 leading to the formation of large particles that scatter light strongly [32]. There was a difference in the
224 range of temperatures where the bicontinuous microemulsion was formed during the heating and
225 cooling processes due to hysteresis effects, such as the finite time required for the samples to reach the
226 incubation temperatures and the presence of an activation energy between the emulsion and
227 microemulsion systems.

228 Optical microscopy images of the samples at different temperatures clearly indicated that they
229 underwent appreciable microstructural changes that were consistent with the turbidity measurements.
230 The optical microscopy images suggested that a water-in-oil emulsion containing relatively large

droplets was formed at 50 °C, a microemulsion phase was formed at 40 °C, and an oil-in-water nanoemulsion containing small droplets was formed at 20 °C (**Figure 2b**). Overall, these results suggest that the PIT of the SOW used in this work was around 30 to 40 °C [42]. It should be noted that measurements of the electrical conductivity of SOW systems are also commonly used to determine their PIT, but we showed in a previous study that there was a good correlation between the PIT determined by turbidity and conductivity measurements for similar systems [44], and so we only used turbidity measurements here.

3.2. Thermal Behavior of Emulsified Oil Phase

The crystallization and melting points of the emulsified lipid phase were determined using DSC. Initially, a nanoemulsion was prepared by heating a SOW mixture above the PIT, and then rapidly cooling to ambient temperature (which is above the crystallization temperature). Then the sample was loaded into a DSC pan, placed in the instrument, and cooled to 5 °C. A heat-cool cycle was then applied to the sample, which involved heating to 60 °C and then cooling back to 5 °C, and the change in heat flow with temperature was monitored using DSC (**Figure 3**). During heating, a single endothermic peak was observed around 26 °C, which can be attributed to the melting of the crystalline octadecane droplets [45]. The melting temperature of the emulsified octadecane was slightly below that of bulk octadecane (28 °C), which can be attributed to the influence of confinement effects on the thermodynamic phase transition temperature [46, 47]. During cooling, a large exothermic peak was observed around 8 °C, which can be attributed to crystallization of the emulsified oil phase. The fact that the emulsified octadecane crystallized at a much lower temperature than the bulk octadecane can be attributed to substantial supercooling effects [48, 49]. Bulk oils usually contain catalytic impurities that promote the formation of nuclei within the oil phase, whereas it is highly unlikely to find one of these impurities inside very small oil droplets. Consequently, crystallization tends to be preceded by heterogeneous nucleation in bulk oils, but by homogeneous nucleation in emulsified oils [50]. In summary, the DSC measurements indicated that LLNs would remain in a liquid state when maintained at temperatures exceeding about 12 °C, and SLNs would remain in a solid state when kept at temperatures below about 23 °C (**Figure 3**).

3.3. Effect of Storage Temperatures on Solid and Liquid Lipid Nanoparticles

SLNs and LLNs were fabricated by controlling the thermal history of lipid nanoparticles formed using the PIT method (**Figure 1**). SLNs were fabricated by cooling SOW systems from a temperature (50 °C) above the PIT to a temperature (5 °C) below the crystallization point to completely crystallize the lipid phase. The SLNs were then stored at temperatures below (5 and 20 °C), around (25 °C) and

263 above (32 °C) their melting temperature. As a control group, LLNs were produced by directly cooling
264 the SOW mixtures from 50 °C to similar storage temperatures (20, 25, and 32 °C) as the SLNs. The
265 effect of storage temperature on the lipid nanoparticles was examined by measuring their mean particle
266 size (**Figure 4**) and studying their microstructure (**Figure 5**) before and after 24 hours incubation.

267 For the SLNs, the mean particle diameters of the nanoparticles were initially relatively small at 5
268 and 20 °C ($d \approx 109$ and 128 nm, respectively) and did not change appreciably after 24 hours of storage.
269 These measurements indicated that it was possible to produce SLNs (*i.e.*, $d < 200$ nm) using the PIT
270 method and that SLNs stored well below their melting temperature were stable to particle growth in the
271 short-term. However, examination of the SOW systems using optical microscopy showed that the
272 SLNs did not exist as individual solid particles as expected (**Figure 1**), but actually formed complex
273 aggregated structures (**Figure 5a**). These structures may have formed due to the relatively high
274 surfactant levels used leading to depletion flocculation [51, 52]. The fact that an increase in particle
275 size was not observed by light scattering can be attributed to the fact that the flocs became dissociated
276 when the samples were diluted prior to making the measurements.

277 When the SLNs were held at higher temperatures there was an appreciable increase in mean
278 particle diameter, which is indicative of particle growth and/or aggregation, which may have occurred
279 due to morphology changes, flocculation, coalescence, or partial coalescence (**Figures 4 and 5b**). The
280 SLNs stored at 25 °C were highly unstable to aggregation, and gelation was observed in the overall
281 system after 24 hours storage, *i.e.*, the samples did not flow when the test tubes were inverted. In
282 addition, the optical microscopy images indicated that the system consisted of large irregularly shaped
283 structures that were associated with each other (**Figure 5b**). This effect may have occurred due to the
284 formation of a network of aggregated lipid particles caused by partial coalescence [24, 53]. Partial
285 coalescence occurs when the lipid droplets are only partly crystalline so that a crystal in one droplet
286 can penetrate into the liquid portion of another droplet [48]. Electron microscopy studies have
287 previously demonstrated the formation of a particle network formation in systems containing partially
288 crystalline hydrocarbon nanoparticles [54]. The fact that the system contained large irregularly shaped
289 particles, rather than small spherical lipid particles similar to those in the initial samples, suggests that
290 there was a large morphology change associated with droplet crystallization. A recent study reported
291 that hydrocarbon droplets may form a variety of shapes after crystallizing depending on holding
292 temperature and cooling rates [55], which is consistent with our data.

293 Appreciable particle aggregation was also observed when the SLNs were stored at 32 °C, but
294 gelation was not observed in this case (**Figure 5c**). This temperature is appreciably above the melting
295 point of the lipid nanoparticles, and therefore the lipid phase will have been completely liquid. In this

case, the increase in particle size was most likely due to accelerated droplet coalescence at temperatures just below the PIT [56]. When the optimum curvature of the surfactant monolayer favors an O/W emulsion, but is still fairly close to unity, then the droplet surfaces are highly mobile and prone to coalescence [57].

For the LLNs, the lipid nanoparticles exhibited a relatively small amount of growth when stored at 20 °C for 24 hours as seen in the light scattering (**Figure 4**) and optical microscopy images (**Figure 5a**). Small droplets were present immediately after nanoemulsion formation (0 hours) but their dimensions increased after 24 hours incubation. This effect may have occurred because the storage temperature was not sufficiently below the phase inversion temperature, which led to coalescence.

Extensive particle growth and gelation were observed when the samples were stored at 25 °C (**Figures 4 and 5b**). One might not have expected partial coalescence to occur for the LLNs because the droplets were initially completely liquid. However, droplet coalescence may have occurred during storage since the temperature was still fairly close to the PIT, which led to an increase in droplet size, and thus the chance of finding catalytic impurities within the oil droplets increased [48]. As a result, heterogeneous nucleation (rather than homogeneous nucleation) occurred within the lipid phase leading to the formation of octadecane crystals that promoted partial coalescence and gelation. The LLNs stored at 32 °C showed an appreciable increase in particle diameter with no gelation (**Figures 4 and 5c**), which can again be attributed to the fact that the storage temperature was quite close to the PIT, which promoted accelerated droplet coalescence [56].

3.4. Storage Stability of Solid and Liquid Nanoparticles

Finally, we measured the long-term storage stability of the lipid nanoparticles found to be the most stable in the short-term stability studies: SLNs at 5 and 20 °C and LLNs at 20 °C. DSC was used to confirm the physical states of the lipid nanoparticles in the LLNs and SLNs at 20 °C at the beginning of the storage studies. The two samples were heated from 20 to 60°C in the DSC instrument to monitor if any melting transition occurred (**Figure 6**). During heating, an endothermic peak associated with lipid phase melting was observed for the SLNs, but no peak was observed for the LLNs, which confirmed that the SLNs initially contained solid particles whereas the LLNs initially contained liquid ones. To monitor the storage stability of the SLNs and LLNs, the change in mean particle size and particle size distribution was recorded over time. The SLNs were relatively stable to particle growth throughout 8 days of storage at both at 5 and 20 °C, with little increase in mean particle diameter (**Figure 7**) or change in particle size distribution (**Figure 8a and 8b**). Completely solid lipid nanoparticles would be expected to be stable to both coalescence and partial coalescence, which would

328 account for this effect. Conversely, the LLNs exhibited an appreciable increase in mean particle size
329 during storage (**Figure 7**) and the particle size distribution changed appreciably (**Figure 8c**). As
330 discussed earlier, the increase in droplet size for the LLNs during storage can be attributed to droplet
331 coalescence when the storage temperature is not sufficiently far below the PIT [57]. These results
332 highlight the critical importance of storage temperature for ensuring the stability of lipid nanoparticles
333 fabricated using the PIT method.

334 4. Conclusions

335 We have shown that both LLNs and SLNs can be produced by the PIT method using model
336 surfactant (C₁₂E₄) / oil (octadecane) / water systems by altering their thermal history. The effect of
337 storage temperature on the stability of the lipid nanoparticles was examined by holding them at
338 different temperatures and monitoring changes in their particle size. The lipid nanoparticles were
339 unstable when the storage temperature was above the melting temperature of the emulsified lipid
340 phase, which was attributed to rapid coalescence at temperatures just below the PIT. This problem
341 may be overcome by using an SOW system that has a PIT temperature that is appreciably above the
342 intended storage temperature of the lipid nanoparticles. The lipid nanoparticles tended to aggregate
343 and gel when the storage temperature was around the melting temperature of the emulsified lipid
344 phase, **which was attributed to an appreciable change in particle morphology and partial coalescence.**
345 **Using a lipid phase that is not partially crystalline at the intended storage temperature may alleviate**
346 **this problem.** SLNs stored at temperatures well below the melting temperature of the lipid phase were
347 relatively stable to particle growth during storage. This effect may be because completely solid
348 particles are stable to both coalescence and partial coalescence. In summary, this study provides some
349 useful insights into the formation and storage of LLNs and SLNs **fabricated** using the PIT method.
350 These lipid nanoparticles may be used as colloidal delivery systems for lipophilic active ingredients for
351 industrial applications, such as pharmaceuticals, nutraceuticals, or supplements.

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Figure 1: Schematic representation of formation of SLNs and LLNs using the PIT method. LLNs are formed by cooling SOW mixtures below the PIT but above the droplet crystallization temperature, whereas SLNs are formed by cooling below the PIT and droplet crystallization temperature.

Figure 2a: Temperature dependence of the turbidity of the surfactant/oil/water system (20 wt. % octadecane, 6 wt. % Brij 30, 74 wt. % pH 3 buffer solution) during heating and cooling at a controlled rate of $1^{\circ}\text{C min}^{-1}$.

Figure 2b: Temperature dependence of the microstructure of the surfactant/oil/water system (20 wt. % octadecane, 6 wt. % Brij 30, 74 wt. % pH 3 buffer solution) at 50 °C, 40 °C and 20 °C upon cooling.

Figure 3: DSC profiles of emulsified oil phases of the surfactant/oil/water system during heating and cooling at a controlled rate of $20\text{ }^{\circ}\text{C min}^{-1}$.

Figure 4: Mean particle diameters of emulsions with different thermal histories and storage temperatures after 0 and 24 hours. Emulsions were prepared by cooling rapidly from 50 to 5 °C to form SLNs, and then heating to 5, 20, 25, 32 or by cooling directly from 50 °C to 20*, 25*, 32* to form LLNs. For both groups, the emulsions at 25 °C became gels after 24 hours storage. Statistical differences of the means are shown between 0 hour and 24 hours for each sample (A, B) and among different samples at 0 hour (a, b, c) or 24 hours (a*, b*, c*) were tested. Means with different letters are significantly different ($P < 0.05$).

(a)

$50^{\circ}\text{C} \rightarrow 5^{\circ}\text{C} \rightarrow 25^{\circ}\text{C}$

$50^{\circ}\text{C} \rightarrow 25^{\circ}\text{C}$

0 hr

24 hr

(b)

Figure 5: Optical microscopy images of surfactant/oil/water mixtures with different thermal histories taken at 0 hours or after 24 hours storage. The scale bars are 100 μm in length.

Figure 6: DSC profiles of lipid nanoparticle suspensions during heating at a controlled rate of 20 °C min⁻¹. SLNs were prepared by cooling from 50 to 5 °C and then heating to 20 °C (solid), while LLNs were prepared by cooling directly from 50 to 20 °C (liquid).

Figure 7: Changes in mean particle diameter of SLNs and LLNs during storage at 20°C.

Figure 8: Changes in the particle size distribution of SLNs or LLNs during storage: SLNs were prepared by cooling from 50 to 5 °C (a), and then heating to 20 °C (b), while LLNs were prepared by cooling directly from 50 to 20 °C (c).