Distribution of a Model Bioactive within Solid Lipid Nanoparticles and Nanostructured Lipid Carriers Influences its Loading Efficiency and Oxidative Stability

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Abstract

The overall goal of this study was to characterize the distribution of a model bioactive encapsulant in the lipid domain of SLNs and NLCs and its relationship with loading efficiency and reactivity of the model encapsulant with oxidative stress agents. Distribution of a model bioactive (beta-carotene) was compared to that of fluorescent dye (Nile red) in SLNs, 10% NLC, 30% NLC, 50% NLC, 70% NLC (the number represents the percentage of liquid lipid within the total lipid amount) and emulsions. Fluorescence imaging shows that the distribution of Nile red in the lipid domain of colloidal carriers was similar to that of beta-carotene in all formulations. Based on the combination of imaging observations and loading efficiency measurements, the results demonstrate that beta- carotene was excluded from the lipid domain in both SLNs and NLCs. The extent of exclusion decreased, while uniformity in the distribution of encapsulant in the lipid domain of colloidal carrier increased with an increase in percentage of liquid lipid content of NLCs. Oxidative stability of the encapsulated beta-carotene in SLN and NLCs (at least until 30% liquid lipid composition) was significantly lower compared to that in emulsion. Only for the NLCs with 50 and 70% liquid lipid content, oxidative stability of the encapsulated compound was significantly higher than that in emulsions. Overall, the results demonstrate that differences in loading efficiency and oxidative stability of beta-carotene in SLNs and NLCs may be explained by the differences in the intra-particle distribution of beta-carotene.

Key words

Imaging; Solid lipid nanoparticles (SLNs); Nanostructured lipid carriers (NLCs); Emulsions; Loading efficiency; Oxidative stability

1. Introduction

Lipid based carriers have been widely used for encapsulation and delivery of hydrophobic compounds in food, pharmaceutical and cosmetic industries (Fathi et al., 2012; Muller et al., 2002b; Sagalowicz and Leser, 2010; Tamjidi et al., 2013). Among the various approaches used to classify these carriers, one of the common approaches is based on the physical state of the lipid phase. Based on this approach, lipid carriers that are commonly used in diverse applications can be classified as solid lipid nanoparticles (SLNs) with a solid lipid phase, emulsions with a fluid lipid phase and nanostructured lipid carriers with a combination of solid and liquid lipids, respectively (Muller et al., 2002a; Pardeike et al., 2009). Identification of optimal formulations among these diverse lipid carriers has been of significant research interest. Previous studies have indicated that the SLN and NLC based lipid carriers show improved stability against coalescence compared to emulsions (Mehnert and Mader, 2012; Muller et al., 2002b). In addition, prior studies have compared these formulations based on a variety of factors, including (a) encapsulation efficiency; (b) chemical stability; and (c) controlled and triggered release of the encapsulated ingredients (Fathi et al., 2012; Mehnert and Mader, 2012; Muller et al., 2002b; Tamjidi et al., 2013; Teeranachaideekul et al., 2007a; Tikekar and Nitin, 2011).

Based on these criteria, prior studies have indicated significant differences in the encapsulation efficiency (Mehnert and Mader, 2012; Muller et al., 2002a, b); oxidative stability (Muller et al., 2002b; Tikekar and Nitin, 2011) and controlled release (Mehnert and Mader, 2012; Muller et al., 2002a, b; Teeranachaideekul et al., 2007b) among emulsions, SLNs and NLCs. Distribution of encapsulants within the lipid domain has been proposed as one of the leading

factors influencing these differences among lipid based colloidal carriers (Dan, 2014; Pardeike et al., 2009). Despite potential role of the distribution of encapsulants within the lipid phase on their encapsulation efficiency, oxidative stability and release, there is limited direct evidence of the variations in the distribution of the encapsulants within these lipid-based particles. Furthermore, there is limited understanding of differences in the oxidative barrier properties among SLNs, NLCs and emulsions. Recent evidence of variations in the distribution of the encapsulants among SLNs and selected compositions of NLCs and their impact on the reactivity of these encapsulants was presented using surrogate dyes such as Nile red and peroxy radical sensitive BODIPY dyes or EPR probes such as PTMIO (Tikekar and Nitin, 2011, 2012; Yucel et al., 2013). It is widely recognized that the properties of the surrogate dyes such as their partition coefficient, solubility in lipid domains, and the ability to form crystalline states may not match the properties of the target encapsulants such as bioactive compounds. For example, the log P_{ow} value of Nile red dye (a lipid imaging dye used in a prior study) is ~ 4 (Turner and Guy, 1997), while the log P_{ow} value of beta-carotene (provitamin A pigment) is ~ 17 (Decker et al., 2005). In addition, bioactive compounds such as beta-carotene can form crystalline structures (Zhou et al., 1996) that can have a limited solubility in either lipid or aqueous phase. This complex behavior cannot be easily mimicked by the surrogate dyes. Furthermore, there is significant interest in optimization of NLC compositions for improving encapsulation efficiency, controlled release and/or improved oxidative stability of the bioactives (Tamjidi et al., 2013), but there is limited understanding of the differences in the distribution of encapsulants within these NLCs and its impact on encapsulation efficiency and oxidative stability of the encapsulant. Thus, measuring the distribution of encapsulants can be critical for understanding the differences in the functional

properties of these formulations, such as controlled release and oxidative stability of the encapsulants.

The overall motivation for this study was to characterizing the distribution of a model bioactive encapsulant within the lipid domain of SLNs and NLCs and its relationship with loading efficiency and reactivity of the model encapsulant with oxidative stress agents. With this motivation, the specific goals of this study were to: (1) directly visualize, compare and correlate the distribution of a model dye and a model bioactive within the lipid domain of SLNs, 10% NLC, 30% NLC, 50% NLC, 70% NLC (the number represents the percentage of liquid lipid incorporated within the total lipid amount) and emulsion; and (2) evaluate the effect of variations in the distribution of the model bioactive compound within the lipid domain of the colloidal carriers on its loading efficiency and oxidative stability.

Eicosane and glyceryl trioctanoate (GT) were selected as the solid and liquid lipids, respectively. The % of solid and liquid lipids used for the NLC formulations selected for this study have been used in some prior studies to evaluate the physicochemical properties and release profiles of various encapsulants (Jenning et al., 2000b; Souto et al., 2004; Teeranachaideekul et al., 2008). A fluorescence imaging approach was used to visualize the distribution of a model dye (Nile red) and a model bioactive compound (beta-carotene) within the lipid domain of the colloidal carriers. Beta-carotene has antioxidant activity and has been proposed as a cancer prevention agent (Burton and Ingold, 1984; Omenn et al., 1996). However, limited water solubility and poor oxidative stability limits its broad application in diverse products. The unique and novel aspects of this study are: (1) imaging based evidence for the differences in the distribution of a selected bioactive and a dye molecule within the lipid domain of SLNs, NLCs and emulsion formulations. As discussed earlier, the prior studies including the

recent studies have either postulated about differences in the distribution of encapsulants within the lipid domain of colloidal particles (Jenning and Gohla, 2001; Jennings et al., 2000a; Salminen et al., 2016; zur Muhlen et al., 1998) or provided an indirect evidence for it (Jores et al., 2003; Teeranachaideekul et al., 2008); (2) a systematic comparison among SLN, NLCs and emulsion for characterizing influence of the lipid composition on the loading efficiency and oxidative stability of the encapsulants. The observed effects on the loading efficiency and oxidative stability of the encapsulants in these colloidal formulations could be specifically attributed to the lipid composition since the colloidal carriers were prepared using identical methods and were stabilized using the same set of emulsifier molecules; and (3) a comparison between the distribution of a model hydrophobic bioactive compound and a fluorescent hydrophobic dye within the lipid domain of the colloidal carriers. The results of this analysis may validate and enable the use of low—cost, fluorescent dyes as surrogates to evaluate lipid particles for encapsulation applications. Success in this approach may enable evaluation of a broad set of bioactive compounds that may not have endogenous fluorescent properties.

2. Materials and methods

2.1. Materials

Bile salts, eicosane, glyceryl trioctanoate (GT), Nile red, beta-carotene, tetrahydrofuran (THF) and 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) were purchased from Sigma-Aldrich Incorporated (St. Louis, MO). High-melting lecithin (Phospholipon[®] 80 H) was a gift from American Lecithin Inc. (Oxford, CT). Ultrapure water (16M Ω -cm) was obtained from an in-house water filtration system.

2.2. Preparation of SLNs, NLCs and emulsions

The aqueous phase was prepared by dispersing high-melting lecithin and bile salts in ultrapure water to achieve the final concentration of 2 w/v % and 1 w/v % of the aqueous phase, respectively. The total lipid content for all SLNs, NLCs and emulsions was set at 10 w/v % of the aqueous phase. For SLNs, the lipid phase consisted of 100% eicosane. NLCs were prepared by blending liquid (glyceryl trioctanoate) and solid (eicosane) lipids in various proportions (10:90, 30:70, 50:50, and 70:30 liquid: solid) to form 10%, 30%, 50%, and 70% NLCs, respectively. Emulsions contained only GT as the lipid phase. The lipid phase was heated to 70 °C to ensure a complete melting of eicosane. Nile red (12 µg/g total lipid) or beta-carotene (1 mg/g total lipid) was mixed with the lipid phase. The amount selected for beta-carotene was based on the solubility limit of beta-carotene in bulk triglyceride (Borel et al., 1996). The aqueous phase was also heated to 70 °C using a hot plate. The lipid phase was mixed with the aqueous phase and dispersed using a hand-held disperser (Ultra-Turrax model T25, IKA Works, Wilmington, NC) set at 9500 rpm for 2 min. The coarse emulsion was then sonicated for 30 s with a probe-tip sonicator (Qsonica Q55 ultrasonic processor, Newton, CT) at 50% of the maximum amplitude to form stable SLNs, NLCs and emulsion. Sodium azide (0.1 w/v %) was added to SLNs, NLCs and emulsion to prevent the microbial growth and the samples were stored at 4 °C overnight to enable solidification of eicosane.

2.3. Particle size measurements

Hydrodynamic diameters of SLN and NLC particles and emulsion droplets was measured using a particle size analyzer (Model: Malvern Nano Series, Malvern Instruments, Inc., Westborough, MA). The settings for the analyzer were- material type: oil, particle refractive index = 1.45, dispersant type: water, dispersant refractive index= 1.33, temperature: 25 °C. Particle size measurements were analyzed based on the number average droplet size distribution.

2.4. Differential scanning calorimetry (DSC)

DSC (Model: Pyris 1, Perkin Elmer, Waltham, MA) was performed to identify the melting and solidification temperatures of the emulsified eicosane. SLN and NLC samples (approximately 5 mg) were placed in an aluminum pan for DSC measurements. An empty aluminum pan was used as a control. The sample was heated from 25 °C to 90 °C at the rate of 10 °C min⁻¹ and then cooled to 5 °C at the same rate. This process was repeated twice to ensure consistency in the measurement of melting and solidification temperatures.

2.5. Fluorescence imaging

Fluorescence confocal imaging was conducted to characterize the distribution of the encapsulated Nile red or beta-carotene within the lipid phase of SLNs, NLCs and emulsions. Coarse lipid particles and emulsions were prepared for imaging purposes. After the formation of coarse particles and coarse emulsions, the samples were diluted 10 times with ultrapure water and mounted on a glass slide, covered with a cover slip and stored at 4 °C for 1 h, after which fluorescence imaging was performed using a Zeiss LSM 510 Meta confocal microscope. Confocal images were obtained using a 100× oil immersion objective. The samples encapsulating Nile red were imaged using a 543 nm laser excitation and the fluorescence emission signal was collected using a band pass 565-615 nm filter. The samples encapsulating beta-carotene were imaged using a 488 nm laser excitation and the fluorescence emission signal was collected using a band pass 515-560 nm filter. To characterize the distribution of the encapsulated Nile red or beta-carotene within SLNs, NLCs and emulsions, the samples were optically sectioned using a z-scanning mode. The z-scans of the samples were acquired with a z-

spacing of 1.0 μm . The 3-D reconstruction of images was performed using a 'montage' function in Metamorph[®] image processing software (Molecular devices Inc., Carlsbad, CA).

2.6. Loading efficiency of beta-carotene in SLNs, NLCs and emulsions

SLN, NLC and emulsion samples containing beta-carotene were passed through a hollow fiber filter with a pore size of 0.2 μm to remove the micelles formed from the excess surfactant that may incorporate a small fraction of the added beta-carotene. This step enabled us to specifically remove these micelles and measure the beta-carotene loaded exclusively within the lipid particles. Beta-carotene was extracted using THF from the concentrated formulations after filtration through a hollow fiber filter. The concentrated formulation (200 μL) was mixed with 1 mL THF and the mixture was centrifuged at 14000 g for 10 min. The supernatant (1 mL) was placed in a cuvette and the absorbance was measured at 450 nm using a UV-Vis spectrophotometer (GENESYS 10S Series, Thermo Scientific, Rockford, IL). A blank sample was prepared using 1 mL of THF and 200 μL of ultrapure water. All the measurements were carried out in triplicate.

2.7. Stability of beta-carotene in SLNs, NLCs and emulsions

The oxidative stability of the encapsulated beta-carotene was measured by characterizing the changes in the absorbance of beta-carotene as a function of incubation time. SLN, NLC and emulsion samples containing beta-carotene were incubated with and without 20 mM AAPH at 22°C in the dark for 96 h. The stability of beta-carotene in particles or droplets was also compared to that in lecithin-bile salts macromolecular aggregates known as mixed micelles (Mazer et al., 1980). The mixed micelles were prepared using the same emulsifiers used to form particles or droplets. Beta-carotene was added to THF at the concentration of 1 mg/mL. The

solution was dried in a glass vial under a stream of nitrogen, then placed in a vacuum desiccator for 1 h. The lecithin-bile salts mixture was added to the glass vial after drying in order to form the mixed micelles.

To measure the stability of beta-carotene, samples were disrupted periodically at 0, 6, 18, 24, 48, 72 and 96 h by mixing 200 μ L of mixed micelles, SLN, NLC or emulsion sample with 1 mL of THF. The mixture was centrifuged at 14000 g for 10 min. The supernatant (1 mL) was placed in a cuvette and the absorbance was measured at 450 nm using a UV–Vis spectrophotometer (GENESYS 10S Series, Thermo Scientific, Rockford, IL). A blank sample was prepared using 1 mL THF and 200 μ L ultrapure water. All the measurements were carried out in triplicates.

2.8. Statistical analysis

Statistical analysis was carried out using Microsoft Excel 2007 (Microsoft Corp., Bellevue, WA, USA). Student's t-test was used to identify significant differences between the treatments ($\alpha=0.05$).

3. Results and discussion

3.1. Particle size measurements

Table 1 compares the mean particle diameter of the SLNs, NLCs and emulsions loaded with beta-carotene. The mean particle diameter for all the formulations ranged between 200-350 nm. The PDI (polydispersity index) and FWHM (full width at half maximum) values associated with the size distribution of SLN, 10% NLC, 30% NLC, 50% NLC, 70% NLC and emulsion are also shown in **Table 1**. Based on these values, it is evident that there was a significant overlap in

the particle size distribution of SLNs and NLCs and that the % content of solid lipid in SLNs and NLCs did not significantly influence the particle size ($p > 0.05$). However, the average particle size of SLNs and all NLC formulations was larger than that of emulsions. These results are in agreement with the results of prior studies (Jores et al., 2003; Teeranachaideekul et al., 2008). The relatively large particle size of SLNs and NLCs may be due to the characteristic anisometric shape of SLNs and NLCs that can influence the light scattering measurements and these shape differences can influence the particle size of lipid carriers as proposed by some prior studies (Jores et al., 2003; Teeranachaideekul et al., 2008).

3.2. Differential scanning calorimetry (DSC) for SLNs and NLCs

DSC was used to characterize the melting and solidification temperatures of the emulsified eicosane in SLNs and NLCs. **Figure 1** shows the representative melting and solidification curves of 30% NLC and **Table 2** shows that the average melting temperature, melting peak height, melting enthalpy, solidification temperature, solidification peak height and solidification enthalpy for SLN, 10% NLC, 30% NLC, 50% NLC and 70% NLC. Results in **Figure 1** illustrate the characteristic super-cooling (based on differences in melting and solidification temperatures) of emulsified eicosane in 30% NLC composition. Similar differences in melting and solidification temperatures were also observed in SLNs and other NLC formulations (**Table 2**). These results are consistent with the literature (Ghosh et al., 2006; Gulseren and Coupland, 2008) and our prior studies (Tikekar and Nitin, 2011, 2012). Based on the results in **Table 2**, it is evident that solid lipid fraction was present in all the SLN and NLC formulations as eicosane melted at temperatures between 31 and 37 °C and solidified at temperatures between 11 and 22 °C. The results in **Table 2** also show that the melting temperature, melting peak height and melting enthalpy decreased as the percentage of the liquid

lipid fraction increased in these formulations. Similar to the melting results, the solidification temperature, solidification peak height and solidification enthalpy also decreased with an increase in the percentage of the liquid lipid content. This is due to the presence of GT in NLCs that reduces the formation of eicosane crystals with the increase in liquid lipid (GT) content (Ban et al., 2014; Teeranachaideekul et al., 2008). These results are similar to the results from some prior studies where similar trends in the melting and solidification curves of NLCs were observed (Anantachaisilp et al., 2010; Ban et al., 2014; Teeranachaideekul et al., 2008).

3.3. Fluorescence imaging to visualize the distribution of encapsulated Nile red within SLNs, NLCs and emulsions

To visualize the distribution of Nile red using optical imaging, coarse particles (both SLNs and NLCs) and coarse emulsion droplets were imaged using confocal microscopy. **Figures 2(a) – (f)** show confocal sections of a representative SLN, 10% NLC, 30% NLC, 50% NLC, 70% NLC particle and emulsion droplet (magnified view), respectively. Confocal sections of the representative particles obtained at a z-scale interval of 1 μm and a 3-dimensional projection image was reconstructed from the individual confocal sections of a single representative particle. **Figure 2(a)** shows that the SLN had significantly lower fluorescence intensity in the center of the lipid phase compared to the edges, indicating that a significant amount of Nile red was excluded from the SLN. These results are in agreement with some prior studies (Jores et al., 2003; Yucel et al., 2013) that suggested the expulsion of the encapsulated molecules from SLNs using electron spin resonance (EPR) measurements. The results are also consistent with our prior studies where exclusion was also observed in SLNs encapsulating surrogate dyes (Tikekar and Nitin, 2011, 2012).

The distribution of Nile red within 10% and 30% NLCs is shown in **Figure 2(b)** and **Figure 2(c)**. The images show that the distribution of Nile red within NLCs was not homogeneous within the lipid phase of these NLC particles. Together this data set in **Figures 2(b-c)** indicates that exclusion of Nile red from the solid domain within a NLC particle results in a heterogeneous distribution of Nile red and its enrichment within distinct domains composed of liquid lipid. As the liquid lipid content increased to 50% and 70% in NLCs (**Figure 2(d)** and **Figure 2(e)**), the distribution of Nile red became relatively more uniform compared to that in SLNs, 10% and 30% NLCs. Furthermore, the distribution of Nile red within these NLC particles (50 and 70% liquid lipid content) was similar to that within emulsions, where a homogeneous distribution was observed (**Figure 2(f)**).

The imaging results clearly demonstrate the differences in the distribution pattern of encapsulated Nile red among SLNs, NLCs and emulsions. Nile red was excluded from SLNs, while it was localized within distinct liquid lipid region in 10% and 30% NLCs. The distribution of Nile red became more uniform with an increase in the content of liquid lipid and resembled that observed in 100% liquid lipid emulsions. The results are in agreement with a prior study that measured the distribution of a model compound using Raman spectrometry and found that the model compound was mostly soluble in the liquid lipid and the distribution of the compound become more uniform with an increase in the liquid lipid content in NLCs (Anantachaisilp et al., 2010).

3.4. Fluorescence imaging to visualize the distribution of encapsulated beta-carotene within SLNs, NLCs and emulsions

The next goal was to characterize the distribution of a model bioactive, beta-carotene, within SLNs, NLCs and emulsions using confocal microscopy. Beta-carotene results can then be

compared with the distribution of Nile red. **Figures 3(a) – (f)** show confocal sections of a representative SLN, 10% NLC, 30% NLC, 50% NLC, 70% NLC particle and emulsion droplet (magnified view), respectively, obtained at a z-scale interval of 1 μ m. Based on the optical sectioning, a 3-dimensional projection image was reconstructed from the individual confocal sections of a representative particle/droplet. **Figure 3** shows that exclusion of beta-carotene was also observed in SLNs and its distribution became more uniform with an increase in the liquid lipid content in NLCs. Results in **Figure 3** demonstrate that despite their different chemical structures, the distribution of beta-carotene was similar to that of Nile red in all the formulations. These results indicate that for the selected composition of the SLN and NLCs, exclusion of the encapsulated compound was independent of the relative hydrophobicity of the encapsulated compounds. To the best of our knowledge this is a novel finding that has not been reported in the prior literature. These results also indicate that Nile red may be a suitable surrogate molecule to evaluate the distribution of hydrophobic encapsulants within encapsulation systems. It is particularly attractive as a surrogate tracer due to its relatively high photo stability and a high fluorescence quantum yield (Kucherak et al., 2010).

3.5. Loading efficiency of beta-carotene in SLNs, NLCs and emulsions

Based on the imaging results, we hypothesized that the exclusion of beta-carotene in SLNs and NLCs would affect its loading efficiency in these colloidal carriers with respect to emulsions. To test this hypothesis, the loading efficiency of beta-carotene in SLNs, NLCs and emulsion was measured (**Figure 4**). Results in **Figure 4** show that SLNs was only able to load 20% of the beta-carotene originally added to the melted eicosane prior to the formation of particles. As the liquid lipid content increased, the amount of loaded beta-carotene increased. For emulsions prepared with 100% liquid lipid, the loading efficiency reached close to 100%. This

was expected since the total amount of beta-carotene selected for loading was based on the maximum solubility of beta-carotene in bulk triglycerides as described in the materials and methods section. These results demonstrate that the amount of encapsulated bioactive in lipid particles is related to the fraction of the liquid lipid present within the SLNs, NLCs and the emulsion. The results are consistent with some prior studies where it was observed that NLCs show a high encapsulation efficiency of the encapsulants compared to SLNs and the percentage of encapsulated compounds increased with an increase in the liquid lipid content (Hu et al., 2005; Jennings and Gohla, 2001; Tikekar and Nitin, 2012).

3.6. Stability of beta-carotene in SLNs, NLCs and emulsions

Oxidative stability of encapsulated bioactives in SLN, NLC and emulsions was evaluated using a radical mediated oxidation approach. This approach represents the relative reactivity of the encapsulated compound in the selected formulations and the results of these measurements are indicative of the accelerated oxidation processes in the presence of reactive oxygen species. AAPH was used as a peroxy radical generator in this study, due to its relatively long half-life (approximately 175 h) in the aqueous solution (Zimowska et al., 1997). Long half-life of the AAPH enables a relatively constant generation of peroxy radicals for an extended time period. Stability of beta-carotene was measured based on the changes in absorbance of beta-carotene at 450 nm. The supplementary information section illustrates the correlation between the changes in UV-Vis absorbance of beta-carotene at 450 nm and reduction in its antioxidant potential as measured using the ferric reducing ability of antioxidant (FRAP) assay.

Table 3 compares the oxidative stability of beta-carotene encapsulated in SLNs, NLCs, emulsions and mixed micelles in the absence (**Table 3(a)**) and presence (**Table 3(b)**) of AAPH. The rationale behind measuring the oxidative stability of beta-carotene within mixed micelles

was that it was likely that the beta-carotene excluded from the lipid particles could be stabilized by the mixed micelles formed from the excess emulsifier present in the aqueous phase. Furthermore, due to the dynamic equilibrium or exchange of emulsifier molecules between mixed micelles and colloidal lipid dispersions, the two phases cannot be completely separated without destabilizing the formulations.

In the absence of AAPH, beta-carotene was stable in all the formulations with at least 90% remaining in all the samples after 96 h, including the SLNs and the micelles. However, more than 90% of beta-carotene encapsulated within the mixed micelles degraded within the first 6 h in the presence of AAPH, indicating that beta-carotene solubilized within these mixed micelles was highly susceptible to degradation. Although SLNs performed better than the mixed micelles, approximately 90% of beta-carotene was degraded just after 24 h of exposure. 10% NLCs performed better than SLNs and 90% beta-carotene was degraded only after 72 h of AAPH treatment. However, as the liquid lipid content increased above 10% within NLCs, the stability of beta-carotene improved significantly. At 72 h of AAPH treatment, only 45%, 35% and 20% of beta-carotene was degraded in 30%, 50% and 70% NLCs, respectively. Thus, the solid lipid in SLNs provided limited barrier to peroxy radicals as compared to high liquid lipid content NLCs (50% and 70% NLCs).

This improvement in the stability of the encapsulated beta-carotene in NLCs as compared to SLNs can be explained by the fact that a significant exclusion of the encapsulated beta-carotene from the solid lipid phase of SLNs. Comparison of the stability of the encapsulated beta-carotene among different NLC formulations demonstrates that the stability of beta-carotene improved as the liquid lipid content increased. This is attributed to the fact that the beta-carotene encapsulated within NLCs containing higher fraction of liquid lipid was largely localized inside

the lipid particle with only a small fraction present at the surface. Thus, the effective surface area of exposure of the encapsulated bioactive in high liquid lipid content NLCs was significantly smaller than NLCs with lower liquid lipid content (such as in 10% NLCs). Moreover, for SLNs and low liquid lipid content NLCs, a large amount of beta-carotene may be stabilized in the form of mixed micelles due to its exclusion from the lipid phase of the particle, thus increasing the combined rate of oxidation of beta-carotene as observed in this experiment. Interestingly, for the emulsion sample that contained 100% liquid lipid, the oxidative stability of beta-carotene was significantly lower than that for the 50% and 70 % NLCs after 96 h of storage, and it was comparable to that in 30% NLCs. This may be due to the fact that in 50% and 70% NLCs, beta-carotene was retained in the liquid compartments surrounded by a protective barrier of solid lipid network, resulting in improved oxidative stability of encapsulated bioactives (Cornacchia and Roos, 2011). For the selected composition of lipids (eicosane and GT), oxidative stability of the encapsulated beta-carotene in SLN and NLCs (at least until 30% liquid lipid composition) was significantly lower compared to that in emulsion. For the 50% and 70% NLCs (50 and 70% lipid content), oxidative stability of the encapsulated compound was significantly higher than that in emulsions. Overall, oxidative stability of an encapsulant was in the order $SLN < NLCs \text{ (at least 30\% lipid composition)} < \text{emulsion} < NLC \text{ (50 and 70\% liquid lipid composition)}$. This is a novel and a significant finding as oxidative stability is one of key attributes in many lipid based formulations for applications in cosmetic, pharmaceutical and food products. To the best of our knowledge, there is no prior study that has demonstrated this significant finding systematically by comparing across diverse lipid based formulations.

Stability of beta-carotene in SLNs has been studied before (Helgason et al., 2009; Jennings and Gohla, 2001; Qian et al., 2013; Salminen et al., 2016). Qian and others compared the

degradation rate of beta-carotene in SLNs and liquid lipid nanoparticles prepared using a blend of cocoa butter and hydrogenated palm oil and found that the degradation rate was higher in SLNs compared to liquid nanoparticles due to potential exclusion of beta-carotene from the SLNs (Qian et al., 2013). Helgason and others (Helgason et al., 2009) and Salminen and others (Salminen et al., 2016) evaluated the effect of surfactant properties on the stability of beta-carotene in SLNs alone. Consistent with our observation, these previous studies hypothesize that exclusion of encapsulant from the lipid phase significantly increases its degradation rate. In this study, we observed that in the absence of oxidative stress no significant degradation of beta-carotene was observed in both lipid particles and the phospholipid micelles. In the presence of oxidative stress induced by AAPH, beta-carotene encapsulated in mixed-micelles degraded more rapidly as compared to SLN, but within 24 hours of AAPH treatment more than 90% reduction of the beta-carotene had degraded in both mixed micelles and SLN. Thus, SLNs only provide a partial protection for beta-carotene as compared to NLCs in the presence of oxidative stress. Some of the prior studies have hypothesized that initiation of lipid crystallization in SLNs at the droplet interface and its subsequent progression to the center of the lipid particle can lead to the entrapment of bioactives and these entrapped bioactives may be protected from pro-oxidants (Helgason et al., 2009; Salminen et al., 2016). These studies also indicate that initiation of crystallization may depend both on the physico-chemical properties of the interface and the encapsulated bioactive compound. In this study, the results demonstrate similar exclusion of both beta-carotene and Nile red dye from SLNs, although the log P values and the chemical structure of beta-carotene and Nile red are significantly different. Similarly, in this study the results did not indicate effective entrapment of beta-carotene in SLNs. These differences from the hypothesis proposed in previous studies may result due to differences in the physico-chemical

properties of eicosane and the use of a combination of bile salts and phospholipids from the interfacial composition. In contrast to triglycerides used in prior studies, crystallization of the eicosane is monotropic and its crystallization behavior in colloidal particles is not different from its crystallization behavior in the bulk phase (Yoon et al., 1989). Furthermore, the presence of bile salts as a co-emulsifier may also influence the physical properties of the interface and impact initiation of crystallization at the emulsion interface as has been proposed in the literature. Overall, the results of this study demonstrate that the distribution of the encapsulated bioactive affects its oxidative stability in SLNs, NLCs and emulsions. The results also demonstrate that despite significant differences in molecular properties, both the selected bioactive and the dye molecule have similar distribution in SLNs and NLCs. Further studies are required to improve understanding of the role of lipid and interfacial composition on crystallization of lipids in SLNs and NLCs.

4. Conclusions

Based on the experimental observations, exclusion of the encapsulated compounds (both Nile red and beta-carotene with diverse log P values) was visualized in SLNs using confocal microscopy. Exclusion of the encapsulated compounds was also observed in the case of NLCs, particularly 10 and 30% NLCs as the encapsulation efficiency of the compounds in NLCs was significantly reduced compared to that in emulsion. Together, these results indicate that for the selected composition of the SLN and NLCs, exclusion of the encapsulated compound was independent of the relative hydrophobicity of the encapsulated compounds. For the selected composition of lipids (eicosane and GT), oxidative stability of the encapsulated beta-carotene in SLN and NLCs (at least until 30% liquid lipid composition) was significantly lower compared to that in emulsion. For the 50 and 70% NLCs (50 and 70% liquid lipid content), oxidative stability

of the encapsulated compound was significantly higher than that in emulsions. Overall, oxidative stability of an encapsulant was in the order: SLN < NLCs (at least until 30% liquid lipid composition) < emulsion < NLC (50 and 70% liquid lipid composition). Overall, the results demonstrate that the differences in the distribution of the encapsulant localized within SLNs and NLCs can explain the differences in its loading efficiency and oxidative stability.

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Table 1: Number based particle diameter, PDI and FWHM of SLNs, 10% NLC, 30% NLC, 50% NLC, 70% NLC and emulsions. Each value is an average of three independent measurements $\pm$ standard deviation (standard deviation not shown for PDI values).

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Figure 3: Distribution of beta-carotene within a representative (a) SLN, (b) 10% NLC, (c) 30% NLC (d) 50% NLC, (e) 70% NLC particle or (f) an emulsion droplet at different z positions (z spacing = 1 μm) and 3-d reconstruction of the distribution of beta-carotene within the particle or emulsion droplet (scale bar = 5 μm).

Figure 4: Loading efficiency of beta-carotene in SLNs, 10% NLC, 30% NLC, 50% NLC, 70% NLC and emulsions. Each value is an average of three independent measurements $\pm$ standard deviation. Different letters represent statistically significant difference ($p < 0.05$).

Table 1

Sample	Number based particle size (nm)	PDI	FWHM (nm)
SLN	358.4 ± 40.0	0.216	101.7 ± 32.3
10% NLC	285.4 ± 39.2	0.132	93.1 ± 21.4
30% NLC	281.5 ± 47.2	0.111	87.0 ± 24.4
50% NLC	295.1 ± 15.4	0.149	94.6 ± 21.7
70% NLC	312.2 ± 33.6	0.195	91.5 ± 26.1
Emulsion	205.4 ± 15.5	0.193	79.4 ± 7.5

Table 2

	SLN	10% NLC	30% NLC	50% NLC	70% NLC
Melting temperature (°C)	37.4 ± 0.09	36.1 ± 0.01	34.9 ± 0.04	33.6 ± 0.19	31.3 ± 0.12
Melting peak height (mW)	7.90 ± 0.11	4.65 ± 0.05	3.08 ± 0.01	2.30 ± 0.40	0.58 ± 0.03
Melting enthalpy (J/g)	27.5 ± 0.66	22.8 ± 0.21	16.5 ± 0.03	14.7 ± 0.10	5.70 ± 0.05
Solidification temperature (°C)	22.1 ± 0.10	18.1 ± 0.09	15.6 ± 0.01	12.6 ± 0.19	11.3 ± 0.21
Solidification peak height (mW)	-8.37 ± 0.17	-4.38 ± 0.02	-3.72 ± 0.02	-2.82 ± 0.29	-0.97 ± 0.12
Solidification enthalpy (J/g)	-31.5 ± 0.41	-22.6 ± 0.37	-21.3 ± 0.04	-13.9 ± 0.16	-7.04 ± 0.01

Table 3(a)

Time (h)	Micelles	SLN	10% NLC	30% NLC	50% NLC	70% NLC	Emulsion
0	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a
6	90.6 ± 0.6^a	96.4 ± 1.4^{bc}	95.0 ± 1.9^{bc}	98.4 ± 1.3^{bd}	99.2 ± 0.8^d	91.6 ± 3.0^{ab}	100 ± 1.0^d
18	92.9 ± 1.9^a	92.8 ± 0.9^a	95.7 ± 0.8^{ab}	97.6 ± 1.8^{bcd}	99.2 ± 0.8^c	93.5 ± 1.6^a	97.4 ± 0.4^d
24	95.3 ± 1.3^{ac}	92.0 ± 0.9^b	94.3 ± 0.4^a	96.8 ± 1.8^c	95.9 ± 0.9^c	92.2 ± 3.9^{abc}	96.9 ± 1.5^c
48	89.8 ± 2.5^a	91.5 ± 0.4^a	90.7 ± 0.4^a	95.9 ± 0.4^b	95.9 ± 3.4^{bc}	94.8 ± 3.7^{bc}	97.4 ± 1.1^c
72	85.9 ± 1.2^a	90.6 ± 2.7^b	91.4 ± 1.2^b	93.5 ± 2.2^{bc}	97.5 ± 1.6^c	95.5 ± 2.0^c	96.1 ± 1.6^c
96	89.1 ± 2.0^a	90.1 ± 3.0^a	92.1 ± 1.6^{abd}	95.2 ± 1.8^{bc}	96.7 ± 1.5^c	94.8 ± 1.3^c	95.3 ± 0.6^{cd}

Table 3(b)

Time (h)	Micelles	SLN	10% NLC	30% NLC	50% NLC	70% NLC	Emulsion
0	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a
6	9.12 ± 2.5^a	93.9 ± 3.2^{bd}	99.2 ± 1.0^{ce}	97.4 ± 1.1^{cde}	97.5 ± 1.9^{bcde}	95.3 ± 3.2^{bcd}	100.4 ± 1.3^e
18	6.32 ± 0.8^a	31.1 ± 0.6^b	87.7 ± 2.6^c	99.1 ± 0.6^d	95.8 ± 1.9^e	97.3 ± 0.9^e	91.0 ± 1.1^c
24	5.96 ± 2.3^a	12.1 ± 0.6^b	82.8 ± 3.6^c	96.6 ± 4.6^d	97.3 ± 2.8^d	95.3 ± 5.3^d	88.6 ± 0.7^e
48	6.31 ± 0.9^a	6.81 ± 4.4^a	31.9 ± 1.5^b	80.3 ± 3.4^c	85.8 ± 3.7^{cd}	87.3 ± 0.7^d	77.5 ± 0.7^c
72	6.67 ± 1.9^a	7.57 ± 1.1^a	9.84 ± 1.4^a	56.1 ± 1.9^b	65.8 ± 1.9^c	80.7 ± 1.6^d	57.1 ± 0.8^b
96	4.21 ± 1.1^a	6.82 ± 1.0^b	9.83 ± 1.1^c	33.3 ± 1.9^d	50.8 ± 1.5^e	59.3 ± 1.3^f	40.8 ± 1.1^g