Cellular Uptake of \(\beta\)-Carotene from Protein Stabilized Solid Lipid Nanoparticles Prepared by Homogenization–Evaporation Method

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ABSTRACT: With a homogenization–evaporation method, \(\beta\)-carotene (BC) loaded nanoparticles were prepared with different ratios of food-grade sodium caseinate (SC), whey protein isolate (WPI), or soy protein isolate (SPI) to BC and evaluated for their physicochemical stability, \textit{in vitro} cytotoxicity, and cellular uptake by Caco-2 cells. The particle diameters of the BC loaded nanoparticles with 0.75% SC or 1.0% WPI emulsifiers were 75 and 90 nm, respectively. Mean particle diameters of three BC loaded nanoparticle nanoemulsions increased less than 10% at 4 \(\degree\)C while they increased more at 25 \(\degree\)C (10–76%) during 30 days of storage. The oxidative stability of BC loaded nanoparticles encapsulated by proteins decreased in the following order: SC > WPI > SPI. The retention rates of BC in nanoparticles were 63.5%, 60.5%, and 41.8% for SC, WPI, and SPI, respectively, after 30 days of storage at 25 \(\degree\)C. The BC’s chemical stability was improved by increasing the concentration of protein. Both the rate of particle growth and the total BC loss at 25 \(\degree\)C were larger than at 4 \(\degree\)C. The color of BC loaded nanoparticles decreased with increasing storage in the dark without oxygen, similar to the decrease in BC content of nanoparticles at 4 and 25 \(\degree\)C. Almost no cytotoxicity due to BC loaded nanoparticles cellular uptake was observed, especially when diluted 10 times or more. The uptake of BC was significantly improved through nanoparticle delivery systems by 2.6-, 3.4-, and 1.7-fold increase, respectively, for SC, WPI, and SPI, as compared to the free BC. The results of this study indicate that protein stabilized, BC loaded nanoparticles can improve stability and uptake of BC.

KEYWORDS: \(\beta\)-carotene, nanoparticles, stability, Caco-2, toxicity, uptake

INTRODUCTION

\(\beta\)-Carotene (BC) is the vegetable source of vitamin A (VA). While the importance of retinol to vision is well-known, BC is also the precursor of ligands necessary for the activity of nuclear receptors that regulate energy metabolism. Some studies have shown that BC reduces weight gain and may have an effect on obesity. VA also is inversely associated with indicators of aging. VA is lower in patients with Alzheimer’s disease and slows the deposit of amyloid \(\beta\)-protein in the brain of transgenic mouse models injected with VA. VA and its precursor BC have an important role in many metabolic and developmental, growth-related processes.

Increasing uptake of BC by dietary supplementation is difficult to achieve because it is insoluble in water, only very slightly soluble in food oils, and easily oxidized or isomerized. A wide variety of emulsion delivery systems have been studied for the oral delivery of BC, other lipids, and water insoluble drugs. Water dispersible nanoemulsions are of particular interest because their high surface area may have the potential of increasing the bioavailability of lipophilic substances and can be incorporated into optically transparent products due to their weakly light scattering properties. The stability of encapsulated bioactive molecules has been shown to be significantly improved by nanoemulsions encapsulated by modified starches. Nanosize also confers a high stability against particle aggregation and gravitational separation. Furthermore, using nanoparticles prepared by the solvent evaporation method rather than using fats as a carrier for lipid-soluble bioactive compounds allows preparation of an emulsion system with a high load of the bioactive components without having high lipid content, which may be beneficial to consumers attempting to limit fat intake in their diet.

The emulsification–evaporation technique has been successfully used to prepare BC nanodispersions with Tween 20, a nonionic synthetic emulsifier and sodium caseinate. Previous studies investigated the physicochemical properties of BC emulsion process parameters, formation, properties, and stability of oil-in-water (O/W) BC emulsions. Emulsifiers reduce the interfacial tension between the oil and water phases and reduce the amount of input energy required to disrupt the oil phase into nano- or microsized droplets; thus, the proper selection of emulsifier for the application is critical. For human oral delivery the use of synthetic emulsifiers such as Tween 20–80 may be subject to safety concerns and oxidative stability.

The food proteins, sodium caseinate (SC), whey protein isolate (WPI), and soy protein isolate (SPI), are generally recognized as safe (GRAS) and are good emulsifiers. Adsorption to the surface of lipid droplets is stabilized by their relatively high hydrophobic amino acid content. The major caseins, \(\alpha\)-s1 and \(\beta\), have molecular weight ranges of 22–
24 kDa. Caseinates are flexible proteins that readily adsorb to lipid surfaces with coverage of 1 mg protein/m². β-Lactoglobulin, the major protein component of WPI, has a molecular weight of about 18 kDa. Although β-lactoglobulin and caseinates have similar interfacial energy lowering properties,14 globulins are more compact and slower to reorganize upon adsorption resulting in a higher amount for surface coverage, 2–3 mg/m². SPI is also a globulin but much larger, 200–350 kDa. The surface charge upon adsorption is highly dependent on pH. At acidic and neutral pH encountered in the digestive system, dairy and SPI proteins are highly cationic or anionic, respectively. The release of protein encapsulated lipids may also be assisted by digestive enzymes. In vitro digestion by gastric pepsin did not significantly increase release of lipids encapsulated by SPI, WPI, or β-lactoferrin, but intestinal pancreatin significantly accelerated release of emulsion stabilized by β-lactoglobulin and WPI.15 Differences in size, conformation, hydrophobic amino acid content, and ionic amino acid content contribute to different emulsion size, protein layer thickness, stability to aggregation, and stabilization of the lipid core to oxidation. Proteins (like SC, WPI, and SPI) are thought to be able to better protect BC and lipids against oxidation and degradation in encapsulated systems3,16 compared to small molecule surfactants (like Tween 20), because of the free radical scavenging and metal chelating properties of cysteine, tyrosine, tryptophan, phenylalanine, and histidine.17

Although protein emulsifiers are GRAS, there are concerns regarding the safety and potential cytotoxicity of nanosystems because they may be inhaled or absorbed via pinocytosis by the intestinal epithelial cells.18 Hu et al. investigated the cytotoxicity of chitosan—caseinophosphopeptide nanocomplexes by MTT assay and found no significant toxicity.19 He et al. showed that the viability of cells exposed to food protein stabilized nanoemulsions was greater than 85% at 3 mg/mL concentration, while other traditional emulsifiers (like Tween 80) caused a significant decrease in cell viability.15 To date, there have been no reports about the cellular cytotoxicity and in vitro uptake of BC loaded nanoparticles with Caco-2 cells.

In the present study, SC, WPI, and SPI were used as emulsifiers to develop food-grade, relatively stable nano- and microparticles for oral delivery of BC. The effects of emulsifiers and the concentrations on physicochemical stability of BC loaded nanoparticles during storage were evaluated. We hypothesize that although the residual BC of the nanoparticles prepared by the solvent evaporation is likely to be a solid, uptake by Caco-2 cells will still occur because of the high surface area. The cytotoxicity and cellular uptake of prepared BC loaded nanoparticles in an in vitro digestive system were also investigated.

**MATERIALS AND METHODS**

**Materials.** BC (>97.0%, UV) was purchased from Sigma-Aldrich (St. Louis, MO). Sodium caseinate (SC) powder (protein content >95% and 1.0% fat on dry basis by manufacturer) was obtained from Fonterra Co-operative Group (Auckland, New Zealand). Whey protein isolate (WPI; Hilmar 9410, 93.0% protein, 0.2% lactose, 1.0% fat, and 2.0% ash, on a dry basis, and 4.5% water) was obtained from Hilmar Food International, Inc., Livingston, CA). SPI was extracted in our laboratory from dehulled and defatted soybean meal by the alkali-solution and acid-isolation method (protein content >93% and 0.96% fat content on dry basis by measurement).20 Dulbecco’s modified Eagle’s medium (DMEM) (containing 4.5 g/L d-glucose and GlutaMAX), penicillin and streptomycin (100×), fetal bovine serum (FBS), TrypLE Select, Hanks’ balanced salt solution (HBSS), and phosphate buffer solution (PBS) (10×) were purchased from GIBCO (Grand Island, NY). The human colon carcinoma cell lines (Caco-2 cells, ATCC, Manassas, VA) were grown in DMEM. Cells after 25–40 passages were used in the uptake study. All other chemicals and reagents were used HPLC-grade and obtained from Sigma-Aldrich (St. Louis, MO).

**Methods.** BC Loaded Nanoparticle Preparation. SC, WPI, and SPI were dispersed (0.10–1.50%, w/w) separately in 5 mM phosphate buffer pH 7.0 containing 0.02 wt % sodium azide antimicrobial agent. The dispersions were stirred for 2 h and refrigerated overnight to maximize dissolution. Then, 0.1% β-caroteine (BC) (w/w) solutions were prepared in ethyl acetate. BC loaded nanoparticles were prepared according to the method described by Tan and Nakajima21 with minor changes. Water, 100 mL, was saturated with 8.3 g of ethyl acetate prior to formulating the protein solution in order to prevent precipitation of BC during homogenization. The BC in ethyl acetate solution was combined with the ethyl acetate saturated protein solution at a ratio of 1:9. The mixtures were homogenized for 10 min at 8000 rpm using an Ultra-Turrax homogenizer (T25, Ika-Werke, Staufen, Germany) to form a coarse emulsion before additional homogenization using a two-stage valve homogenizer (APV-1000 high-pressure homogenizer, Wilmington, MA) for one cycle at 70 MPa. After the homogenization, ethyl acetate was removed from the emulsions on a 30 °C. Temporator at 40 °C. The residual ethyl acetate was determined by headspace gas chromatography and found to be less than 0.001% (data not shown), less than that by other investigators.22 Samples of 5 mL of each nanoparticles were stored in 10 mL amber glass vials flushed with nitrogen at 4 and 25 °C for analysis of BC retention during 30 d storage.

**Measurement of Surface Tension and CMC.** Surface tension was measured using the pendant plate method with the DataPhysics DCAT21 dynamic contact angle meter and tensiometer (DataPhysics Instruments, Germany) according to the method described by Mao et al.23 The critical micelle concentration (CMC) of SC in this study was determined by using DataPhysics DCAT21 similar to the method previously described.23 Briefly, several concentrations of protein nanoparticles were prepared by dilution of the protein stock solution (20 mg mL⁻¹) with sterile ultrapure water. The CMC is the point at which the surface tension no longer increases with increasing protein concentration. Temperature was kept constant at 25.0 ± 0.2 °C with a circulating water bath system.

**Particle Diameter and ζ-Potential Analysis.** The characteristics of BC loaded nanoparticle solutions were determined by measuring the mean particle diameter, diameter distribution, and ζ-potential of nanoparticles by dynamic light scattering technique using a Malvern Zetasizer (Nano-ZS; Malvern Instruments, Worcestershire, U.K.). Refractive indices of 1.47 for BC and 1.33 for phosphate buffers were used. To reduce multiple scattering effects, the nanoparticles dispersions were diluted 100 times with 5 mM phosphate buffer pH 7.0 prior to the measurements that were performed in triplicate.

**Particle diameter measurements to determine the effect of storage on BC loaded nanoparticle solutions stabilized by the three proteins were conducted at 0, 16, and 30 d at 4 and 25 °C.**

**Color Evaluation.** The color of nanoparticles of BC and its isomers depends on their concentration, stability, and particle diameter. The color parameters (L*, a*, b*) were determined using a tristimulus colorimeter Ultra Scan PRO (Hunternab, Japan). L* values are a measure of lightness, a* indicates degree of greenness (for negative a* values) and degree of redness (for positive a* values), and b* also ranges from negative to positive values indicating degree of blueness to yellowness, respectively. The color of the nanoparticle samples all prepared with 1.0% (w/w) proteins was determined by measuring the reflectance from the surface of a transparent quartz cuvette. All measurements were repeated three times. Color changes (color differences) were calculated using the following formula:

\[
\Delta E^* = \sqrt{\left( L^* - L_0^* \right)^2 + \left( a^* - a_0^* \right)^2 + \left( b^* - b_0^* \right)^2 }^{1/2} 
\]

Here, subscript 0 indicates color at start of storage period.
Transmission Electron Microscopy (TEM) Analysis. TEM was used to observe differences in internal structure of the nanoparticles and to confirm diameter by dynamic light scattering. Samples were prepared by the conventional negative-staining method. Nanoparticles were placed on copper grids and negatively stained with 2% (w/v) phosphotungstic acid for several minutes at room temperature. Grids bearing nanoparticles were analyzed with a Hitachi H-700 transmission electron microscope (Hitachi, Tokyo, Japan).

Cytotoxicity of BC Loaded Nanoparticles on Caco-2 Cells. Cell viability, a commonly used indicator of in vitro cytotoxicity, was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylessrazolium bromide (MTT) assay. The colorimetric MTT assay measures the activity of cellular enzymes that reduce MTT to its insoluble purple formazan form. Caco-2 cells were incubated in DMEM containing 10% FBS, 1X nonessential amino acid (NAA), and 1X penicillin and streptomycin. Caco-2 cells in DMEM were seeded at a density of 1.2 × 10^5 cells/well onto 96-well plates and incubated (Sanyo, Osaka, Japan) at 37 °C in 90% humidity and 5% CO_2. After 48 h incubation, the media was discharged, and BC loaded nanoparticles diluted with DMEM were added to the wells. After 24 h incubation at 37 °C the nanoparticles containing media were replaced with 100 μL of DMEM containing MTT (10 μL, 5 mg/mL in 1X PBS, pH 7.2) and incubated for 2 h at 37 °C. Finally, the supernatant was removed and 100 μL of dimethyl sulfoxide (DMSO) added. The 96-well plates were analyzed with a Multilabel microplate counter (Victor3, PerkinElmer, Waltham, MA) at 570 nm. Cell viability was calculated by the percentage of absorbance relative to control (treated with DMEM only).

Cellular Uptake of BC Loaded Nanoparticles. Caco-2 cells were incubated in DMEM containing 10% FBS, 1X nonessential amino acid (NAA), and 1X penicillin and streptomycin at 37 °C in a 90% humidity and 5% CO_2, incubator (Sanyo, Oska, Japan) onto 12-well plates. Caco-2 cells were seeded at a density of 1.2 × 10^6 cells/well. The media was changed every two days. After five days, the cell monolayers were observed by optical microscopy (Leica, IL) to ensure that the cells reached about 90% confluence. Caco-2 cell monolayers were washed with DMEM three times. Both protein encapsulated BC loaded nanoparticles and BC in THF/DMSO (1:1, v/v) solution (as control) were diluted in DMEM to a final BC concentration of 5 μg/mL. A 1 mL portion of the BC encapsulated nanoparticle and control in THF/DMSO were added to separate wells containing washed Caco-2 cells. The final concentration of THF was less than 0.1%. Following 24 h incubation at 37 °C in a CO_2, incubator, the supernatants were removed, and the cell monolayers were washed three times with precooled PBS solution to stop uptake and wash the surface of cell monolayers. After that, 0.5 mL of 10% ethanolic PBS solution was added to dissociate cell monolayers. Cell suspensions were obtained with cell scrapers. A 0.4 mL portion of cell suspension was extracted with solvent and analyzed for BC content by HPLC (procedure given below), and the remaining 0.1 mL was used for protein content determination.

Protein Content Analysis. Caco-2 cell protein content was determined using the bicinchoninic acid (BCA) assay at 560 nm with bovine serum albumin as the standard.

Extraction and Quantification of BC from Nanoparticles and Caco-2 Cells. The BC in nanoparticles was extracted with 3 mL of ethanol/n-hexane (1:2, v/v) three times. The hexane fractions were combined and brought up to 10 mL for HPLC analysis. The effect of storage on BC loaded nanoparticle dispersion stabilized by the three proteins was conducted at 0, 2, 9, 16, 23, and 30 days at both temperature conditions.

A 25 μL portion of 5 μg/mL trans-β-Apo-8’-carotenal in ethanol was added to 0.4 mL Caco-2 cell suspension as an internal standard. The cell suspensions (0.4 mL) were extracted three times with 3 mL of ethanol/n-hexane (1:2, v/v). Organic fractions were removed and combined with transfer pipets, and the extract was dried under a stream of nitrogen gas at 40 °C. The BC extract was dissolved in 0.1 mL of methanol/dichloromethane (1:1, v/v) containing 0.1% BHT for HPLC analysis. The recovery of trans-β-Apo-8’-carotenal from Caco-2 cells was greater than 94%.

HPLC quantification of BC was performed using an Agilent 1100 HPLC system with DAD UV-vis absorption detector (Agilent, Santa Clara, CA), according to the published method. BC quantitation range (0.1 μg/mL to 100 ug/mL) was established for HPLC analysis of BC in nanoparticles and Caco-2 cells. A polymeric carotene C_10 reverse-phase analytical column (250 mm × 4.6 mm i.d., 5 μm, YMC, Inc., Wilmington, NC) was used to separate BC with a flow rate of 1 mL/min at room temperature. The guard column was a reverse-phase C_18 column (50 mm × 3.0 mm i.d., 5 μm, YMC, Inc.). The injection volume was 20 μL, and the detection wavelength was 450 nm. The chromatography conditions were as follows: solvent A, methanol/acetonitrile/H_2O (84:14:2, v/v/v); solvent B, dichloromethane. The solvent gradient program was 80% A/20% B at 0 min and was changed linearly to 45% A/55% B in 15 min, and maintained for 5 min, and followed by a linear return to 80% A/20% B at 25 min.

RESULTS AND DISCUSSION

Particle Diameter. The particle diameter distributions of solid BC nanoparticles encapsulated by SC, WPI, and SPI at the concentrations resulting in the smallest mean particle diameter are shown in Figure 1, and the mean diameter and polydispersity index determined by dynamic light scattering (DLS) are shown in Table 1. The loading capacity of BC in nanoparticles was 1.07%, 1.05%, and 1.06% for SC, WPI, and SPI, respectively. For all three different protein solid lipid nanoparticles, the efficiencies of encapsulation of BC were 99.1%, 98.8%, and 98.7%, respectively, for SC, WPI, and SPI at 1.0% (w/w), respectively, indicating BC was fully encapsulated by three proteins. Although evaporation of the solvent resulted in solid lipid nanoparticles, DSC and X-ray diffraction did not show crystalline cores (results not shown). The SC, WPI, and SPI used contained 1% residual lipids (Materials and Methods section). The ethyl acetate used to solubilize BC and saturate the protein solution most likely also extracted the residual lipids and BC formed a mixture that was not crystalline.

Figure 1. Particle diameter distributions of SC (1.0%, w/w), WPI (1.0%, w/w), and SPI (1.0%, w/w) encapsulated BC loaded nanoparticles.
or simply facilitates uptake. Increasing the emulsifier concentration of SC, WPI, and SPI from 0.10% to 1.00% generally resulted in a significant ($P < 0.05$) decrease in particle diameter (Table 1). The Z-average particle diameter of 0.1% (w/w) SC stabilized nanoparticles was 162.4 nm (Table 1), and some unemulsified BC-lipid solids could be observed after evaporation, illustrating that BC was not fully emulsified. When SC was increased to 0.25% (w/w), the mean diameter decreased drastically ($P < 0.05$) to 125.8 nm. However, the particle distribution was now bimodal with peaks at about 100 and 30 nm. Increasing concentrations of SC increased the relative amounts of the 30 nm peak at the expense of the 100 nm. This suggests that the amount of SC limits the formation of the smaller particles due to their higher surface area. At 0.75% (w/w) SC, the particle diameter reached its minimum (75.0 nm). The critical micelle concentration (CMC) of SC in this study was determined to be 0.688%. When the concentration of SC exceeded the CMC, SC self-associated to form micelles leading to a slight ($P > 0.05$) increase in diameter of BC loaded nanoparticles to 77.8 nm at 1.0% (w/w). Continuing to increase the SC concentration to 1.5% (w/w) resulted in a significant increase of particle diameter to 121.3 nm ($P < 0.05$),

Table 1. Effect of Different Concentrations of Emulsifiers on the Particle Diameter of BC Loaded Nanoparticles (Mean ± STD, $n = 3$)

<table>
<thead>
<tr>
<th>conc (%)</th>
<th>SC</th>
<th></th>
<th></th>
<th></th>
<th>WPI</th>
<th></th>
<th></th>
<th></th>
<th>SPI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D$ (nm)</td>
<td>PDI</td>
<td></td>
<td></td>
<td>$D$ (nm)</td>
<td>PDI</td>
<td></td>
<td></td>
<td>$D$ (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td>0.10</td>
<td>162.4 ± 3.7e</td>
<td>0.098 ± 0.008a</td>
<td></td>
<td></td>
<td>190.9 ± 3.5e</td>
<td>0.165 ± 0.010a</td>
<td></td>
<td></td>
<td>493.4 ± 10.4e</td>
<td>0.315 ± 0.001b</td>
</tr>
<tr>
<td>0.25</td>
<td>125.8 ± 2.0d</td>
<td>0.206 ± 0.016b</td>
<td></td>
<td></td>
<td>131.1 ± 2.8d</td>
<td>0.218 ± 0.012b</td>
<td></td>
<td></td>
<td>450.6 ± 6.5d</td>
<td>0.297 ± 0.031ab</td>
</tr>
<tr>
<td>0.50</td>
<td>121.7 ± 2.9c</td>
<td>0.310 ± 0.008c</td>
<td></td>
<td></td>
<td>125.1 ± 2.8c</td>
<td>0.156 ± 0.023a</td>
<td></td>
<td></td>
<td>421.8 ± 2.9c</td>
<td>0.356 ± 0.020c</td>
</tr>
<tr>
<td>0.75</td>
<td>75.0 ± 1.5a</td>
<td>0.305 ± 0.124c</td>
<td></td>
<td></td>
<td>113.6 ± 3.2b</td>
<td>0.231 ± 0.006bc</td>
<td></td>
<td></td>
<td>402.3 ± 7.4b</td>
<td>0.360 ± 0.010c</td>
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<td>1.00</td>
<td>77.8 ± 0.2a</td>
<td>0.386 ± 0.003d</td>
<td></td>
<td></td>
<td>89.7 ± 1.7a</td>
<td>0.245 ± 0.002c</td>
<td></td>
<td></td>
<td>371.8 ± 6.8a</td>
<td>0.286 ± 0.003a</td>
</tr>
<tr>
<td>1.25</td>
<td>103.1 ± 2.6b</td>
<td>0.449 ± 0.152e</td>
<td></td>
<td></td>
<td>90.1 ± 1.5a</td>
<td>0.351 ± 0.007d</td>
<td></td>
<td></td>
<td>397.5 ± 5.0b</td>
<td>0.471 ± 0.004e</td>
</tr>
<tr>
<td>1.50</td>
<td>121.3 ± 2.0c</td>
<td>0.357 ± 0.026d</td>
<td></td>
<td></td>
<td>88.5 ± 2.5a</td>
<td>0.340 ± 0.020d</td>
<td></td>
<td></td>
<td>400.1 ± 3.2b</td>
<td>0.416 ± 0.006d</td>
</tr>
</tbody>
</table>

*Values are the mean and standard deviation of triplicates. Values in the same column with different letters (a–e) are significantly different ($p \leq 0.05$).

Figure 2. Change of Z-average diameters of three protein encapsulated BC loaded nanoparticles over time (30 days) under storage at 4 °C (A, C, E) and 25 °C (B, D, F). A and B refer to SC, C and D refer to WPI, and E and F refer to SPI. Data are represented as the mean ± STD ($n = 3$).
higher temperatures and attributed the temperature effect to gravitational separation and dissociation of colloidal calcium phosphate micelles arising from the disruption of internal hydrophobic interactions and dissociation of colloidal calcium phosphate in interparticle bridges leading to aggregation and association. Dickinson has also suggested that at the molecular level pressure-induced disintegration of casein micelles arises from the disruption of internal hydrophobic interactions and dissociation of colloidal calcium phosphate micelles resulting in interparticle bridges leading to aggregation and association.30

The mean particle diameter of WPI nanoparticles gradually decreased from 190.9 to 89.7 nm when the concentration of WPI increased from 0.10% to 1.0% (w/w). The particle diameter of the nanoparticles did not change with increasing WPI concentration from 1.0% to 1.5% (w/w). These results suggest that the surplus WPI in solution did not form multiple adsorption layers or aggregate at the surface of droplets due to net negative charges on the free protein and adsorbed proteins.

SPI is composed of the following two major proteins: 7S and 11S with molecular weights of approximately 200 000 and 350 000 Da, respectively. When SPI was used as an emulsifier, the particle diameter of the nanoparticles was approximately two times larger than when using SC or WPI. This could be attributed to the poor emulsifying ability of SPI, and fewer hydrophobic amino acids on the surface to bind to the lipid core in comparison with milk proteins. Because SPI does not efficiently stabilize the newly formed droplets, coalescence occurs and results in larger particle diameter.

The PDI value is a measure of the variability in the distribution of particle diameter. All samples had a relatively narrow distribution with a PDI value less than 0.471. As protein concentration increased, the SC and WPI stabilized nanoparticles exhibited an increased PDI, possibly due to the formation of an increasing population of smaller particles. The PDI of the SPI encapsulated nanoparticles did not change with increasing protein concentration, and the particle size did not decrease as much as the milk proteins.

Particle Diameter of BC Loaded Nanoparticles During Storage. The diameter stability of nanoparticles is important for their utilization in foods because texture and appearance depend on structural stability. The particle diameters increased less than 10% at 4 °C, and in general, there was a greater increase in particle diameter at 25 °C (10−76%) during the 30 day storage period (Figure 2). The stability to storage is probably due to the repulsive forces due to the negative surface charges at pH 7 (ζ-potential Table 3). Yang et al.2 also noted that the diameter increments of nanoparticles were greater at higher temperatures and attributed the temperature effect to more rapid movement at higher temperature that increased gravitational flocculation. Although the initial diameters of the particles encapsulated by SC were smaller at the start of storage, the diameter increase was greater than those encapsulated by the globule proteins WPI and SPI at all concentrations after 30 days. The larger WPI and SPI particles are expected to increase in diameter with storage due to their more rapid rise to the surface and subsequent aggregation or flocculation. While flocculation due to gravitational separation probably occurs, it is slow compared to other mechanisms. In the case of SC emulsions, increasing protein concentration allows for intermolecular bridging of adsorbed proteins by proteins not adsorbed to the lipid surface. In conclusion, storage temperature may be an important factor to determine nanoparticles’ physical stability. There were no obvious BC lipid solids or sediments in the stored BC nanoemulsion samples indicating that they were fairly stable.

Effect of Emulsifier Type and Concentrations on BC Chemical Stability. Samples of BC dissolved in ethyl acetate were used as the control. Compared to the control samples, BC in nanoparticles showed significantly improved chemical stability and higher retentions at both temperatures (4 and 25 °C) (Figure 3), indicating nanoparticles were a good delivery system in enhancing stability of encapsulated lipophilic molecules. The order of chemical stability of BC loaded nanoparticles was the following: SC > WPI > SPI (Table 2). The chemical stability of corn oil, containing 70% polyunsaturated linoleic acid, emulsions was in the following order: SC > WPI > SPI.33 The decrease in BC concentration was fit to a zero-order kinetic reaction model (Figure 3) as suggested by Cornacchia and Roos.34 The rate of loss was greater at 25 °C (Table 2). The regression coefficients (R²) are from 0.896 to 0.998 and the intercepts are from 0.919 to 1.00 (Supporting Information, Table 1S).

The oxidative stability of the BC core is affected by two predominant properties of the encapsulating protein: the binding of metal ions and the thickness of the encapsulating layer. Binding of transition metal ions by cysteine, tyrosine, tryptophan, phenylalanine, and histidine from the aqueous phase can reduce free radical oxidation; however, if the binding site is at the interfacial layer, proximity of transition metals may increase oxidation. The phosphoserine residues in casein increased stability by binding pro-oxidative iron and copper ions.32 WPI also has about 8 mol binding sites for iron per mol of protein.33 Hwang et al.34 reported that there was a positive

Table 2. Slopes of the Linear Plots Describing BC Loss (w/w/day) with Changing Formulation

<table>
<thead>
<tr>
<th>Protein [w/w]</th>
<th>SC</th>
<th>WPI</th>
<th>SPI</th>
</tr>
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<tbody>
<tr>
<td>0.10</td>
<td>4.23 × 10⁻³</td>
<td>2.04 × 10⁻²</td>
<td>4.15 × 10⁻³</td>
</tr>
<tr>
<td>0.25</td>
<td>3.77 × 10⁻³</td>
<td>1.83 × 10⁻²</td>
<td>6.03 × 10⁻³</td>
</tr>
<tr>
<td>0.50</td>
<td>3.38 × 10⁻³</td>
<td>1.54 × 10⁻²</td>
<td>3.73 × 10⁻³</td>
</tr>
<tr>
<td>0.75</td>
<td>2.89 × 10⁻³</td>
<td>1.33 × 10⁻²</td>
<td>3.34 × 10⁻³</td>
</tr>
<tr>
<td>1.00</td>
<td>2.18 × 10⁻³</td>
<td>1.24 × 10⁻²</td>
<td>2.42 × 10⁻³</td>
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<tr>
<td>1.25</td>
<td>2.53 × 10⁻³</td>
<td>1.14 × 10⁻²</td>
<td>2.46 × 10⁻³</td>
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<tr>
<td>1.50</td>
<td>2.14 × 10⁻³</td>
<td>1.12 × 10⁻²</td>
<td>2.13 × 10⁻³</td>
</tr>
</tbody>
</table>

Table 3. Droplet ζ-Potential (Mean ± STD, n = 3) of BC Loaded Nanoparticles at pH 7.0 Stabilized by SC, WPI, and SPI at 1.0%

<table>
<thead>
<tr>
<th>Emulsifiers</th>
<th>SC</th>
<th>WPI</th>
<th>SPI</th>
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</thead>
<tbody>
<tr>
<td>ζ-potential (mV)</td>
<td>−35.7 ± 0.4b</td>
<td>−29.9 ± 0.5a</td>
<td>−37.8 ± 0.4c</td>
</tr>
</tbody>
</table>

*Values are the mean ± STD of triplicates. Values in the same column with different letters (a−c) are significantly different (p ≤ 0.05).
relationship between the carboxyl group content of ethylenediaminetetraacetic dianhydride (EDTAD)-modified SPI and metal-binding ability.

Casein, in excess, can form a thick interfacial layer around carrier oil droplets extending from 10 to 15 nm compared to 2−3 nm for whey proteins.35 The greater thickness may reduce the interaction between BC and pro-oxidants in aqueous phase.

At low protein concentrations (0.1−1.0%, w/w), the BC lipid surface may not be fully covered by the emulsifier, and direct contact with metal ions, pro-oxidants, and dissolved oxygen may increase BC oxidation. Increasing the protein content (SC, WPI, SPI) in solution resulted in a decrease in the loss rate of BC (Table 2). Most likely, oxidation was initiated by dissolved oxygen during the high speed mixing step.

Impact of Emulsifiers on Total Color Change ($\Delta E^*$). The color and appearance are important visual factors for food choice by consumers.36 The apparent color of carotenoids varies (red/orange/yellow) with concentration, oxidation, and particle diameter. The lightness ($L^*$) of three BC nanoparticles increased during storage, indicating the color fading and BC loss. The decrease of $a^*$ value suggested the decrease of redness of BC nanoparticles, while the yellowness ($b^*$) increased with increasing storage (Supporting Information, Table 2S). The degree of total color change, as expressed by $\Delta E^*$, with storage temperature was consistent with the decrease of BC. The color changes at 25 °C were greater than at 4 °C (Figure 4), and the order was SC < WPI < SPI at both temperatures. The BC loaded nanoparticles produced in this study had better physicochemical stability than those previously reported by Qian et al.,3 perhaps due to solid lipid cores which have been reported to have lower susceptibility to degradation than BC in solution.37

TEM of BC Loaded Nanoparticles. The shape of the BC nanoparticles stabilized by three proteins (SC, WPI, and SPI) was observed by transmission electron microscope (TEM). As shown in Figure S, the three nanoparticles were spherically shaped and uniformly dispersed, suggesting BC was fully encapsulated by proteins. The TEM images of BC loaded nanoparticles prepared by the solvent displacement technique emulsified by gelatin and Tween 2038 were also spherical.

Cell Toxicity of BC Loaded Nanoparticles. BC loaded nanoparticle toxicity was evaluated by the MTT assay which is a commonly used, sensitive colorimetric method to determine cell viability.39 The cell viability was determined at different dilution times (DT) by the MTT assay (Figure 6). Pure β-carotene was used as a control. Pure β-carotene is insoluble in water, so β-carotene dissolved in THF/DMSO (1:1) was diluted in DMEM as control to study the toxicity of β-carotene. No cytotoxicity was observed with Caco-2 cells treated with pure β-carotene at the same concentrations in nanoparticles. At DT = 0 (initial protein concentration = $C_0 = 10$ mg/mL), the cell viabilities treated with the three BC loaded nanoparticles were 84 ± 8% for SC, 80 ± 7% for WPI, and 83 ± 7% for SPI, respectively. The viabilities of Caco-2 cells when exposed to food protein (WPI and SPI) stabilized nanoemulsions at 3 mg/mL were about 85%,15 which was similar to our finding. He et al.15 also reported that the food proteins had a better biocompatibility, compared to egg phosphatidylcholine (EPC)
and other surfactants. In addition, at DT = 10 (0.1 × C₀) or greater, all the cell viabilities were above 95% indicating that the BC loaded nanoparticles were nontoxic when the concentrations of protein emulsifiers were below 1 mg/mL. The results suggest that protein-based nanoparticles are biocompatible.

**BC Loaded Nanoparticle Uptake in Vitro.** Cellular accumulation of micellar BC was found to be proportional to up to 30 h of incubation time. A 24 h period was chosen for the uptake of BC nanoparticles with Caco-2 cells. The rate and extent of nanoparticle uptake is determined by the properties of the particle, like, diameter, shape, charge, and interfacial characteristics. The uptake of BC loaded nanoparticles prepared by homogenization–solvent evaporation was greater than BC in THF/DMSO suspension (Figure 7). This was surprising since in both cases, BC nanoparticles versus BC suspension THF/DMSO at 5 μg/mL, BC was in solid form. However, BC in THF/DMSO may have precipitated in the crystalline form whereas BC in the nanoparticles, by X-ray diffraction, appears to be in an amorphous solid. We hypothesize that a mixture composed of residual lipids extracted by ethyl acetate from SC, WPI, and SPI prevented crystallization. The nanoparticles were also smaller in diameter. The cellular uptake of BC in THF/DMSO suspension was 264 ± 37 pmol/mg protein (Figure 7). While the uptake values of SC, WPI, and SPI BC loaded nanoparticles were 687 ± 69, 891 ± 142, and 452 ± 48 pmol/
mg protein, respectively, which was a 2.6-, 3.4-, and 1.7-fold increase, respectively. The cellular uptake values among the three nanoparticles were significantly different (P < 0.05) and increased in the following order: SPI < SC < WPI. Differences in particle diameter of the BC core may be the main reason for differences in uptake. The BC core diameter is probably related to encapsulated nanoparticle diameter although there will be differences in the thickness of the adsorbed protein layers. However, the mean diameter of SPI encapsulated BC loaded nanoparticle was 372 ± 7 nm, and much larger than SC stabilized (77.8 ± 0.2 nm) or WPI stabilized BC loaded nanoparticles (89.7 ± 1.7 nm). Besides the increased surface area of smaller particles nanosized objects, the release properties of the lipid may be affected by its crystallinity and distribution within the particle.

Although transport by scavenger receptor SR-BI is believed to be the conventional means of BC absorption, the literature also reports that nanosize droplets (d < 200 nm) may be directly transported across the epithelium cell layer by paracellular mechanism, passive diffusion or an “endocytosis” mechanism. There was a significant negative correlation between BC uptake and the nanoparticles’ negative surface charge (ζ-potential) (P < 0.05). ζ-Potential is influenced by ionic strength, and the measurements were made in 5 mM (pH 7.0) phosphate buffer while the concentration of the main sources of ions in cell culture media was about 150 mM. Therefore, our ζ-potential measurements may not be reliable although Dagliesh et al. found that the ζ-potential for casein became more negative by only 1.5 mV going from 100 mM to 20 mM NaCl. Previous research has indicated that surface charge is an important factor in permeation of nanoparticles. The surface of enterocytes (Caco-2 monolayers) is coated with negatively charged mucin layer. The negative electrical charge of the surface of three protein encapsulated BC loaded nanoparticles may interact with the mucin layer through electrostatic repulsion, restricting the uptake of BC. The largest ζ-potential value of SPI nanoparticles also had the least BC uptake content. These results indicated that changing surface charge may be an effective method to improve cellular uptake.

In summary, our study shows that BC loaded nanoparticles can be prepared and stabilized by food grade proteins. The toxicity of nanoparticles appears to be low at about 10 mg BC/100 mL and insignificant when diluted 10 times or more. The evaporation method produced spherical solid BC cores probably mixed with residual protein lipids. The protein encapsulated nanoparticles had increased uptake of BC compared to BC in THF/DMSO suspension. Oxidative stability was highest in BC loaded nanoparticles stabilized by SC proteins due to the different interfacial characteristics at the droplet surface and the amino acid compositions. The intrinsic antioxidative capacity of proteins contributed to oxidative stability. This study contributes to interest in designing new delivery systems to increase stability and uptake of lipophilic nutrients in the food industry.

**REFERENCES**


