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Ca^{2+} Cross-Linked Alginic Acid Nanoparticles for Solubilization of Lipophilic Natural Colorants

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The increased tendency toward healthy lifestyles has promoted natural food ingredients to the detriment of synthetic components of food products. The trend followed into the colorant arena, with consumers worried about potential health problems associated with synthetic colorants and demanding food products that use natural pigments. The goal of this study was to entrap a lipophilic natural pigment (β-carotene) in a water-soluble matrix made of Ca^{2+} cross-linked alginic acid, to allow its use as a colorant in water-based foods. The effects of different synthesis parameters such as type of solvent, alginic acid concentration, and calcium chloride concentration on nanoparticle characteristics (i.e., size, zeta potential, and morphology) were evaluated. The particle stability was assessed by measuring aggregation against pH, oxidation, and particle precipitation as a function of time. The particle synthesized measured 120−180 nm when formed with chloroform and 500−950 nm when synthesized with ethyl acetate. The particles were negatively charged (−70 to −80 mV zeta potential) and were stable at pH values ranging from 3 to 7. The presence of calcium was prevalent on the particles, indicating that the divalent ions were responsible for cross-linking lecithin with alginic acid and forming the matrix around the β-carotene pockets. The addition of calcium increased nanoparticle density and improved β-carotene protection against oxidation. It is concluded that the method proposed herein was capable of forming water-soluble nanoparticles with entrapped β-carotene of controlled functionality, as a result of the type of solvent and the amounts of alginate and Ca^{2+} used.

KEYWORDS: Colorant; nanoparticles; alginic acid; lecithin; cross-linking; β-carotene

INTRODUCTION

Synthetic, nature-identical, and natural colorants are important components of food, as color is directly related with the perception of food quality by consumers. Approval of new synthetic colorants produced by chemical synthesis (i.e., Sunset Yellow) is very difficult due to the strict safety requirements imposed by the U.S. FDA and the increasing public rejection of those components linked to allergic reactions and potential carcinogenesis (*1*). Nature-identical colorants are a better alternative; also produced by chemical synthesis, natural-identical colorants have structures identical to those of pigments found in nature (i.e., β-carotene, canthaxanthin) (*2*). Natural colorants (i.e., β-carotene, bixin, antocyanins, and curcumin) derived from natural sources by extraction and purification methods are most preferred by health-conscious consumers. There are several natural pigments that have been used in the past to impart color and seasoning to food (i.e., paprika oil or carotenoids), but their use was restricted to hydrophobic environments because of their low solubility in water. Water-based products mainly used synthetic water-soluble colorants to ensure a uniform color of the food. Besides their low water solubility, many natural pigments are considerably less stable as compared to synthetic colors and are more expensive (*3*). The uncontested advantage of pigments, however, is that they not only impart color but also have well-documented health benefits related to the prevention and treatment of cancer, atherosclerosis, and other common diseases (*4*, *5*).

The interest in natural colorants such as β-carotene triggered research and development of different protocols and systems designed to entrap lipophilic natural molecules in water-dispersible systems, such as liposomes, vesicles, microparticles, emulsions, and others (*6*−*9*). A system designed to dissolve pigments in water must meet several criteria for food use. The system should be stable (with minimum precipitation or oxidation), natural (only natural components should be used to form the system), able to impart a uniform color (readily dispersible in water), easy to manipulate (least sensitive to temperature, light, and other parameters), reproducible (batch by batch), versatile (able to impart different colors as function of the concentration), scalable (able to be produced at large scale), and inexpensive.

Emulsions have been most extensively used as water-soluble systems for lipophilic components and additives of choice (i.e., antioxidants, antifoaming agents) (*10*). The use of hydrocolloids

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(i.e., gelatin, chitosan, pectin, and others) as stabilizing agents for emulsions formed with the aid of surfactants (i.e., polysorbitol family surfactants, proteins, lecithin, and others) was shown to favor emulsion stability and functionality (*11*−*13*). For the entrapment of β-carotene specifically in systems capable of solubilizing the component in water, similar methods were used (*14*−*17*). Microparticles made with different hydrocolloids or polymers have been systematically used as vehicles for β-carotene, as described in a number of patents. Typically, carotenoids were processed in a fluidized bed coating machine, and a protective coating of sugar or polyhydric alcohol (sorbitol), starch, maltodextrin, or gelatin was added to improve the water solubility of the entrapped components (*18*−*20*).

Nanodispersion of β-carotene using nanoprecipitation and emulsion evaporation techniques were proposed by Nakajima's research group (*21*−*23*). β-Carotene was co-entrapped in poly(lactic-*co*-glycolic acid) and poly(lactic acid) with α-tocopherol, in the presence of emulsifiers (gelatin and Tween 20), using acetone as an organic solvent to form particles that ranged in size from 60 to 200 nm and retained their size for 5 months. Chu et al. (*22*) used the emulsion evaporation technique with hexane as an organic solvent to entrap β-carotene in different proteins (i.e., sodium caseinate, soy protein isolate and concentrate, and whey protein isolate, concentrate, and hydrolysate). Of the proteins studied, sodium caseinate showed the most promising results in terms of particle size (20−200 nm) for preparations with 10% w/w of organic phase and 5% wt of sodium caseinate. No studies of stability over time were presented. The polyglycerol ester of fatty acids as nonionic emulsifier was investigated in conjunction with the emulsion evaporation method. The size of the particles ranged from 85 to 132 nm, with the higher degree of glycerol polymerization leading to the formation of smaller particle size (*24*). The principal limitation of this method for colorant delivery purposes was the low β-carotene content (0.1−0.3 wt %) entrapped in the system.

The use of different block copolymers has been studied with the purpose to entrap β-carotene with higher efficiencies and narrower particle size distribution. Pan et al. (*25*) used casein graft dextran to entrap and protect β-carotene. The particles presented a size ranging from 200 to 300 nm and were stable against dilution, pH changes, ionic strength, and $FeCl_3$ oxidation. Another polymer used to entrap β-carotene, the block copolymer poly(ethylene glycol)-*block*-poly(ε-caprolactone) (*26*), resulted in a particle size ranging from 75 to 275 nm. The method used was flash nanoprecipitation of the block copolymer, which self-assembled into micelles due to its amphiphilic properties. However, the polymers used in these studies were synthetic or chemically modified, which may restrict their use in the food industry.

Lipid carrier particles called solid lipid nanoparticles (SLNs) or nanostructured lipid carriers (NLCs) have been highlighted as viable delivery systems for bioactive components, including β-carotene. Typically, the system can be formed by a mixture of triacylglycerides, fatty acids, and surfactants. The main steps of the synthesis process involve controlled temperature changes (hot to cold), homogenization, and microfluidization to form particles in the nanometer size range (*27*). The entrapment of β-carotene using SLNs was performed by Hetschel et al. (*28*) using a mixture of lipids and polyoxyethylene sorbitan monooleate (Tween 80) as a surfactant. The particle size obtained ranged from 50 to 1000 nm with a mean size of 200 nm, as a function of the amount of surfactant used in the synthesis. The stability of the synthesized particles was higher when stored at 4−8 °C in the presence of α-tocopherol as an antioxidant, as compared with 20 °C (*28*). The low concentration of β-carotene may be a limitation of this method for some applications in the food industry.

As highlighted above, multiple studies were performed and reported on entrapment of β-carotene in various delivery systems with the goal of improving its water solubility. The application of several proposed systems in the food industry is limited due to the non-GRAS (Generally Recognized as Safe) status of one or more of the components used. Another limitation is the low β-carotene loading in these systems. We hypothesized that nanoparticles made of alginic acid Ca^{2+} cross-linked with lecithin can entrap and solubilize β-carotene in water for colorant purposes and that the addition of calcium ions improves the protection of β-carotene against oxidation by cross-linking the alginic acid with the lecithin−β-carotene core (**Figure 1**).

A novel method is proposed herein to efficiently entrap β-carotene in a water-soluble system made with food grade components by a combination of emulsion−evaporation and cross-linking steps. Nanoparticles with entrapped β-carotene in an alginic acid nanostructure cross-linked by divalent calcium ions were synthesized and characterized to assess the effect of type of solvent, and amount of alginic acid and calcium chloride, on the size, surface properties, and stability of the particles.

MATERIALS AND METHODS

Materials. Alginic acid (from brown algae), calcium chloride (>99%), and β-carotene (type I, approximately 95% UV) were purchased from Sigma Chemical Co. (St. Louis, MO). Lecithin (laboratory grade) and buffer solution (pH 7 with potassium phosphate monobasic and sodium hydroxide) were acquired from Fisher Scientific (Pittsburgh, PA). Ethyl acetate and chloroform (HPLC grade) were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ).

Nanoparticle Synthesis. Nanoparticle synthesis consisted of a combination of emulsion−evaporation and cross-linking steps. An emulsion was first formed from an organic phase (formed with ethyl acetate or chloroform) that contained the β-carotene and an aqueous phase that contained the surfactant. The organic phase was formed by dissolving 10 mg of β-carotene in 1 mL of organic solvent. The aqueous phase (10 mL) was formed with water saturated with ethyl acetate (to avoid diffusion of the organic solvent in water when ethyl acetate was employed as the organic solvent) and 200 mg of lecithin. When chloroform was used, the aqueous phase contained water and lecithin only. The organic phase (1 mL) was added to the aqueous phase and sonicated with a Sonic VC 750 (Sonics & Materials Inc., Newton, CT) for 10 min (39% amplitude in pulse mode 4 s on and 2 s off) in an ice bath at 4−8 °C. Next, $CaCl_2$ solution (1 mL, 2.5 and 5 mg/mL) was added under sonication. The final concentrations for $CaCl_2$ were 0.15 and 0.29 mg/mL (1.35 and 2.61 mM, respectively). After $CaCl_2$ addition, 6 mL of buffer solution (sodium and potassium phosphate 0.05 M, pH 7) of alginic acid (at different concentrations: 0.2, 1, 5, and 10% w/v) was added to the emulsion under sonication for 7 min in pulse mode (37% amplitude, pulse mode of 4 s on and 2 s off) at low temperatures (4−8 °C) to avoid solvent evaporation and particle aggregation. Finally, the emulsion was placed in a Buchi R-124 rotoevaporator (Buchi Corp., New castle, DE), and the organic solvent (ethyl acetate or chloroform) was removed. Evaporation was performed for 6 min for chloroform and for 10 min for ethyl acetate under 755 mmHg of vacuum and nitrogen injection for fast solvent evaporation. The nanoparticle suspension was collected and stored under refrigerated, dark conditions until further analysis.

Size, Size Distribution, and Zeta Potential. The size of the synthesized nanoparticle and size distribution (polydispersity index, PI) were evaluated by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, U.K.). The PI was calculated on the basis of cumulative analysis as a dimensionless number ranging from 0 to 1. Values >0.7 usually indicate that the particles have a very broad size distribution. If the PI is <0.1, the sample is considered to be monodisperse. Zeta potential measurements were accomplished with the same Malvern Zetasizer Nano ZS with an MPT-2 autotritator in order to study aggregation of particles against pH. The samples were diluted to a final concentration range of 0.2−0.32 mg/mL prior to the measurements being taken.

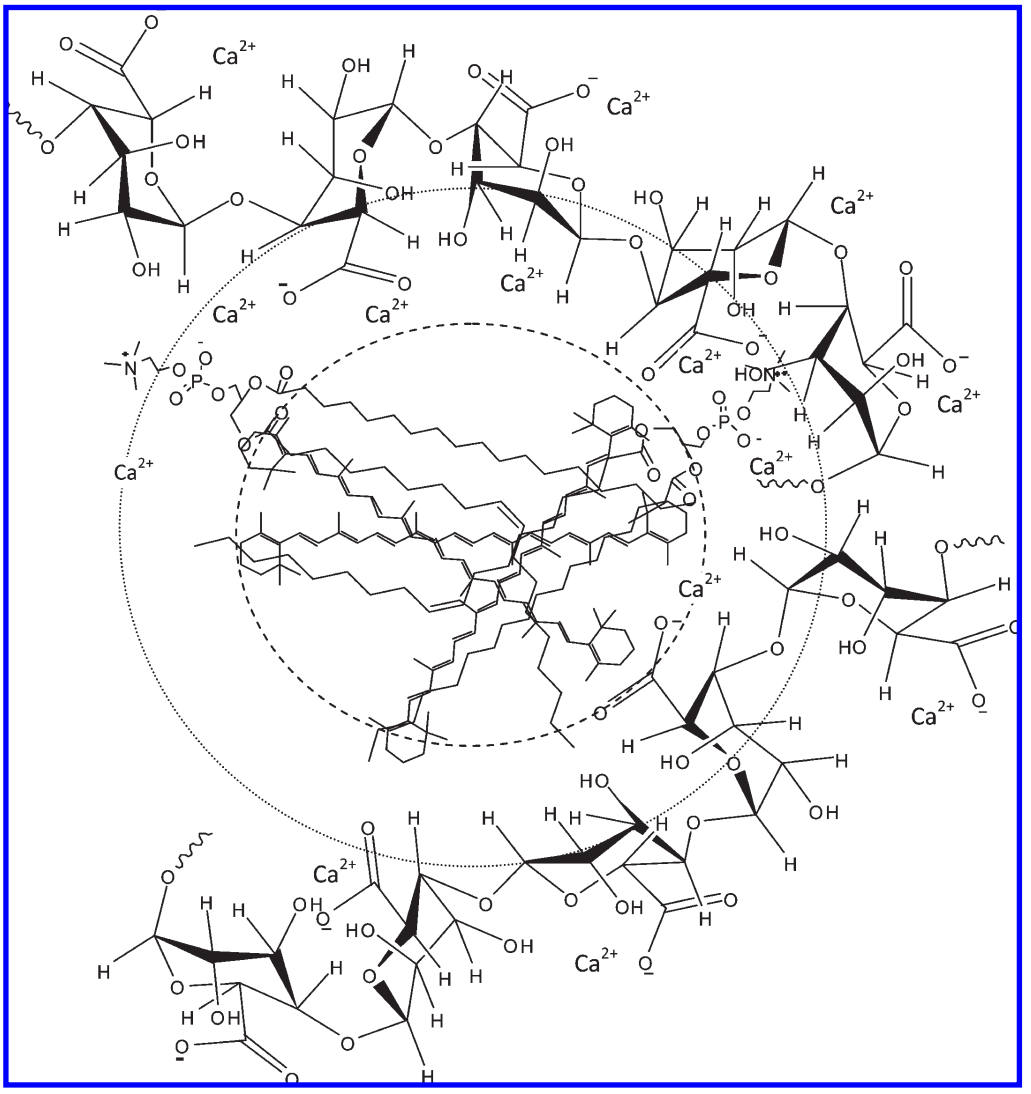

Figure 1. Theoretical core−shell structure of the colorant nanoparticles. β-Carotene−lecithin core is cross-linked to alginic acid by calcium ions.

Morphology of Colorant Nanoparticles. The particle morphology was studied by transmission electron microscopy (TEM) using a JEOL 100-CX (JEOL USA Inc., Peabody, MA). A droplet of the suspension was placed on a carbon grid, and the excess was removed. Uracyl acetate was placed over the dry sample on the carbon grid, and the excess was removed prior to TEM analysis. The effect and distribution of calcium on the nanoparticle morphology were studied by TEM using a JEOL 2100F (JEOL USA Inc.). Elemental analysis was performed in parallel to determine Ca^{2+} distribution in the sample using scanning transmission electron microscopy (STEM) (Gatan, Warrendale, PA) and electronic data system (EDS) (Edax, Mahwah, NJ). The images are an overlay of an STEM DF image with Ca EDS X-ray signal distribution. Both were obtained with the electron beam spot size of 0.7 nm, and the frame size is 256 × 200 pixels.

Stability. Stability against oxidation of the β-carotene particles was evaluated by placing the samples in 3 mL cuvettes under light at room temperature. The variation in the sample absorbance measured at 550 nm using a Geminys 6 spectrophotometer (Thermo Scientific, Waltham, MA) was used as an indicator of β-carotene degradation by oxidation. Precipitation was evaluated by measuring the decrease in the absorbance of the supernatant at 550 nm after sample centrifugation at different velocities. Samples were centrifuged using an Allegra 64R centrifuge (Beckman Coulter, Fullerton, CA) at revolutions per minute ranging from 4000 to 30000 rpm (1145−64396*g*).

Statistical Analysis. The statistical analysis was performed with SAS (Cary, NC). The ANOVA proc mixed procedure with Tukey adjustment was used, and significant differences were declared at a p value of 0.05. All of the analyses were performed on data acquired from two experiments and three measurements per experiment.

RESULTS AND DISCUSSION

Solvent Effect on Particle Size. The effect of the organic phase (ethyl acetate and chloroform) on nanoparticle size was determined at a constant calcium chloride concentration of 0.15 mg/mL and various concentrations of alginic acid (0.07−3.5 mg/mL). When chloroform was used as the organic solvent, particles were significantly smaller (**Figure 2a**) than the particles synthesized with ethyl acetate ($p < 0.05$). Chloroform-synthesized particles measured between 120 and 220 nm at alginic acid concentrations of 0.07−3.5 mg/mL, whereas the size of the ethyl acetate formed particles ranged from 500 to 950 nm for the same alginic acid concentrations (**Figure 2a**). Particle size increased with increasing alginic acid concentrations when ethyl acetate was used as a solvent. The size of the particles synthesized with chloroform was not significantly different across the alginic acid concentrations studied.

The difference in particle size detected when different solvents were used in the organic phase was attributed to the β-carotene solubility in the two solvents. Chloroform can solubilize β-carotene at concentrations up to 2 mg/mL, whereas the solubility of β-carotene in ethyl acetate is only 0.5 mg/mL at room temperature (20 °C) (*29*). At a β-carotene concentration in the organic phase of 10 mg/mL (used in all experiments), its solubility

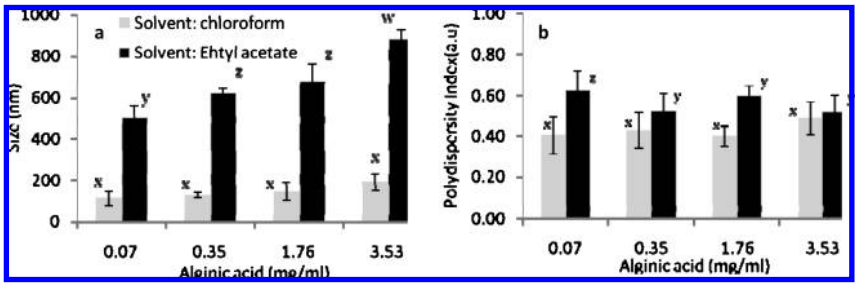

Figure 2. (**a**) Particle size and (**b**) polydispersity index as a function of different alginic acid concentrations for particles synthesized with ethyl acetate and chloroform in the organic phase at a calcium chloride concentration of 0.15 mg/mL. Different letters indicate significant differences between samples.

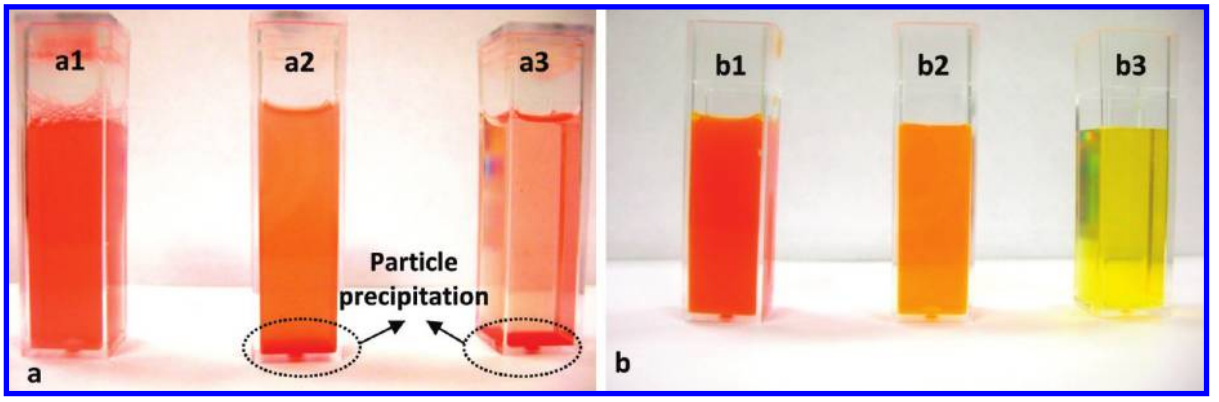

Figure 3. Precipitation and color shades for particles synthesized with (**a**) ethyl acetate and (**b**) chloroform prepared with a $CaCl_2$ concentration of 0.15 mg/mL and an alginic acid concentration of 0.35 mg/mL. (**a**) Precipitation of the freshly synthesized particles (a1) was observed after 6 h (a2) and 24 h (a3). (**b**) No visible precipitation in the system was observed after 24 h. The dark red color of the sample b1 (not diluted) changed to orange (b2) when diluted at 1:20 v/v and to light yellow (b3) when diluted at 1:100 v/v.

in ethyl acetate was exceeded. β-Carotene did not completely dissolve; crystals were dispersed in the organic phase. Their presence in the organic phase, even if of small size (due to sonication), was responsible for the larger size of the nanoparticles formed following evaporation of the organic solvent when ethyl acetate was used as compared to the size of the nanoparticles formed in the presence of chloroform.

Particles synthesized with ethyl acetate not only were larger but also presented a significantly higher PI, with values ranging from 0.5 to 0.65 as compared with 0.4−0.55 when chloroform was used as the organic solvent, with no significant effect provided by the presence of alginic acid in different concentrations (**Figure 2b**). The decrease in the PI achieved by switching the solvent to chloroform was not sufficient to indicate formation of a monodisperse sample. The polydispersity of the chloroform-synthesized sample did not affect its visual appearance indicated by a uniform color distribution, and no precipitation was observed over a 24 h period, due to gravitation.

The difference in size between the ethyl acetate- and chloroform-synthesized nanoparticles was responsible for different precipitation behaviors as a function of time (**Figure 3**). The sample freshly synthesized with ethyl acetate (a1) precipitated in a matter of hours; precipitation was noticeable after 6 h (a2), and it became more pronounced after 24 h (a3). The color of the suspension changed as a function of time due to precipitation, from orange-red to transparent red (**Figure 3a**). The sample synthesized with chloroform (**Figure 3b**) did not show precipitation after 24 h (b1) due to smaller particle size (particles synthesized with chloroform measured 140 nm in diameter, whereas particle synthesized with ethyl acetate were >600 nm in diameter for this specific study) and complete solubilization of β-carotene in chloroform. Different shades of color of the chloroform-synthesized samples were achieved by dilution, from dark red (b1) to orange (b2) with a 1:20 v/v dilution and from orange (b2) to yellow (b3) with a dilution of 1:100 v/v (**Figure 3b**).

In general, the characteristics of the particles synthesized with chloroform as an organic solvent were more desirable in terms of size and polydispersity as compared with those made with ethyl acetate. Therefore, subsequent studies were performed solely on chloroform-synthesized particles.

Effect of Alginic Acid and $CaCl_2$ Concentration on Particle Characteristics. Figure 4 shows the size, PI, and zeta potential for particles synthesized with chloroform, under all alginic acid and calcium chloride concentrations studied. The particle size ranged from 110 to 150 nm for alginic acid concentrations of 0−1.76 mg/mL at all three $CaCl_2$ concentration studied (0, 0.15, and 0.29 mg/mL). The differences in size as a result of the addition of $CaCl_2$ were not found statistically significant. However, when the size of the particles was compared for different amounts of alginic acid, it was found that the particles synthesized with 3.53 mg/mL alginic acid were significantly larger as compared with particles made at all other alginic acid concentrations tested (**Figure 4a**).

The PI was not significantly different for alginic acid concentrations of 0, 0.07, and 0.35 mg/mL (**Figure 4b**). The only significant difference observed was between the samples synthesized with 1.76 and 3.53 mg/mL of alginic acid and those made with less alginic acid. All samples were polydisperse with PI values >0.1.

Zeta potential was very negative (−70 to −80 mV) for all samples, which concurred with the negative charges of alginic acid (carboxylic acid groups) and lecithin at the sample pH of 7 (**Figure 4c**). Zeta potential observed for the higher alginic acid concentration was around −80 mV, but this value was not significantly different when compared with the other zeta potentials measured. The high value of zeta potential indicated that the

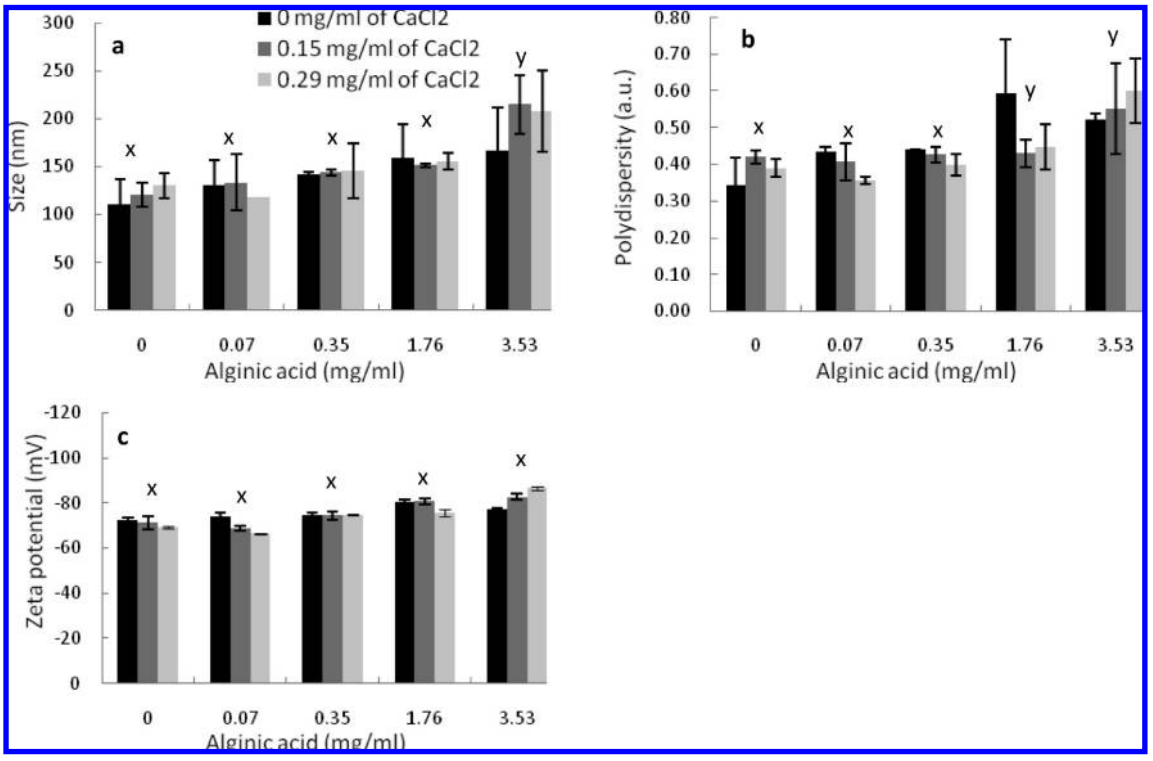

Figure 4. (**a**) Particle size, (**b**) PI, and (**c**) zeta potential (at pH 6.95) for particles made with different alginic acid and calcium chloride concentrations using chloroform as an organic solvent. Samples with different letters are significantly different.

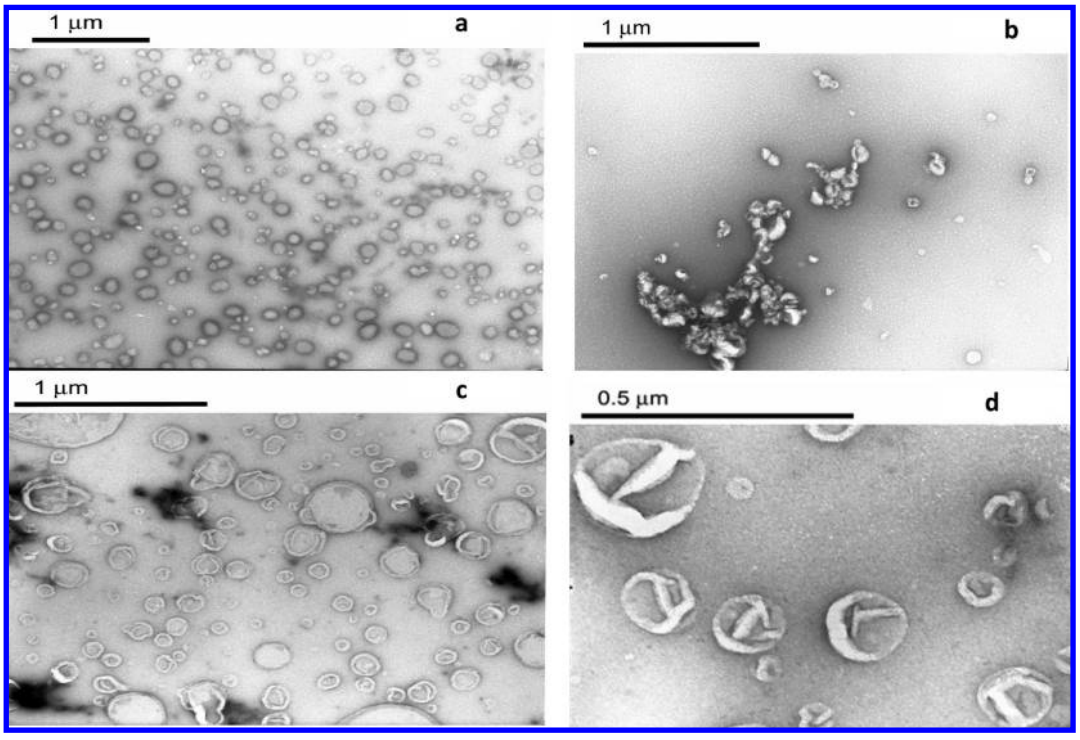

Figure 5. (**a**) Particles synthesized without $CaCl_2$, (**b**) particles synthesized with 0.15 mg/mL $CaCl_2$, and (**c**, **d**) particles synthesized with 0.29 mg/mL $CaCl_2$. Alginic acid concentration was 0.35 mg/mL.

particle susceptibility to aggregation due to electrostatic interactions was low. It is accepted in the literature that for zeta potential values >30 mV or <-30 mV, electrostatic repulsions between particles minimize their aggregation (*30*). At a zeta potential of -80 mV, as is the case observed in this study, agglomeration due to electrostatic interaction was avoided. Nanoparticle cluster formation observed (as discussed below) was due to cross-linking of algininc acid molecules associated with adjacent nanoparticles in the presence of the divalent calcium ions and not to electrostatic attraction forces between the particles.

Morphology. Particles synthesized without calcium chloride presented a regular spherical shape (**Figure 5a**), as determined by

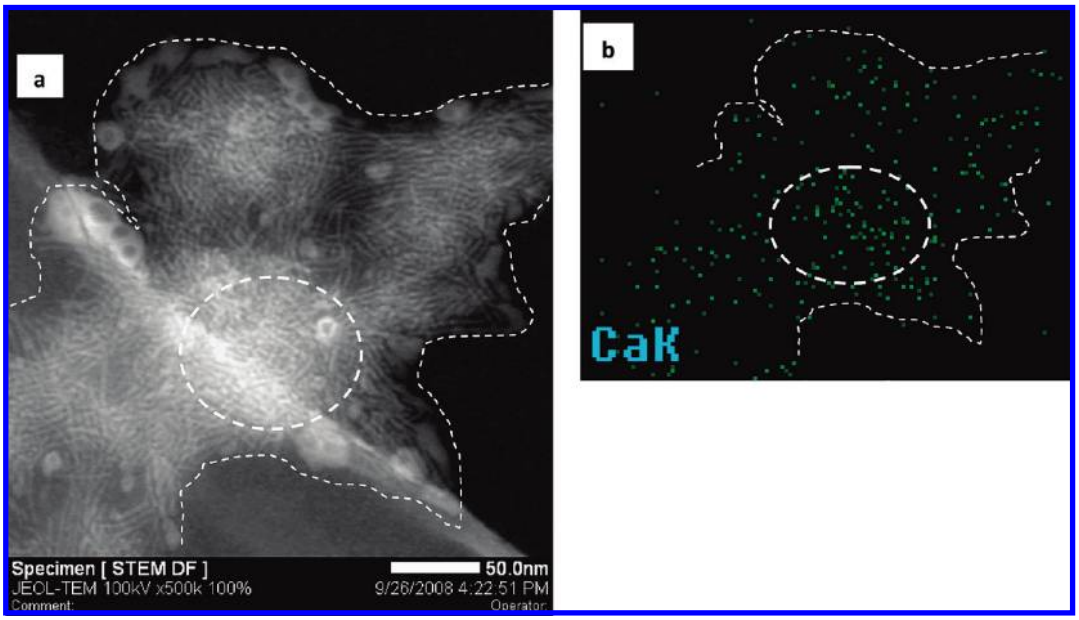

Figure 6. STEM picture of a particle synthesized with 0.29 mg/mL $CaCl_2$ and 0.35 mg/mL alginic acid: (**a**) individual particle with alginic acid filaments surrounding β-carotene−lecithin; (**b**) calcium spectrum showing prevalent spatial distribution of calcium on the particle.

TEM. When calcium chloride was added at a concentration of 0.15 mg/mL (**Figure 5b**), the particle morphology was altered by aggregation. The samples synthesized with 0.29 mg/mL calcium chloride (**Figure 5c**) presented a more regular "donut" shape and less aggregation as compared with the samples with 0.15 mg/mL, but larger particle sizes were detected. The change in morphology suggested that calcium chloride interacted with alginic acid and lecithin to form irregular particles with sections with more, and others with less, cross-linking (**Figure 5c**,**d**).

Calcium Effect. The addition of calcium chloride was performed to cross-link lecithin with alginic acid, improving β-carotene protection against oxidative degradation. STEM analysis (**Figure 6a**) showed a particle with irregular shape formed of alginic acid filaments surrounding the β-carotene−lecithin core. The calcium ions randomly cross-linked the alginic acid strands, promoting formation of particles of irregular shapes. The calcium spectrum (green dots in **Figure 6b**) was confined to the particle surface; calcium was not present outside the particle structure. These findings suggested that the divalent calcium ions played their expected role as cross-linking agent. The bulk addition of calcium chloride solution during synthesis promoted a random clustering by electrostatic interactions between lecithin, alginic acid, and calcium ions. The nonuniform cross-linking was reflected as well in the TEM pictures (**Figure 5**), exhibiting particles with different morphologies. The morphology of the nanoparticles differed from the theoretical model presented in **Figure 1** (where a defined core and shell structure is shown) in that the shape was irregular, cross-linking was not uniformly distributed on the surface of the particle, and multiple β-carotene pockets were present within each particle.

Particle Stability against pH. The pH effect on nanoparticle stability was analyzed by titration with HCl and NaOH to the desired pH, at which measurements of size and zeta potential were taken. The target was to find the pH value at which zeta potential increased to a value where a significant particle size increase due to aggregation can be expected. The study was conducted with samples synthesized with 0.15 mg/mL calcium chloride at four alginic acid concentrations of 0, 0.07, 0.35, and 1.76 mg/mL. At pH 7, all samples showed lower zeta potential values (−70 to −80 mV). When the pH was decreased from pH 7 to 3, zeta potential and particle size remained constant (**Figure 7**). At pH close to 3 or lower, zeta potential increased to values of −15 mV and particle size increased to values of 170−180 nm for all alginic acid concentration tested. These results suggested that the system was stable at pH >3 at which the high zeta potential promoted interparticle repulsion. Under these circumstances, the use of the particles at pH <3 would not be recommended.

Particle Stability against Oxidation. The effect of $CaCl_2$ on the size and PI of the particles was found to be negligible as shown in the previous sections. It was of interest to study the effect of the presence of Ca ions on the particle cross-linking as reflected on the degradation profiles of β-carotene due to oxidation. It was established that the more $CaCl_2$, the slower the rate of β-carotene degradation (**Figure 8**). The samples prepared without calcium chloride and alginic acid, in the form of emulsion droplets, exhibited similar degradation rates as compared with the sample synthesized with alginic acid and without $CaCl_2$, which implied that the effect of alginic acid on β-carotene oxidation was negligible. The addition of calcium chloride (0.15 mg/mL) resulted in a decrease in the slope of the degradation curve (**Figure 8a**). The data suggested that the addition of $CaCl_2$ favored packing, which reduced the degradation of β-carotene. Complete inhibition of degradation could not be obtained at the calcium levels studied, but it was retarded.

β-Carotene entrapped in the particles degraded according to first-order kinetics when $CaCl_2$ was used and followed second-order kinetics in the absence of calcium (**Figure 8b**). The sample without $CaCl_2$ and the control (no $CaCl_2$ or alginic acid added) presented similar kinetics of degradation; samples formed with 0.15 and 0.29% $CaCl_2$ were also very close in behavior. The degradation constant k for the control was 0.0395 h^{-1} and the k for the sample without $CaCl_2$, but in the presence of alginic acid, was 0.0371 h^{-1}; the k values were not significantly different (p value >0.05 for the slopes of degradation). The addition of calcium chloride changed the kinetics of degradation of β-carotene to a first-order reaction (**Figure 8b**). When the synthesis was performed with 0.15 mg/mL $CaCl_2$, the k value was 0.021 $(\text{absorbance} \times \text{hour})^{-1}$. The addition of 0.29 mg/mL $CaCl_2$ resulted in a degradation constant k value of 0.018 $(\text{absorbance} \times \text{hour})^{-1}$ that was not significantly different from

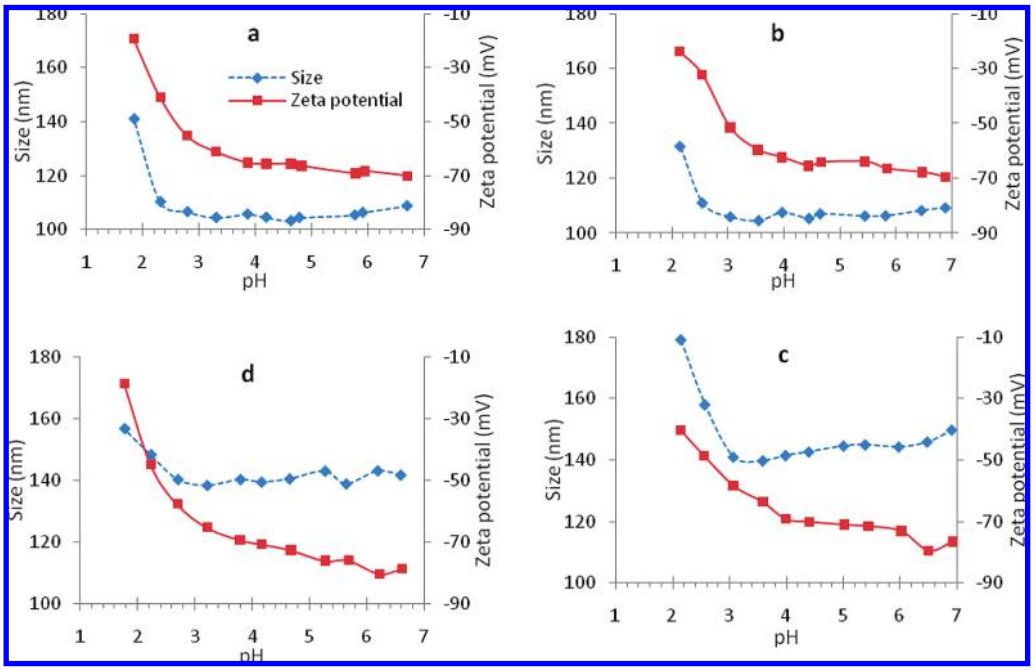

Figure 7. Stability of particles against pH. Particle size and zeta potential were measured at different pH values. All samples were synthesized at a $CaCl_2$ concentration of 0.15 mg/mL and at (**a**) 0, (**b**) 0.07, (**c**) 0.35, and (**d**) 1.76 mg/mL of alginic acid.

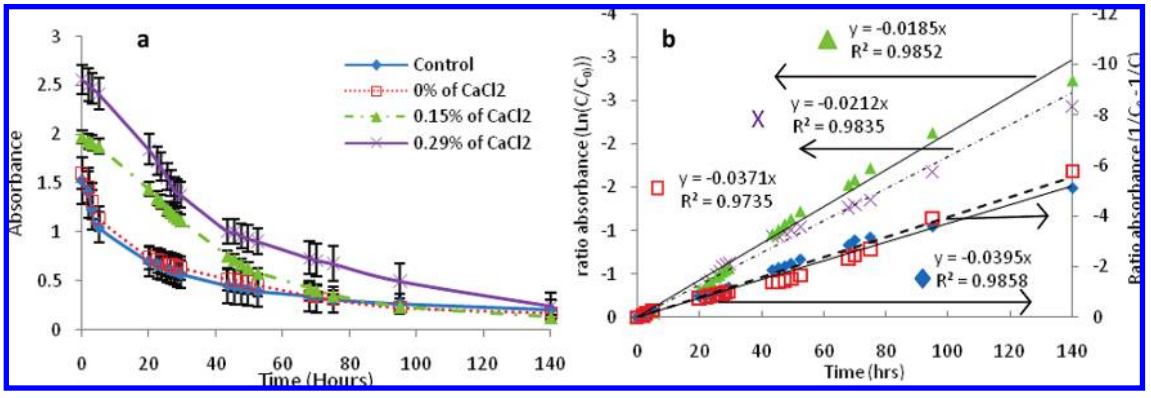

Figure 8. (**a**) Degradation of β-carotene entrapped in alginic acid particles; (**b**) kinetics of β-carotene degradation under room temperature, in the presence of air. The control was prepared following the same processing steps, except that no $CaCl_2$ and alginic acid were added. The other three sets of samples were synthesized with alginic acid, at different $CaCl_2$ concentrations added. Absorbance was measured at 550 nm.

the 0.15 mg/mL $CaCl_2$ sample (p value >0.05 for the slopes of degradation).

Particle Precipitation. The tendency for particles to precipitate was studied by measuring the decrease in absorbance of the supernatant as the particles were centrifuged at different speeds. Precipitation of the emulsion droplets and the particles prepared without $CaCl_2$ presented a similar trend over the speeds tested from 4000 to 30000 rpm (1145−64396g) and were not significantly different. The decrease in absorbance was not as pronounced as compared with that observed in samples prepared with $CaCl_2$ (**Figure 9**). The differences in initial absorbance values suggested a better entrapment of β-carotene promoted by electrostatic interaction due to the addition of calcium chloride (more carotene−lecithin micelles per alginic acid particles, higher density). The samples prepared with $CaCl_2$ showed a strong decrease in absorbance with increasing centrifugation speed due to formation of higher density particles in the presence of calcium ions. The denser particles precipitated faster than the particles without calcium. The samples prepared with 0.15 mg/mL $CaCl_2$ were not significantly different from the sample synthesized with 0.29 mg/mL $CaCl_2$, but was significantly different from all other samples. At 25000 rpm (44719g) all samples showed a similar absorbance, suggesting precipitation of most nanoparticle clusters and the presence of only fine particles in suspension at that centrifugation speed. The centrifugation data suggested that the presence of $CaCl_2$ increased the particle density due to cross-linking by electrostatic interactions.

In conclusion, a new alginate system for the entrapment of hydrophobic components by using calcium as a cross-linking agent was studied by using β-carotene as a potential colorant. β-Carotene particles were synthesized ranging from 120 to 180 nm when chloroform was used as solvent. The particle size increased to 900 nm when the solvent used was ethyl acetate as a result of the lower solubility of β-carotene in ethyl acetate. Particle morphology changed as a function of the calcium chloride concentration, but size was not affected significantly. Calcium ions were present in the particles promoting cross-linking of alginate (showing a filament structure) with lecithin-stabilized β-carotene micelles. The particles synthesized were stable at pH >3; at lower pH values, aggregation of the particles

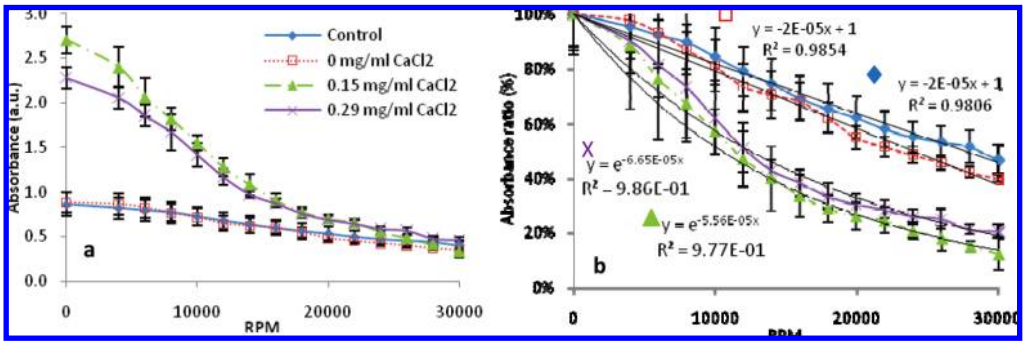

Figure 9. (**a**) Decrease in absorbance profiles of the particle suspensions after centrifugation, as an indication of particle precipitation; (**b**) color change due to particle precipitation. The control was prepared following the same processing steps, except that no $CaCl_2$ and alginic acid were added. The other three sets of samples were synthesized with alginic acid, at different $CaCl_2$ concentrations added. Absorbance was measured at 550 nm.

occurred as a result of a decrease in their zeta potential. Oxidation of β-carotene was retarded in the presence of calcium chloride; the kinetics of β-carotene degradation in the presence of oxygen under room temperature changed from a second-order to a first-order reaction when calcium chloride was added. The addition of calcium chloride, however, promoted nanoparticle precipitation by centrifugation, suggesting a difference in particle density between samples with and without calcium. The random process of calcium chloride addition promoted pronounced or less pronounced cross-linking in certain areas of the particles. The controlled addition of calcium needs to be addressed to avoid the random nanoparticle shape and subsequently improve the stability of the entrapped β-carotene against oxidation.

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