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Encapsulation of Epigallocatechin-3-gallate (EGCG) Using Oil-in-Water (O/W) Submicrometer Emulsions Stabilized by ι-Carrageenan and β-Lactoglobulin

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Oil-in-water (O/W) submicrometer emulsions stabilized by ι-carrageenan and β-lactoglobulin were successfully prepared by high-pressure homogenization (HPH), with the goal to develop biocompatible carriers for the active component of green tea, epigallocatechin-3-gallate (EGCG). The effects of pressure and the number of cycles on the physical properties of emulsions, such as droplet sizes, microstructure, and rheological properties were investigated. The increase in both processing pressure and the number of HPH cycles resulted in a decrease in droplet sizes and viscosities. A submicrometer O/W emulsion with a droplet size of about 400 nm was used to encapsulate EGCG. The results showed that, when EGCG concentration was up to 0.5% in the emulsion, EGCG could be successfully encapsulated in the O/W emulsions stabilized by ι-carrageenan and β-lactoglobulin. Within 14 days, emulsion droplet sizes showed negligible changes. However, when EGCG concentration was >0.5%, significant instability of the O/W emulsions due to the binding between EGCG and β-lactoglobulin was observed, as evidenced by the largely increased droplet sizes from light scattering and the appearance of large aggregates in the optical images. Moreover, EGCG encapsulated in an O/W submicrometer emulsion revealed an enhanced in vitro anticancer activity compared to the free EGCG. This study provides a novel encapsulation formulation to increase the biological efficacy of EGCG.

KEYWORDS: EGCG; O/W submicrometer emulsions; β-lactoglobulin; ι-carrageenan; high-pressure homogenization; in vitro anticancer model

INTRODUCTION

Tea is the most popular beverage next to water, consumed by over two-thirds of the world's population for thousands of years. It is rich in natural antioxidants, which are believed to have health-promotive functions (*1*). The antioxidant components in tea leaves include methylxanthines and polyphenols, especially flavonols of the catechin type. The major green tea polyphenols are epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epicatechin (EC), and galocatechin (GC). Among these components, EGCG accounts for about 60−70% of total tea catechins (*1*) and has shown the most protective activities. Inhibitions of EGCG against skin, stomach, colon, and lung carcinogenesis as well as the growth of human prostate and breast tumor in athymic mice have been reported (*2*). In vitro study has shown the modulation of gene expression of EGCG (*3*). EGCG can induce cell cycle in the G0/G1 phase to inhibit the proliferation of the tumor cells (*4*). EGCG can also affect signal transduction, resulting in growth inhibition, cell cycle arrest, and apoptosis of a cell (*5*).

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On the other hand, the effective tissue concentration of EGCG is very low. The EGCG levels in the tissues and blood corresponded to 0.0003−0.45% of the ingested dose after 60 min (*6*). Another study using [^{3}H]-EGCG showed that following a single initial dose of [^{3}H]-EGCG, only 10% of the initial dose was presented in the blood after 24 h and approximately 1% in the brain, lung, heart, liver, kidney, and other tissues (*7*). However, EGCG has very poor bioavailability, which may be in part explained by Lipinski's rule of 5. That is, if a compound has a molecular weight of >500 and contains 5 or more hydrogen-bonding donors and/or 10 or more hydrogen-bonding acceptors, it is very difficult to pass through transient pores formed in the plasma membrane. Apparently, EGCG has a large molecular size and many hydrogen bond donors and acceptors, resulting in a molecule with a large hydration shell, which is hard to pass through the plasma membrane (*8*). Therefore, it is almost impossible to achieve high EGCG concentrations in most parts of the body except skin and gastrointestinal tract (*9*). Encapsulation systems can be used to overcome these limitations. Attempts to improve bioavailability of EGCG using liposomes (*10*), polyphenol-loaded lipid nanocapsules (*11*), and protein/polyphenol microcapsules (*12*) have been reported. The disadvantage of previous work included the use of either organic solvents or

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non-food-grade synthetic polyelectrolytes. The development of simple, safe, and effective delivery systems is therefore necessary to improve the bioavailability of EGCG. Protein- and polysaccharide-stabilized emulsions can be formed through a layer-by-layer electrostatic deposition method that involves sequential adsorption of protein onto the surfaces of oppositely charged polysaccharide. Therefore, these emulsion-based delivery systems could release in response to a specific pH trigger similar to human digestive system.

In our previous paper, we found the optimum concentrations of β-lactoglobulin and ι-carrageenan for the formation of stable oil-in-water (O/W) emulsions at pH values of both 4.0 and 3.4 were 0.3−0.6 and 0.4−0.7 wt %, respectively (*13*). High-pressure homogenization has been routinely used to engineer nano- or submicrometer nutraceutical dispersions (*14*). In this paper, we have successfully prepared the β-lactoglobulin- and ι-carrageenan-stabilized EGCG O/W emulsions using high-pressure homogenization. The influence of the homogenization parameters, such as processing cycles and pressure, on the physicochemical properties of O/W emulsions was systematically examined using optical microscopy, dynamic light scattering (DLS), and dynamic rheological measurements. The effects of EGCG dose on the stability of EGCG O/W emulsions were investigated through a period of 2 weeks. Finally, the in vitro anticancer activity of EGCG submicrometer emulsion was evaluated using human hepatocellular carcinoma (Hep G2) cell line.

MATERIALS AND METHODS

Materials. EGCG (>95%) and ι-carrageenan (lot 81k1556) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. β-Lactogloblulin (lot BiPro JE 003-3-922) was provided by Davisco Foods Internationals Inc. (Le Sueur, MN). Minimum essential medium, fetal bovine serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Dibasic sodium phosphate, poly(ethylene glycol) (MW = 400), citric acid, acetic acid, and standard NaOH (0.5 N) solution were also purchased from Sigma-Aldrich. Canola oil (Mazola, USA) was purchased from a local supermarket. All solutions were prepared with DI water and filtered with 25 mm 0.45 μm Nalgene glass fiber syringe filters (Whatman Inc., Florham Park, NJ).

Preparation of Emulsions Using β-Lactoglobulin and ι-Carrageenan. A stock buffer solution was prepared by dispersing 100 mM citric acid and 200 mM dibasic sodium phosphate solution in DI water. Both solutions were mixed at a given concentration to make pH 3.4 citrate phosphate buffer solutions. The preparation of the emulsions was carried out in two steps. First, β-lactoglobulin and oil droplets were mixed using a high-speed homogenizer (Ultra-Turrax T-25 basic, IKA Works Inc., Wilmington, NC) at 24000 rpm for 4 min, and then ι-carrageenan solution was added to the mixtures of β-lactoglobulin and oil droplets during high-speed homogenization at 24000 rpm for another 4 min. The final concentrations of canola oil, β-lactoglobulin, and ι-carrageenan were 0.5, 0.4, and 0.6 wt %, respectively.

High-Pressure Homogenization. The biopolymer-stabilized O/W emulsions were prepared as described above. Subsequently, a high-pressure homogenizer (EmulsiFlex-C3, AVESTIN Inc., Ottawa, Canada) operated at 5000, 10000, and 15000 psi for six cycle times, respectively, was used to prepare submicrometer O/W emulsions of different sizes.

EGCG Submicrometer Emulsions. EGCG was first dissolved in 1 mL of poly(ethylene glycol)−water (1:2 v/v) solution and then diluted 2 times into water, which generated EGCG solutions with specific concentrations. EGCG solution was added dropwise into 5 mL of canola oil under high-speed homogenization of 24000 rpm for 4 min. Such high-speed homogenization condition was used for all of the EGCG submicrometer emulsions before high-pressure homogenization. Finally, the emulsion was homogenized at 15000 psi pressure for three cycles to form stable and homogeneous O/W emulsion. The final concentrations of β-lactoglobulin, ι-carrageenan, and canola oil were 0.4, 0.6, and 5%, respectively.

Optical Microscopy. Emulsions were gently agitated in a glass vial before analysis to ensure the homogeneity of the emulsions. The microstructures of the emulsions were then observed using a Nikon TE-2000 inverted microscope (Nikon Corp., Japan).

Dynamic Light Scattering. The hydrodynamic radius of the emulsion was measured using BIC 90 plus particle size analyzer (Brookhaven Instrument Corp., New York). The emulsions were diluted with citrate phosphate buffer prior to the measurement to prevent multiple scattering and then placed in the cuvette holder. All measurements were made at a fixed scattering angle of 90° and at 25.0 ± 0.1 °C. The light source of the particle size analyzer is a solid state laser operating at 658 nm with 30 mW power, and the signals were detected by a high-sensitivity avalanche photodiode detector. The normalized field−field autocorrelation function $g(q,t)$ is obtained from the intensity−intensity autocorrelation function, $G(q,t)$, via the Sigert relationship

$$\alpha g(q,t) = [G(q,t)/A - 1]^{1/2} \quad (1)$$

where A is the experimentally determined baseline and α is the contrast factor, which is <1 due to the fact that only a fraction of dynamic scattering intensity falls within the correlator window and also the fact that a finite size pinhole is used in the experiment. For all particle size measurements, the measured baseline A is in agreement with the theoretically calculated baseline to 0.01%.

The diffusion coefficient D was calculated according to $D = \tau^{-1}q^{-2}$, where q is the amplitude of scattering vector defined as $q = (4\pi n/\lambda)\sin(\theta/2)$, n is the solution refractive index, λ is the laser wavelength, and θ is the scattering angle. The diffusion coefficient D can be converted into mean emulsion droplet diameter d using the Stokes−Einstein equation

$$d = \frac{kT}{3\pi\eta D} \quad (2)$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity.

Cumulant analysis method was used in our size measurements, where $g(q,t)$ was decomposed into a distribution of decay rate Γ ($= 1/\tau$) given by

$$g(q,t) = \int G(\Gamma)\, e^{-\Gamma t}\, d\Gamma \quad (3)$$

The first two moments of the distribution $G(\Gamma)$ are

$$\Gamma = Dq^2 \quad (4)$$

$$\mu_2 = (D^2 - D^{*2})q^4 \quad (5)$$

where D^* is the average diffusion coefficient. The polydispersity term defined in the cumulant analysis is

$$\text{polydispersity} = \mu_2/\Gamma^2 \quad (6)$$

Here polydispersity has no unit. It is close to zero for monodisperse or nearly monodisperse samples and larger for broader distribution.

Rheological Measurements. Rheological measurements of O/W emulsions were performed using the Advanced Rheometric Expansion System (ARES, TA Instruments, New Castle, DE) with parallel plate geometry (25 or 50 mm in diameter). Each measurement was conducted at 25 ± 1 °C. For dynamic rheological measurements, viscosity (η^*) was observed at angular frequency ranging from 0.1 to 100 rad/s. For each measurement, the sample was loaded onto the plate for 10 min to allow the stresses to relax and thermal equilibration.

Cell Culture and in Vitro Anticancer Activity Assay. The human hepatocellular caricinoma (HepG2) cell line was a gift from Dr. Mou-Tuan Huang of Rutgers University. Cells were cultured in minimum essential medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin and were maintained at 37 °C with 5% CO_2.

In vitro anticancer assay was performed using methyl thiazol tetrazolium bromide (MTT) method as described previously (*15*). Briefly, HepG2 cells were seeded in 96-well microplates at a density of 10000 cells in 100 μL of medium. The next day, the cells were treated with one of the following four different media, respectively: (1) media with different

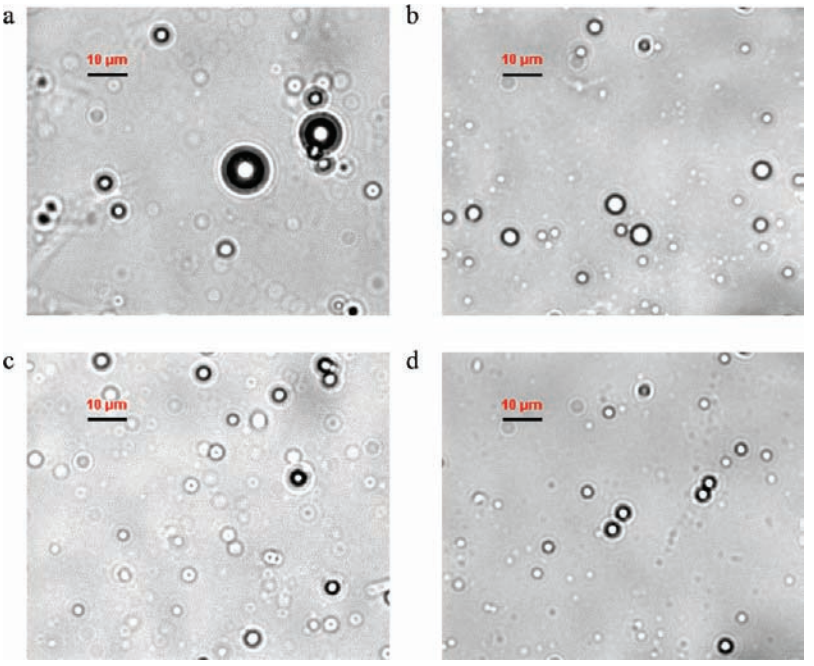

Figure 1. Microstructures of the emulsions stabilized by β-lactoglobulin and ι-carrageenan after high-speed homogenization at 24000 rpm (**a**), followed by one cycle of high-pressure homogenization at different pressures: 5000 psi (**b**); 10000 psi (**c**); and 15000 psi (**d**).

concentrations (50, 25, 12.5, and 6.25 μg/mL) of EGCG dissolved in DMSO; (2) media containing different concentrations of EGCG nanoemulsions; (3) media containing "empty" emulsions with the emulsion concentration corresponding to those used in (2); and (4) media only used as untreated negative controls. After treatment for 24 h, cells were treated with MTT and the formazan crystals formed in each well were dissolved in DMSO. Optical absorbance at 560 and 670 nm was recorded with a Bio-Tek Multi-Mode microplate reader.

Relative viability of cells treated with EGCG in DMSO was expressed as $A_{560}-A_{670}$ normalized by that of the untreated wells, because it was previously established that DMSO at the concentration used did not affect HepG2 growth (*15*). Relative viability of cells treated with EGCG in submicrometer O/W emulsion was calculated as $A_{560}-A_{670}$ normalized by that of the cells treated with the same concentrations of empty emulsion. Data were presented as mean ± standard deviation with four repeats ($n = 4$).

Statistical Analysis. The anticancer effect of DMSO-dissolved and encapsulated-EGCG was compared with t test using SigmaPlot 10.0 software (Systat Software, Inc., Chicago, IL). Cell viabilities were normalized and shown as mean ± standard deviation with four repeats ($n = 4$). ** denotes the difference between the free EGCG treatment and the treatment with encapsulated EGCG was statistically very significant ($p < 0.01$) in that EGCG concentration.

RESULTS AND DISCUSSION

Influence of Homogenization Pressure and Processing Cycles. High-energy emulsification methods, such as high-speed homogenization, high-pressure homogenization, and ultrasonication, as well as low-energy emulsification methods, such as the phase inversion temperature method, can be used to prepare submicrometer and nanoemulsions (*16*). The submicrometer emulsions prepared in this study were typical O/W emulsions. Therefore, this study was conducted to investigate the effect of high-pressure processing parameters on the physical properties of the O/W emulsions to find the optimal formulation. In the present O/W emulsion preparation, high-speed homogenization was first carried out at 24000 rpm to obtain stable O/W emulsions. Subsequently, emulsions were further homogenized with a high-pressure homogenizer at homogenization pressures of 5000, 10000, and 15000 psi to achieve emulsions of smaller droplet sizes. **Figure 1** shows the optical microscopy images of various O/W emulsions, which were prepared by high-speed (24000 rpm) and high-pressure homogenization (5000, 10000, and 15000 psi) after one cycle. It was noted that the premixed emulsion has significantly larger droplet sizes and size distribution. Dramatically smaller emulsion droplets could be observed in high-pressure homogenized emulsions. Due to the detection limit of the optical microscope, the images of emulsion systems with droplets sizes of < 500 nm cannot be observed. Homogenization is one of the most frequently used techniques for preparing stable emulsions. Interactions between two biopolymers may be significantly modified by high-pressure treatment, resulting in changes in the overall physicochemical properties. During sufficiently intense high-pressure treatment, globular proteins, such as β-lactoglobulin, may undergo unfolding and aggregation in aqueous solution (*17*). Pressure-induced unfolding and aggregation of globular protein molecules in the presence of certain polysaccharides provide many opportunities for biopolymer−biopolymer interactions. Changes in the protein−polysaccharide interactions through high-pressure treatment are often manifested in changes of the emulsion properties.

Stokes' law states that the creaming velocity of an emulsion droplet is proportional to the square of its radius. The stability of an emulsion to gravitational separation can therefore be enhanced by reducing the size of the droplet (*18*). In general, emulsions with smaller droplet sizes have greater stability. The main advantage of high-pressure homogenizers over other technologies is that more uniform droplet size distributions can be obtained because the products are subjected to strong shear and cavitation forces that efficiently decrease the diameters of the original droplets (*19*). Homogenization pressure can significantly influence the properties of emulsions as the shear forces and turbulence, both of which are pressure dependent, produced during homogenization can affect the particle size and size distribution (*20*). To obtain the emulsion droplet sizes and polydispersities, we also carried out dynamic light scattering (DLS) measurements. Panels **a** and **b** of **Figure 2** show the DLS results of high-pressure homogenized O/W emulsion prepared at 15000 psi pressure, which were analyzed by cumulant analysis and single stretched exponential fit, respectively.

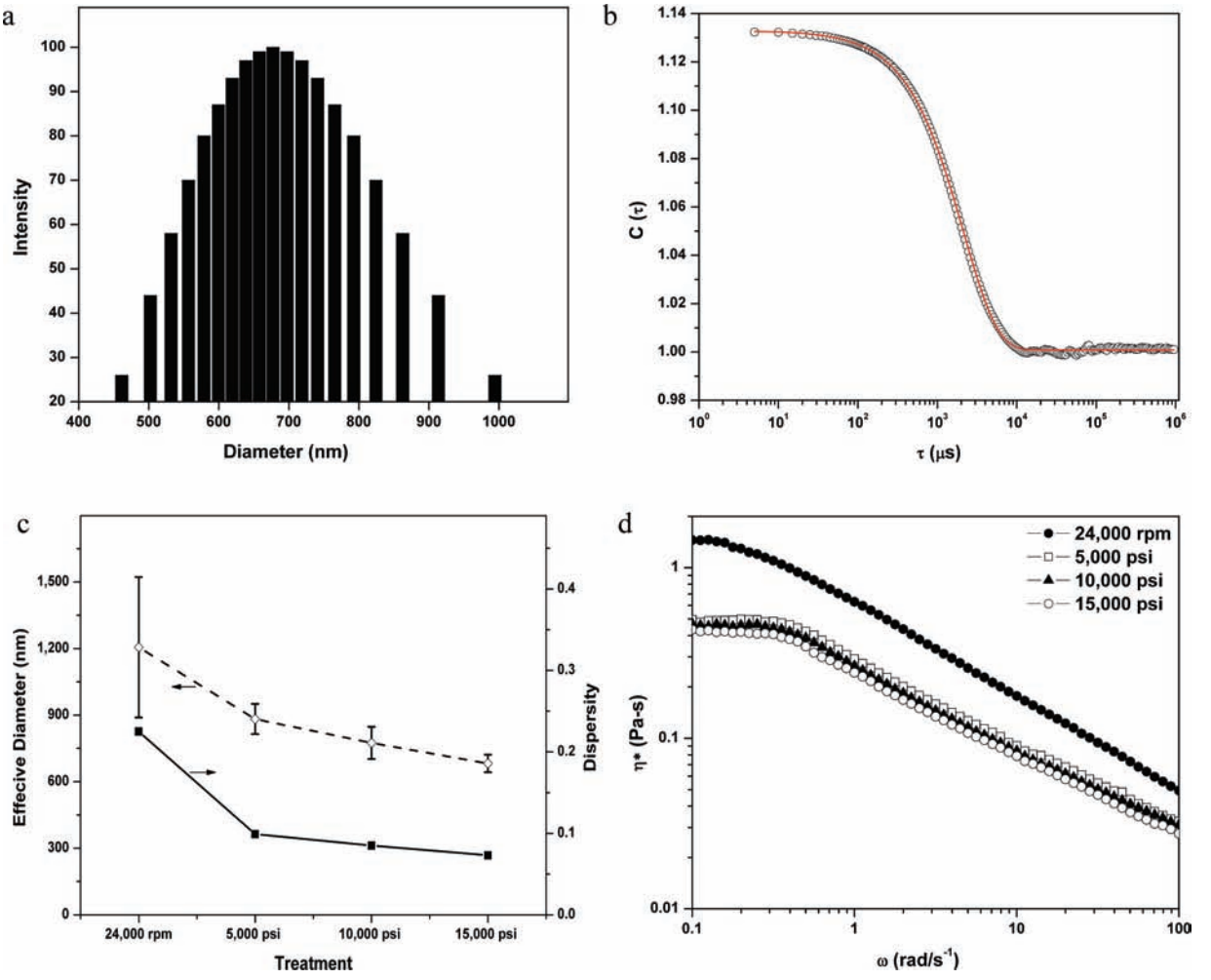

Figure 2. Photon correlation spectroscopy results of emulsion from **Figure 1d** analyzed by cumulant analysis (**a**) and single stretched exponential fit (**b**). The circles are experimental data, whereas the solid line is the fitting curve. (**c**) Plots of particle sizes and polydispersities versus processing conditions and (**d**) dynamic viscosity versus angular frequency for the emulsions stabilized by β-lactoglobulin and ι-carrageenan after high-speed homogenization at 24000 rpm (●), followed by one cycle of high-pressure homogenization at different pressures: 5000 psi (□); 10000 psi (▲); and 15000 psi (○).

The mean emulsion diameters and polydispersities of different emulsions analyzed by these two data analysis methods showed comparable droplet sizes and polydispersities and decreased as the homogenization pressure increased (**Figure 2c**). Similar homogenization pressure effects on emulsion droplet sizes were also reported by other groups using different emulsifiers (*16*, *21*, *22*). Under high homogenization pressures, part of the energy is dissipated as heat, which may lead to breakdown of adjacent oil droplets and result in rheological properties comparable to those of untreated emulsions. Homogenization of emulsions at high pressure decreased droplet size, resulting in a looser interdroplet packaging. Therefore, the expected rheological behavior would be lower emulsion viscosity with increasing pressure. It was also noted from **Figure 2d** that the viscosity of the emulsion under high-pressure homogenization decreased significantly compared to the untreated emulsion, but decreased slightly as the pressure increased. The lower viscosity mixture allows the production of emulsions with smaller oil droplets (*18*). Our results also indicated that high-pressure homogenization was required to prepare stable submicrometer O/W emulsions.

It is known that the number of homogenization cycles can affect the physiochemical properties and stability of the emulsions. To investigate the effects of high-pressure homogenization cycles on the physical properties of O/W emulsions, the microstructures and particle sizes of biopolymer-stabilized emulsions were determined by optical microscopy and DLS, respectively. One notes that the emulsion prepared in the first cycle flocculated more extensively and less homogeneous than those prepared in the fourth, fifth, and sixth cycles (**Figure 3**). Our optical microscope images indicated that emulsion droplets changed significantly at the first three cycles (**Figure 3**). The effects of homogenization cycles on the droplet sizes of O/W submicrometer emulsions are also presented in **Figure 4a**. As expected, increasing the number of homogenization cycles resulted in a decrease in both the droplet size and polydispersity. The emulsion droplet size after the first cycle of high-pressure processing cycle was about 682 nm and decreased progressively as the number of processing cycles increased. After six cycles of high-pressure homogenization, the droplet sizes were reduced to about 426 nm, which is ~62% lower than the initial size (**Figure 4a**). The viscosities of emulsions were also dramatically modified by high-pressure homogenization cycle. As seen from **Figure 4b**, the viscosity of the emulsion was obviously decreased by high-pressure homogenization, especially at the first three cycles. However, after passing the emulsion through the homogenizer three times, subsequent passes had little effect on the particle size and viscosity. Furthermore, no improvement on the size distribution was observed after six cycles of homogenization. These results are in broad agreement with those reported by Tan and Nakajima (*14*).

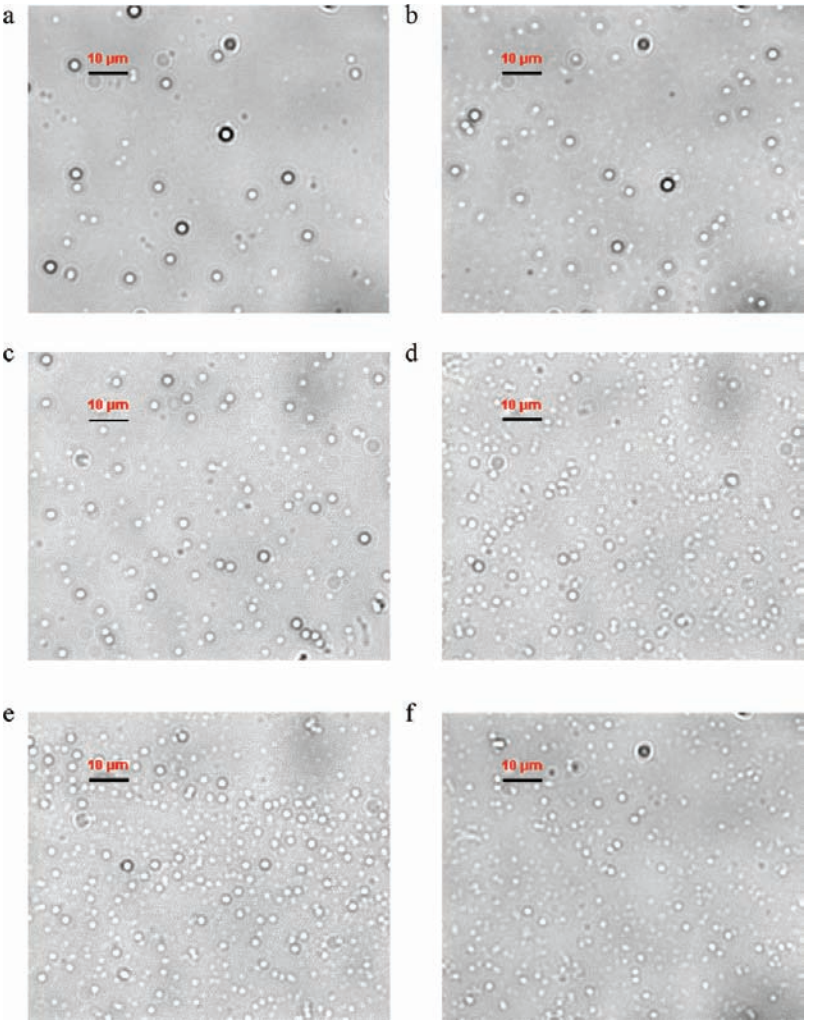

Figure 3. Optical microscopy images of the emulsions stabilized by β-lactoglobulin and ι-carrageenan at various high-pressure cycles: (**a**) one cycle; (**b**) two cycles; (**c**) three cycles; (**d**) four cycles; (**e**) five cycles; (**f**) six cycles. The pH is fixed at 3.4.

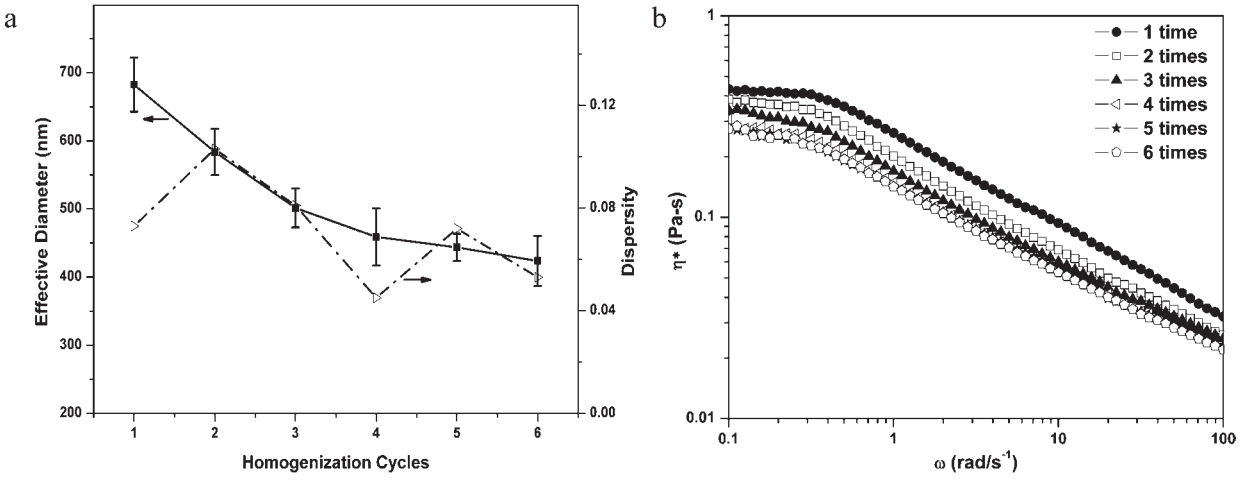

Figure 4. Plots of particle sizes and polydispersities versus homogenization cycles (**a**) and dynamic viscosity versus angular frequency (**b**) of the emulsions stabilized by β-lactoglobulin and ι-carrageenan after various high-pressure homogenization cycles at 15000 psi.

Stability of EGCG Submicrometer Emulsion. The submicrometer O/W emulsion prepared by high-speed homogenization at 24000 rpm and then high-pressure homogenization at 15000 psi was used to encapsulate EGCG. Optical images of the EGCG submicrometer emulsions were made immediately after the emulsions were prepared. High-pressure-treated emulsion showed more homogeneous microstructure when compared to untreated emulsion (**Figures 1a** and **5a**−**c**), and the particle size was about 400 nm (**Figure 6a**). In O/W emulsion, the encapsulation efficiency is hypothesized as 100% in matrix, and no EGCG releases from matrix. However, when the EGCG concentration reached 1 and 2%, a significant association of biopolymers was found

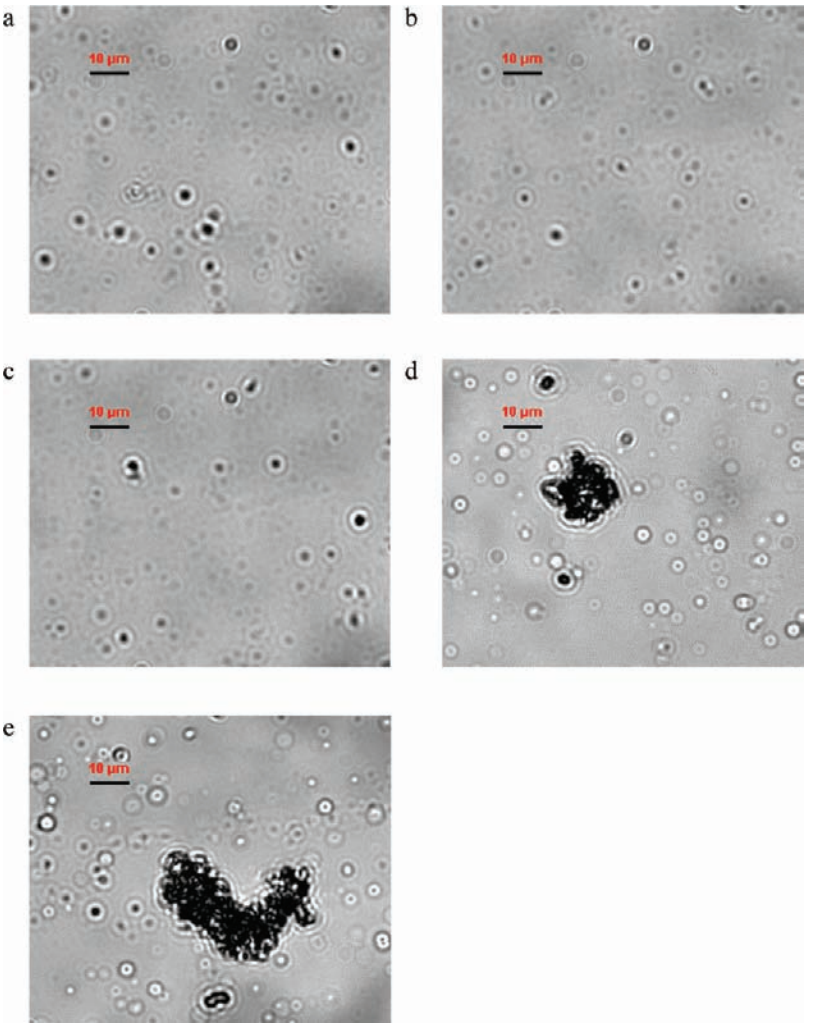

Figure 5. Optical microscopy images of β-lactoglobulin and ι-carrageenan stabilized O/W emulsions containing various EGCG concentrations: (**a**) 0.1%; (**b**) 0.25%; (**c**) 0.5%; (**d**) 1%; and (**e**) 2%. The pH values of the emulsions are fixed at pH 3.4.

(**Figure 5d**,**e**). Meanwhile, particle size was maintained at about 1000 nm when the final concentration of EGCG was enhanced to 1% (**Figure 6a**). Green tea polyphenols are known to be able to interact with proteins (*23*, *24*). Almajano et al. (*25*) found that EGCG could react with β-lactoglobulin. Therefore, if the EGCG concentration reaches $>1\%$, oil cannot fully cover all EGCG. Part of the free EGCG may leak to the water phase and interact with β-lactoglobulin. Although EGCG is partially soluble in water, it was found that 0.5% EGCG could be successfully encapsulated in O/W emulsions, suggesting that O/W emulsions have a relatively high capacity to encapsulate EGCG. **Figure 6b** shows the changes in the particle sizes of EGCG O/W nanoemulsions over a 2 week period of storage. Within 14 days, emulsion droplet sizes showed negligible changes for EGCG nanoemulsions. The consistent particle sizes of these emulsions with time indicate the good stability of EGCG O/W nanoemulsions prepared.

Proteins have been known to interact strongly with polyphenol through hydrogen bonding and hydrophobic interactions. Most antioxidants of interest for foods have one or more phenolic hydroxyl groups, and there are several studies demonstrating that molecules with this structure may bind to proteins (*26*−*29*). Polyphenols may associate with proteins through hydrophobic interactions and hydrogen bonding (*27*), and several phenolic antioxidants have also been shown to bind to bovine proteins (*28*). It is impossible to directly encapsulate EGCG using β-lactoglobulin because EGCG will induce aggregation of β-lactoglobulin. Here we used canola oil and poly(ethylene glycol) to protect EGCG from β-lactoglobulin. Even though the EGCG O/W nanoemulsion containing 0.5% EGCG was stable according to the particle size, we found that there were some precipitates at the bottom of the test tube after storage for 3 days. We speculated that 0.5% final EGCG concentration is a critical concentration that can be encapsulated by O/W emulsion stabilized by β-lactoglobulin and ι-carrageenan.

Proteins are particularly attractive as emulsifiers because they are natural, nontoxic, tasteless, and widely available. They adsorb to the surfaces of the oil droplets created by homogenization of oil−water−protein mixtures, where they facilitate further droplet disruption by lowering the interfacial tension and retard droplet aggregation by forming protective membranes around the droplets. However, protein-stabilized emulsions are highly sensitive to environmental stresses such as pH, ionic strength, and temperature, which have limited their application in many foods (*30*, *31*). Emulsions stabilized by polysaccharides are often more resistant to pH changes, high ionic strength, and elevated temperatures than those stabilized by proteins. This has been attributed to the fact that polysaccharide-stabilized droplets are surrounded by a relatively thick porous polymer layer, which increases the steric repulsion and decreases the van der Waals attraction between droplets (*32*). On the other hand, polysaccharides are usually much less effective at producing emulsions than

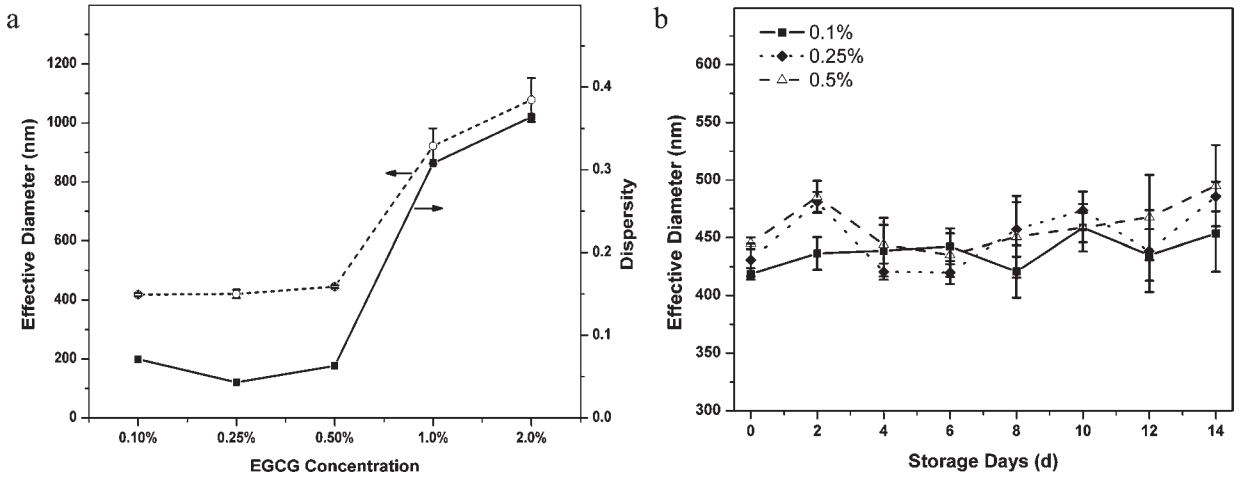

Figure 6. Plot of EGCG concentration versus the particle sizes and size distributions of the emulsions stabilized by β-lactoglobulin and ι-carrageenan (**a**) and plot of particle sizes versus storage days (**b**) for emulsions containing 0.1% (■), 0.25% (◆), and 0.5% (△) EGCG. The pH values of the emulsions are fixed at pH 3.4.

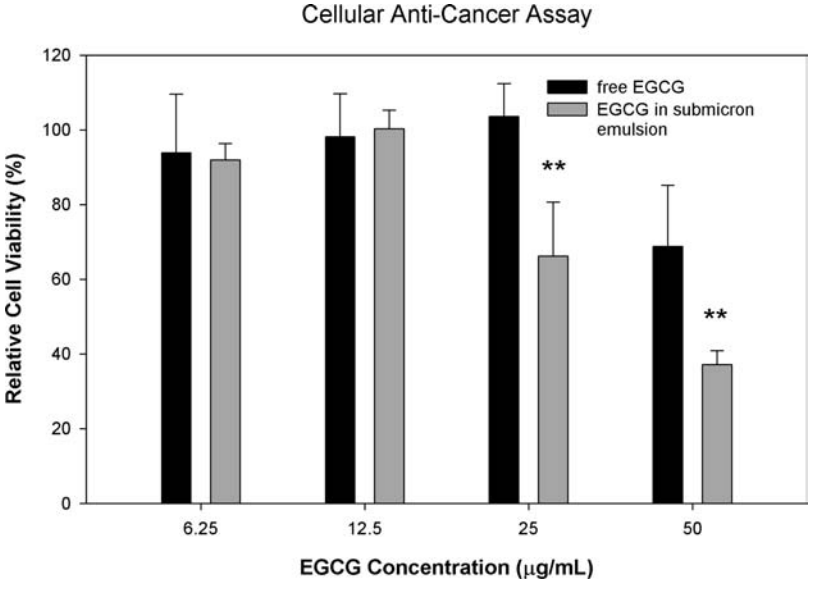

Figure 7. Cellular anticancer assay of free EGCG and submicrometer emulsion encapsulated EGCG on HepG2 cells.

proteins, because they are less surface active. Hence, they must be used in much higher concentrations than proteins to produce emulsions containing small droplets. It would therefore be advantageous to combine the beneficial attributes of proteins and polysaccharides to produce small emulsion droplets with good environmental stability (*32*). This technique is based on layer-by-layer deposition of polyelectrolytes onto oppositely charged surfaces due to electrostatic attraction, which results in the formation of droplets coated by multilayered interfacial membranes. Here, β-lactoglobulin was used as an emulsifier to form an O/W emulsion containing small droplets; with the addition of ι-carrageenan, emulsions containing droplets coated with β-lactoglobulin−ι-carrageenan membranes were thus produced.

In Vitro Anticancer Activity of EGCG Submicrometer Emulsion. It is well-known that EGCG has anticancer activity and is able to induce apoptosis in different cancer cell lines (*33*). To compare the bioactivity of submicrometer emulsion encapsulated EGCG with that of its free form, cellular anticancer experiment was performed on HepG2 cells (**Figure 7**). It was shown that 50 μg/mL free EGCG was able to inhibit cell proliferation, which was similar to other researchers' finding (*34*−*36*). Furthermore, the EGCG submicrometer emulsion showed stronger anticancer effect at concentrations of 25 and 50 μg/mL. This result suggested that EGCG submicrometer emulsion may enhance the bioactivities of EGCG. EGCG is easily oxidized or degraded under basic pH conditions. Compared with free EGCG, which could be considered as a burst release, submicrometer O/W emulsion encapsulated EGCG could be control released and had better stability under basic pH in addition to better permeability or cell absorption.

It is well-known that pH plays a key role in the strength of electrostatic interaction because it determines the sign and the number of charges of a protein. The general picture for the complex between a protein and an anionic polysaccharide is that the primary soluble protein/polysaccharide complexes are initially formed at the first critical pH (pH_c, corresponding to the onset of soluble complex), and then the soluble protein/polysaccharide complexes start to aggregate into insoluble protein/polysaccharide complexes at the second critical pH ($pH_{\varphi 1}$), which ultimately sediment to generate the dense coacervate phase (*37*−*39*). The controlled release properties of EGCG submicrometer

emulsions in simulated human gastrointestinal tract are currently being studied in our laboratory and will be published in a separate paper.

In summary, a relatively simple and effective formulation has been developed to prepare EGCG submicrometer O/W emulsions. This study confirms that high-pressure homogenization is an effective way to prepare submicrometer or nanometer emulsions. The microstructure, particle size, and viscosity of the EGCG O/W emulsions were influenced by homogeinzation pressure and cycles. β-Lactoglobulin- and ι-carrageenan-stabilized O/W emulsions could serve as biocompatible carriers for oral delivery of EGCG, especially at low EGCG concentration (up to 0.5%). EGCG submicrometer emulsion showed improved anticancer effect compared with free EGCG. This emulsion system therefore has the potential to be used as a carrier for sensitive bioactive compounds in food, nutraceutical, and pharmaceutical applications.

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