JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# Preservation of (−)-Epigallocatechin-3-gallate Antioxidant Properties Loaded in Heat Treated β-Lactoglobulin Nanoparticles

Bo Li, Wenkai Du, Jianchang Jin, and Qizhen Du*

Institute of Food and Biological Engineering, Zhejiang Gongshang University, 149 Jiaogong Road, Hangzhou 310035, China

Supporting Information

**ABSTRACT:** (−)-Epigallocatechin-3-gallate (EGCG) was loaded in heat treated β-lactoglobulin (β-Lg) for the preservation of antioxidant activity. The effects of pH (2.5−7.0), the heating temperature of β-Lg (30−85 °C), the molar ratio of β-Lg to EGCG (1:2−1:32), and the β-Lg concentration (1−10 mg/mL) on the properties of β-Lg−EGCG complexes were studied. All four factors significantly influenced the particle size, the ζ-potential, and the entrapment efficiency of EGCG and EGCG loading in β-Lg particles. A stable and clear solution system could be obtained at pH 6.4−7.0. The highest protection of EGCG antioxidant activity was obtained with β-Lg heated at 85 °C and the molar ratio of 1:2 (β-Lg: EGCG). β-Lg−EGCG complexes were found to have the same secondary structure as native β-Lg.

**KEYWORDS:** *nanoparticle, β-lactoglobulin, (−)-epigallocatechin-3-gallate (EGCG), antioxidant activity*

## 1. INTRODUCTION

Catechins are flavanols present in a variety of foods, such as tea, fruits, vegetables, and wine, and they have garnered considerable attention due to their beneficial effects on health.[1] (−)-Epigallocatechin gallate (EGCG), as the most abundant catechin and major biologically active component in green tea (*Camellia sinensis* (L.) O. Kuntze, Theaceae) leaves, is probably the best known and most investigated catechin.[2] Numerous studies have demonstrated its potential activities in antioxidant, antimutagenic, antiobesity, antibacterial, antiviral, and protective effects from many diseases, such as cancer, cardiovascular disease, diabetes, and inflammation.[3,4]

Catechins can be used as natural antioxidants and antimicrobial agents in foods to retard lipid oxidation and extend the shelf life and as functional ingredients in various foods and beverages to provide a health benefit and a healthier appeal to the consumer.[1,5] However, the instability of catechins during food processing and storage limits their commercial applications. It was reported that the stability of tea catechins is closely associated with pH, temperature, and oxygen concentration.[6] Tea catechins are very unstable in neutral and alkaline solutions, and they undergo degradations and epimerizations in aqueous systems during thermal processing.[7] Oxidation and polymerization of catechins give undesired yellowish-brown color in aqueous solution.[8,9] Encapsulation is an effective method to protect the core material from adverse environmental conditions. Various materials such as chitosan, liposomes, and gelatin have been used for catechins micro- and nanoencapsulation to improve the stability and bioavailability of catechins *in vivo* and *in vitro*.[10]

β-Lactoglobulin (β-Lg), a water-soluble protein that forms the major component of ruminant milk whey, was suggested to be a pH and temperature sensitive hydrogel former.[11] It is a small globular protein folded into a calyx formed by eight antiparallel β-strands and a three-turn α-helix located at the outer surface of the β-barrel.[12] At neutral pH and room temperature, β-Lg exists as a dimer, while at pH below 3 or above 8 it dissociates into monomers. Each monomer consists of 162 amino acid residues and has a molecular mass of 18 kDa. Between pH 3.5 and 5.5, β-Lg tends to form octamers.[13] The thermal behavior of bovine β-Lg at different temperatures has been reviewed.[14] Heat treatments make the protein undergo dissociation and conformational modifications. β-Lg is an attractive natural vehicle for biologically active substances due to its ability to bind many compounds, cost effectiveness, abundant availability, and acceptability.[15] It has been used as a carrier molecule for resveratrol, curcumin, and ω-3 polyunsaturated fatty acids to improve their stability and bioavailability.[16−18] Binding of ligands to β-Lg depends on the structure and oligomeric state of the protein.[19] Some small hydrophobic ligands exhibited a higher binding ability to β-Lg in the heat-induced molten globule state compared to unheated β-Lg.[20]

Recently it was reported that thermally induced (75−85 °C) β-Lg at neutral pH exhibited greater affinity for EGCG compared with native β-Lg, and could be used as nanovehicles to enhance EGCG stability.[21,22] However, the detailed impacts of some important process parameters, including pH, heating temperature, molar ratio of β-Lg to EGCG, and β-Lg concentration on the properties of β-Lg−EGCG nanoparticles are not reported. In this study, we examined the effects of the above four factors on particle size, surface charge, entrapment efficiency, EGCG loading, antioxidant activity, and the secondary structure of heat treated β-Lg−EGCG complexes, with the aim of identifying conditions where stable nanoparticles could be formed for the effective preservation of EGCG antioxidant activity.

Received: October 14, 2011
Revised: March 11, 2012
Accepted: March 12, 2012
Published: March 12, 2012

  

## 2. MATERIALS AND METHODS

**2.1. Materials.** Bovine β-lactoglobulin (β-Lg) (>90% purity), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and tripyridyltriazine (TPTZ) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, USA). EGCG (>95% purity) was obtained from Chengdu Biopurity Phytochemicals Ltd. (Chengdu, China). Methanol and acetonitrile of HPLC grade were purchased from Tianjin Shield Company (Tianjin, China). Other reagents used were of analytical grade. Water was filtered through a MILLI-Q water system (Millipore Corp., Bedford, MA, USA).

**2.2. Preparation of β-Lg–EGCG Nanoparticles.** β-Lg–EGCG nanoparticles were prepared according to the procedure reported previously[21] with slight modifications. Briefly, $Na_2HPO_4$–$NaH_2PO_4$ buffer solutions at pH 6.0–7.0 were prepared and the buffer solution at pH 6.0 was adjusted to pH 2.5–5.5 with $H_3PO_4$. β-Lg solution in 30 mM buffer at varying pH containing 0.02% sodium azide ($NaN_3$) as preservative was prepared and stirred overnight at room temperature. EGCG solution was prepared using a 30 mM buffer (pH 2.5) before use. EGCG solution (0.1 mL) at room temperature was added to 1.9 mL of β-Lg solution which had been heated at different temperatures in a water bath for 20 min. After addition of EGCG to the protein solution, the sample was vortexed for 30 s and fast cooled down to room temperature (25–30 °C) under running tap water. The addition of β-Lg or EGCG did not appreciably modify the pH.

Different experimental conditions were used for preparation of β-Lg–EGCG nanoparticles, and four factors were studied in the following order: the pH value of the buffer used for β-Lg solution preparation (pH 2.5, 5.0, 5.5, 6.0, 6.2, 6.4, 6.6, 6.8, and 7.0), the heating temperature (30, 55, 70, and 85 °C), the molar ratio of β-Lg to EGCG (1:2, 1:4, 1:8, 1:16, and 1:32) and the β-Lg concentration (1.0, 2.5, 5.0, and 10.0 mg/mL; i.e., 0.054, 0.135, 0.270, and 0.540 mM) in the final mixture.

**2.3. Particle Size and Charge Measurement.** The average diameter and size distribution of particles were determined by dynamic light scattering (DLS) using a Zetasizer Nano-ZS analyzer (Malvern Instruments, Worcestershire, U.K.). DLS measurements were accomplished at 633 nm, 173°. Particle size distributions were produced from the volume measurements, in which the relative percentage of particles in each size class is based on the volume they occupy. The particle charge data were reported as the ζ-potential, which was measured with laser Doppler velocimetry (LDV) using the same Zetasizer Nano-ZS analyzer.[23]

**2.4. Entrapment Efficiency and EGCG Loading in β-Lg–EGCG Complexes.** A β-Lg–EGCG complex solution (2 mL) was transferred into an Amicon Ultra-3K centrifugal filter device (Millipore Corp., Billerica, MA, USA) made up of a centrifuge tube and a filter unit with a low-binding Ultracel membrane (3000 MWCO). After centrifugation at 4000*g* for 30 min, β-Lg–EGCG complexes remained in the filter unit and free EGCG penetrated through the Ultracel membrane into the centrifuge tube.[24] Free EGCG content in the ultrafiltrate was determined by ultraperformance liquid chromatography (UPLC). EGCG was quantified using a calibration curve, which was prepared by analyzing five concentrations of EGCG standard (12.5–200 μg/mL) vs the peak area of the eluted peak. The linear regression equation (and the correlation coefficient) was $Y = (5 \times 10^{-5})X + 2.2799$ ($R^2 = 0.999$), where $X$ is the peak area and $Y$ is the concentration.

UPLC separation was performed with a Waters ACQUITY UPLC system (Waters, Milford, MA, USA) equipped with a binary solvent delivery manager, a column manager, a sample manager, and a photo array detector (PAD). The mobile phase A was formic acid/acetonitrile/water (0.5:3.0:96.5, v/v/v), and phase B was formic acid/acetonitrile/water (0.5:30.0:69.5, v/v/v). Gradient elution was carried out on a BEH-$C_{18}$ column (1.7 μm, 50 mm × 2.1 mm i.d., Waters, Milford, MA, USA) at a flow rate of 0.3 mL/min. The gradient program was as follows: 0–7 min, 5–50% B; 7–8 min, 50–5% B; 8–11 min, 5% B. The injection volume was 5 μL, the column was kept at 25 °C, and the wavelength was set at 280 nm. The entrapment efficiency of EGCG and the actual EGCG loading in β-Lg particles were calculated using the following equation:

$$\text{amount of entrapped EGCG} = \text{total amount of EGCG} - \text{amount of EGCG in ultrafiltrate} \quad (1)$$

$$\text{entrapment efficiency}(\%) = 100\frac{\text{amount of entrapped EGCG}}{\text{total amount of EGCG}} \quad (2)$$

$$\text{EGCG loading (nmol/mg)} = \frac{\text{amount of entrapped EGCG}}{\text{mass of β-Lg}} \quad (3)$$

**2.5. Turbidity Measurement.** The turbidity of solutions contained in a 10 mm path length quartz cuvette was measured using a Beckman Coulter DU800 spectrophotometer at 600 nm. Distilled water was used as control.[25]

**2.6. Fourier Transform Infrared (FTIR) Spectroscopy.** FTIR spectra of pure β-Lg and β-Lg–EGCG complexes were recorded using a Nicolet 380 FTIR spectrometer (Thermo, USA), equipped with a deuterated triglycine sulfate (DTGS) detector. The spectrometer was continuously purged with dried air. Two milligrams of freeze-dried sample was mixed with 100 mg of KBr and ground gently with an agate pestle and mortar under an infrared lamp and afterward was pressed into a 13-mm diameter disk by applying 15 tons of pressure for 2 min. FTIR spectra were obtained in the wavenumber range from 400 to 4000 $cm^{-1}$ during 100 scans, with 2 $cm^{-1}$ resolution.

For analysis of the secondary structure of β-Lg and β-Lg–EGCG complexes, the spectral region between 1600 and 1700 $cm^{-1}$ was selected. This region contains the amide-I absorption band of the peptide backbone. The FTIR spectra were smoothed with a 13-point Savitzky and Golay function, their baselines were corrected automatically, and Fourier self-deconvolution was performed with the software provided with the spectrometer (Omnic software) using a bandwidth of 18 $cm^{-1}$ and a line narrowing factor $k = 2$. Second derivative calculations and a multiple Gaussian curve-fitting analysis were performed to estimate the number and position of the component bands and the relative percentage of the secondary structural elements using PeakFit Version 4.12 software (SPSS Inc., Chicago, IL, USA).

**2.7. Antioxidant Activity of β-Lg–EGCG Nanoparticles Using the DPPH Assay.** The antioxidant activities of the free EGCG and β-Lg–EGCG nanoparticles were measured in vitro by DPPH assay according to the method reported by Kumari et al.[26] with slight modifications. Briefly, a stock solution of DPPH (0.1 mM) was prepared in absolute ethanol and 0.1 mL of sample solution was added in 2.9 mL of DPPH stock solution. The reaction mixture was incubated in dark at room temperature for 30 min, and the absorbance was measured at 517 nm. The control solution contained the same amount of buffer and DPPH radical. The radical scavenging activity was calculated using the following equation:

$$\text{scavenging activity}(\%) = 100(1 - A_{\text{sample,517nm}}/A_{\text{control,517nm}}) \quad (4)$$

**2.8. Ferric-Reducing Antioxidant Power (FRAP) Assay.** The reducing ability was determined by using the FRAP assay described by Shi et al.[27] The FRAP reagent was freshly prepared from 300 mmol/L acetate buffer (pH = 3.6), 10 mmol/L tripyridyltriazine (TPTZ) made up in 40 mmol/L HCl and 20 mmol/L $FeCl_{zzz3}$. All three solutions were mixed together in the ratio of 10:1:1 (v/v/v) before use. The tested sample solution (0.1 mL) was mixed with 3.0 mL of FRAP reagent, and the absorption of the reaction mixture was measured at 593 nm after 30 min of incubation at 37 °C.

In both antioxidant activity assays described above, β-Lg–EGCG complexes were prepared using 6.5 mM EGCG solution in the pH 2.5 buffer. The β-Lg–EGCG complex solution and free EGCG solution were kept at room temperature, and a little of sample solution was drawn out for DPPH and FRAP assays every 24 h.

**Table 1. Effects of pH on the Particle Size, Volume-Weighted Fraction of the Size Distribution (% volume), ζ-Potential, and Entrapment Efficiency of EGCG and EGCG Loading in β-Lg Particles (Heating Temperature, 70 °C; the Molar Ratio of β-Lg to EGCG, 1:2; β-Lg Concentration, 5 mg/mL)[b]**

| | particle size (nm) (% volume) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | β-Lg–EGCG complexes | | pure β-Lg | | ζ-potential (mV) | | | |
| pH value | peak I | peak II | peak I | peak II | β-Lg–EGCG complexes | pure β-Lg | entrapment efficiency (%) | EGCG loading (nmol/mg) |
| 2.5 | 3.60 ± 0.12 E (100.0 ± 0.0) | | 3.51 ± 0.01 D (100.0 ± 0.0) | | 17.0 ± 0.8 A | 18.9 ± 5.3 A | 31.94 ± 3.05 E | 34.50 ± 3.29 E |
| 5.0 | 404.5 ± 39.6 A (94.8 ± 2.5) | 5432 ± 195 A (5.2 ± 2.5) | 371.6 ± 16.6 A (92.7 ± 2.2) | 5073 ± 135 A (7.3 ± 2.2) | −12.9 ± 2.2 B | −11.8 ± 1.4 B | 54.95 ± 2.92 D | 59.34 ± 2.72 D |
| 5.5 | 349.6 ± 13.9[a] B (96.3 ± 1.4) | 5321 ± 206 A (3.7 ± 1.4) | 153.8 ± 9.9 B (96.1 ± 2.3) | 5193 ± 163 A (3.9 ± 2.3) | −22.2 ± 1.1 C | −20.6 ± 0.5 C | 54.02 ± 1.10 D | 58.34 ± 1.18 D |
| 6.0 | 19.12 ± 1.52[a] C (93.5 ± 2.6) | 3462 ± 103 B (6.5 ± 2.6) | 5.35 ± 0.16 C (100.0 ± 0.0) | | −24.4 ± 0.4 CD | −22.7 ± 1.7 CD | 59.23 ± 0.01 C | 63.97 ± 0.01 C |
| 6.2 | 7.55 ± 0.19[a] D (100.0 ± 0.0) | | 5.69 ± 0.02 C (100.0 ± 0.0) | | −27.1 ± 1.8 D | −24.0 ± 2.8 CD | 63.80 ± 0.40 B | 68.90 ± 0.42 B |
| 6.4 | 6.97 ± 0.20[a] D (100.0 ± 0.0) | | 5.34 ± 0.05 C (100.0 ± 0.0) | | −32.5 ± 1.2[a] E | −25.1 ± 2.8 CD | 71.82 ± 0.06 A | 77.57 ± 0.07 A |
| 6.6 | 7.15 ± 0.26[a] D (100.0 ± 0.0) | | 5.48 ± 0.08 C (100.0 ± 0.0) | | −31.1 ± 1.8[a] E | −24.2 ± 1.7 CD | 70.75 ± 0.08 A | 76.42 ± 0.09 A |
| 6.8 | 6.83 ± 0.04[a] D (100.0 ± 0.0) | | 5.80 ± 0.12 C (100.0 ± 0.0) | | −34.5 ± 2.9[a] E | −26.8 ± 2.0 D | 69.95 ± 1.73 A | 75.54 ± 1.87 A |
| 7.0 | 7.41 ± 0.30[a] D (100.0 ± 0.0) | | 6.11 ± 0.29 C (100.0 ± 0.0) | | −34.4 ± 3.2[a]E | −26.4 ± 2.9 D | 72.66 ± 1.19 A | 78.48 ± 1.29 A |

[a]Significantly different from corresponding pure β-Lg ($p < 0.05$). [b]Values in a column followed by different letters are significantly different ($p < 0.05$).

**2.9. Statistical Analysis.** In this study all samples were prepared and analyzed in triplicate. The data were presented as means ± standard deviations of three determinations. Statistical differences between two groups were evaluated using the Student's *t* test. Multiple comparisons of means were done by LSD (least significant difference) test. A probability value of <0.05 was considered significant. All computations were made by employing the SAS system for windows V8.

## 3. RESULTS AND DISCUSSION

**3.1. Effects of pH on the Characteristics of β-Lg–EGCG Complexes.** The effects of different pH values (2.5–7.0) on the particle size, ζ-potential, and entrapment efficiency of EGCG and EGCG loading in β-Lg particles were studied at the heating temperature 70 °C, the molar ratio 1:2 (β-Lg/EGCG), and the β-Lg concentration 5 mg/mL (0.27 mM) in the final mixture. The temperature 70 °C was chosen as the initial condition since β-Lg dimers at this temperature and neutral pH began to dissociate into monomers and the thiol group and hydrophobic residues were exposed.[28] A molar ratio of β-Lg/EGCG of 1:2 was used because considerable protection to EGCG against oxidative degradation at this ratio has been reported.[21]

The size of particles is important because it has direct relevance with the stability, cellular uptake, in vivo distribution, and drug release of nanomedicines.[29] As shown in Table 1, the particle sizes of β-Lg–EGCG complexes and pure β-Lg between pH 5.0 and 5.5 were significantly higher than that at pH 2.5 and pH 6.0–7.0 ($p < 0.05$). Most particle sizes (>90% volume) were around hundreds of nanometers, and the minor particle sizes (<10% volume) were around 5000 nm. The particles at pH 6.2–7.0 were about twice as large as that at pH 2.5, and their sizes were all less than 10 nm. No significant differences in the particle sizes between pH 6.2 and 7.0 were observed ($p < 0.05$). The particle sizes obtained at near-neutral pH in our study were close to those of β-Lg–EGCG complexes prepared using a similar method and measured with a different dynamic light scattering (DLS) analyzer in the previous study.[21] The particle sizes coincided with the solution turbidity, which was low at pH 2.5 and pH 6.0–7.0 and was high at pH 5.0–5.5 (Figure 1). It is known that β-Lg exists as a dimer at neutral pH and room temperature, dissociates into monomers at pH below 3, and forms octamers between pH 3.5 and 5.5.[13] Soluble aggregates of β-Lg were formed at neutral pH and pH 2.5 upon heating above 70 and 80 °C, respectively.[28] In the case of pH close to the isoelectric pH (pH 5.1) of the "native" protein dimer, large sedimenting protein particulates were formed after

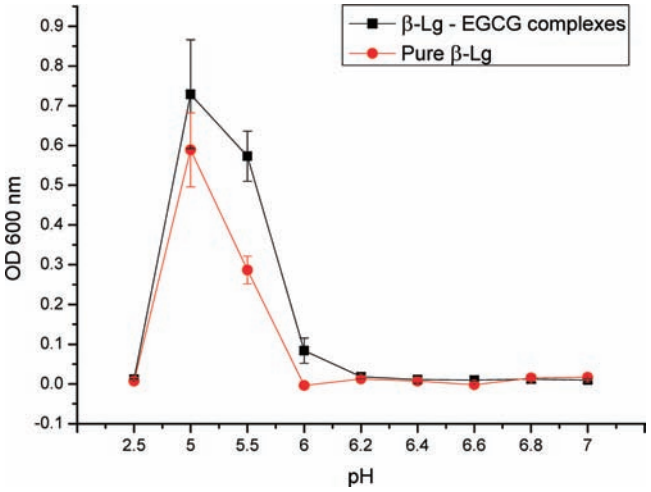


**Figure 1.** Turbidity of β-Lg–EGCG complexes and pure β-Lg solutions at different pH values. Error bars represent standard deviation (heating temperature, 70 °C; the molar ratio of β-Lg to EGCG, 1:2; β-Lg concentration, 5 mg/mL).

Table 2. Effects of Heating Temperature on the Particle Size, ζ-Potential, and Entrapment Efficiency of EGCG and EGCG Loading in β-Lg Particles (pH 7.0; Molar Ratio of β-Lg to EGCG, 1:2; β-Lg Concentration, 5 mg/mL)[b]

| | particle size (nm) | | ζ-potential (mV) | | | |
|---|---|---|---|---|---|---|
| temp (°C) | β-Lg−EGCG complexes | pure β-Lg | β-Lg−EGCG complexes | pure β-Lg | entrapment efficiency (%) | EGCG loading (nmol/mg) |
| 30 | 6.76 ± 0.02[a] C | 4.85 ± 0.08 D | −17.9 ± 1.4[a] A | −8.2 ± 0.5 A | 58.87 ± 2.20 C | 63.58 ± 2.37 C |
| 55 | 6.63 ± 0.16[a] C | 5.42 ± 0.07 C | −27.7 ± 1.1[a] B | −19.2 ± 1.4 B | 59.65 ± 0.61 C | 64.42 ± 0.65 C |
| 70 | 7.41 ± 0.30[a] B | 6.11 ± 0.29 B | −34.4 ± 3.2[a] C | −26.4 ± 2.9 C | 72.66 ± 1.19 B | 78.48 ± 1.29 B |
| 85 | 9.65 ± 0.10[a] A | 6.87 ± 0.18 A | −39.8 ± 1.8[a] D | −28.9 ± 1.0 C | 77.93 ± 0.46 A | 84.16 ± 0.50 A |

[a]Significantly different from corresponding pure β-Lg ($p < 0.05$). [b]Values in a column followed by different letters are significantly different ($p < 0.05$). For the measurement of size distribution, only one peak appeared in all cases and the volume-weighted fraction (% volume) was 100%.

heat treatment.[30,31] The present results are in agreement with these previous reports.

ζ-Potential is an important parameter to reflect the physicochemical and biological stabilities of nanoparticles in suspension. High ζ-potential helps the formulation repel each other, which ensures long-term stability and avoids particle aggregation.[32] As shown in Table 1, the ζ-potential of particles at pH 2.5 was around +20 mV, since the protein has more positive charges on its surface at low pH values. The charge became more negative as the pH increased, as would be expected when the pH moves above the isoelectric point of a protein. The addition of EGCG significantly increased negative charges on the particle surface at pH 6.4−7.0 ($p < 0.05$). In this pH range, the phenolic groups of EGCG can be deprotonated and the generated oxygen center imparts a high negative charge density, which further decreases the ζ-potential value of the complexes.[33] A minimum ζ-potential of ±30 mV is often necessary for good particle stability.[34] The nanoparticles system was stable at pH 6.4−7.0, since the ζ-potentials of β-Lg−EGCG nanoparticles were more than −30 mV.

Table 1 shows that the entrapment efficiency of EGCG increased from 31.94 to 71.82% as the pH value rose from 2.5 to 6.4, and was not significantly different between pH 6.4 and pH 7.0 ($p > 0.05$). This observation was in agreement with the previous reports that the affinity of EGCG or curcumin for β-Lg at neutral pH was greater than that at acidic pH.[17,22] EGCG loading per milligram of proteins was more than 75 nmol/mg between pH 6.4 and 7.0. At acidic pH, β-Lg exists in a closed conformation wherein the hydrophobic cavity is not accessible to ligand binding. At neutral pH, β-Lg has an open conformation, allowing ligands to bind at the hydrophobic cavity.[35] Overall, considering the particle size, solution turbidity, ζ-potential, and entrapment efficiency of EGCG, the β-Lg−EGCG nanoparticles should be prepared at pH 6.4−7.0. It was found that the pH value of tea drinks varied with brands, ranging from 3.3 to 6.5.[6] The pH of the biscuit dough is more on the alkaline side compared with that of the bread dough in the range 5−6.[36] Green tea polyphenols can be used as a cosmetic composition.[37] However, the pH of cosmetic products is almost neutral, since a suitable pH for human skin is considered to be 6.5.[38] Our results indicated heat treated β-Lg could be used as protective nanovehicles for the delivery of EGCG in soft drinks, food, and other possible items with a near neutral pH.

**3.2. Effects of Temperature on the Characteristics of β-Lg−EGCG Complexes.** Temperature is taken as a significant factor influencing the conformational changes of β-Lg.[14] The effects of different heating temperatures (30−85 °C) on the particle size, ζ-potential, and entrapment efficiency of EGCG and EGCG loading in β-Lg particles were studied at pH 7.0, molar ratio of 1:2 (β-Lg/EGCG), and β-Lg concentration of 5 mg/mL (0.27 mM) in the final mixture. As shown in Table 2, the particle size, negative charge, entrapment efficiency, and EGCG loading all increased with the rise of heating temperature from 30 to 85 °C. Higher ζ-potentials (−34.4 to −39.8 mV), entrapment efficiency (72.66−77.93%), and EGCG loading (78.48−84.16 nmol/mg) were obtained when the heat temperature was above 70 °C. All the sample solutions were clear, and their $OD_{600}$ values were less than 0.05 (data not shown). Our results were consistent with the previous report that the association constant for binding of EGCG to β-Lg heated at 75−85 °C was higher than that with the native protein.[21] In native β-Lg the free thiol group and the disulfide bridges are inaccessible to solvent, since they are in a hydrophobic cleft between two β-strands and are covered by the α-helix.[39] Upon heating to approximately 70 °C, β-Lg dimers at neutral pH dissociate into monomers and the thiol group and hydrophobic residues become solvent accessible. Aggregates may then be formed via thiol-disulfide exchange, and to a lesser extent thiol−thiol oxidation and noncovalent interactions.[29] The accessibility of reactive thiol groups and adhesive hydrophobic surfaces may increase the interactions between β-Lg and EGCG.[21] van der Waals interactions and hydrogen bonding probably played major roles in the binding interactions.[40] Higher heating temperature may result in larger aggregates and more accessible binding sites; thus, larger particle sizes and higher entrapment efficiency of EGCG were obtained in this study. More EGCG binding with β-Lg decreased the ζ-potentials due to the negative charge offered by the oxygen center of the phenolic groups.[33]

**3.3. Effects of the Molar Ratio of β-Lg to EGCG on the Characteristics of β-Lg−EGCG Complexes.** The effects of different molar ratios of β-Lg to EGCG on the particle size, ζ-potential, and entrapment efficiency of EGCG and EGCG loading in β-Lg particles were studied at pH 7.0, heating temperature of 70 °C, and β-Lg concentration of 5 mg/mL (0.27 mM). Table 3 shows the particle sizes and negative ζ-potential values increased sharply as the molar ratio of β-Lg to EGCG decreased from 1:0 to 1:32 ($p < 0.05$), indicating the amount of EGCG had an important influence on the characteristics of β-Lg−EGCG complexes. The entrapment efficiency of EGCG decreased and EGCG loading increased with the rise of the molar ratio of β-Lg to EGCG, respectively. All the sample solutions were transparent with $OD_{600}$ values less than 0.05 (data not shown). It is known that, at low polyphenol concentration, polyphenols associate with the proteins to form small soluble complexes, and with increasing polyphenol concentration, each polyphenol-coated protein starts to cross-link with other soluble complexes, where the cooperative weak intermolecular bridging interactions are also carried out by the polyphenols. This leads to the formation of bigger particles and entrapment of more polyphenols,[41] which further decreases the

Table 3. Effects of the Molar Ratio of β-Lg to EGCG on the Particle Size, ζ-Potential, and Entrapment Efficiency of EGCG and EGCG Loading in β-Lg Particles (pH 7.0; Heating Temperature,70 °C; β-Lg Concentration, 5 mg/mL)[a]

| β-Lg/EGCG (molar ratio) | particle size (nm) | ζ-potential (mV) | entrapment efficiency (%) | EGCG loading (nmol/mg) |
|---|---|---|---|---|
| 1:0 (pure β-Lg) | 6.11 ± 0.29 E | −26.4 ± 2.9 A | | |
| 1:2 | 7.41 ± 0.30 D | −34.4 ± 3.2 B | 72.66 ± 1.19 A | 78.48 ± 1.29 E |
| 1:4 | 9.88 ± 0.10 C | −36.6 ± 1.0 B | 71.23 ± 0.15 A | 153.86 ± 0.33 D |
| 1:8 | 17.60 ± 0.08 B | −43.3 ± 1.5 C | 70.33 ± 0.74 A | 303.83 ± 3.20 C |
| 1:16 | 18.63 ± 0.60 B | −49.1 ± 2.0 D | 63.59 ± 0.33 C | 549.38 ± 2.87 B |
| 1:32 | 31.27 ± 0.14 A | −51.9 ± 1.8 E | 51.08 ± 1.74 B | 882.59 ± 29.99A |

[a]Values in a column followed by different letters are significantly different ($p < 0.05$). For the measurement of size distribution, only one peak appeared in all cases and the volume-weighted fraction (% volume) was 100%.

Table 4. Effects of β-Lg Concentration on the Particle Size, ζ-Potential, and Entrapment Efficiency of EGCG and EGCG Loading in β-Lg Particles (pH 7.0; Heating Temperature, 70 °C; Molar Ratio of β-Lg to EGCG, 1:2)[b]

| | particle size (nm) | | ζ-potential (mV) | | | |
|---|---|---|---|---|---|---|
| conc of β-Lg (mg/mL) | β-Lg−EGCG complexes | pure β-Lg | β-Lg−EGCG complexes | pure β-Lg | entrapment efficiency (%) | EGCG loading (nmol/mg) |
| 1.0 | 5.80 ± 0.38 C | 5.32 ± 0.08 C | −31.0 ± 2.1 A | −27.0 ± 2.5 A | 58.20 ± 1.37 C | 62.86 ± 1.48 D |
| 2.5 | 6.37 ± 0.34 C | 5.83 ± 0.41 C | −32.1 ± 3.1 A | −28.1 ± 3.7 A | 61.28 ± 1.64 C | 66.18 ± 1.77 C |
| 5.0 | 7.41 ± 0.30[a]B | 6.87 ± 0.18 A | −34.4 ± 3.2[a] A | −26.4 ± 2.9 A | 72.66 ± 1.19 B | 78.48 ± 1.29 B |
| 10.0 | 8.85 ± 0.61[a] A | 7.13 ± 0.43 A | −35.5 ± 2.4[a] A | −26.1 ± 0.5 A | 89.70 ± 0.96 A | 96.88 ± 1.04 A |

[a]Significantly different from corresponding pure β-Lg ($p < 0.05$). [b]Values in a column followed by different letters are significantly different ($p < 0.05$). For the measurement of size distribution, only one peak appeared in all cases and the volume-weighted fraction (% volume) was 100%.

ζ-potential value of the complexes due to the negative charge offered by EGCG.[33]

**3.4. Effects of β-Lg Concentration on the Characteristics of β-Lg−EGCG Complexes.** The effects of different β-Lg concentrations (1−10 mg/mL) on the particle size, ζ-potential, and entrapment efficiency of EGCG and EGCG loading in β-Lg particles were studied at pH 7.0, heating temperature of 70 °C, and molar ratio of 1:2 (β-Lg/EGCG). As shown in Table 4, the particle size, entrapment efficiency, and EGCG loading all increased with the rise of β-Lg concentration. The ζ-potentials of particles at various β-Lg concentrations were similar ($p > 0.05$). These results indicated the β-Lg concentration had little influence on the system stability and had a positive effect on the utilization rate of proteins within the experimental range.

**3.5. Antioxidant Activity of β-Lg−EGCG Nanoparticles.** DPPH assay and FRAP assay were used to evaluate the influence of complexing with heat treated β-lactoglobulin on the antioxidant activity of EGCG. DPPH radical is a stable free radical that is commonly used as a substrate to evaluate antioxidant activity. The antioxidant can scavenge the radical by hydrogen donation, which caused the decrease of DPPH absorbance at 517 nm. In FRAP assay, the presence of reducers in the test solution results in the increase of absorbance at 593 nm, owing to the formation of a blue colored $Fe^{2+}$−tripyridyltriazine compound from the colorless oxidized $Fe^{3+}$ form. Figure 2 displays the change over time of DPPH radical scavenging capacity and the reducing power of β-Lg−EGCG complexes prepared at various temperatures, pH 7.0, and the molar ratio 1:2 (β-Lg/EGCG) compared to free EGCG, respectively. The two assays both indicated pure β-Lg had little antioxidant activity compared with β-Lg−EGCG complexes, and the antioxidant activity of the latter decreased more slowly than that of free EGCG at neutral pH and room temperature (25−30 °C). This was in agreement with the previous report that complexing with native or heat denatured and then cooled β-Lg provided preservation of EGCG antioxidant activity.[22] Furthermore, our results showed greater protection of EGCG antioxidant activity could be obtained with β-Lg heated at higher temperature within the range investigated. This confirmed the result in section 3.2 that higher heating temperature of β-Lg resulted in greater entrapment efficiency of EGCG. In addition, aggregation of β-Lg at higher temperature may provide a better shield to protect EGCG from adverse environmental conditions.

Figure 3 shows the change over time of DPPH radical scavenging capacity and the reducing power of β-Lg−EGCG complexes prepared at various molar ratios of β-Lg to EGCG, pH 7.0, and heating temperature of 85 °C compared to free EGCG, respectively. A better protective effect on EGCG antioxidant activity was observed at higher molar ratio of β-Lg to EGCG. The protective effects were not obvious at molar ratios of 1:16 and 1:32. Protein−polyphenol complexes are formed by multiple interactions. At higher molar ratio of β-Lg to EGCG, hydrogen bonds between EGCG and heat treated β-Lg play an important role in reinforcing and stabilizing the complexes.[21] As EGCG concentration increased, less binding sites on β-Lg were accessible and hydrophobic interaction plays a major role in the binding interactions, indicating that the association of EGCG with β-Lg is principally a surface phenomenon, and so the complexes are unstable.[41]

**3.6. FT-IR Studies.** The amide I band, between 1600 and 1700 $cm^{-1}$, is the most useful for infrared spectroscopic analysis of the secondary structure of proteins.[42] Supporting Information Figure 1 shows the FT-IR spectra of β-Lg−EGCG complexes (molar ratio of 2:1) and pure β-Lg after various heat treatments at pH 7.0. Supporting Information Figure 2 shows the amide I spectrum (1600−1700 $cm^{-1}$), fitted band components, and the second derivative spectrum of samples. Ten major bands associated with the conformation of proteins were distinctly observed in the amide I region. These bands, mainly attributed to the C═O stretching vibration and to a small extent to the C−N stretching vibration of the peptide bonds, were assigned to different secondary structure elements

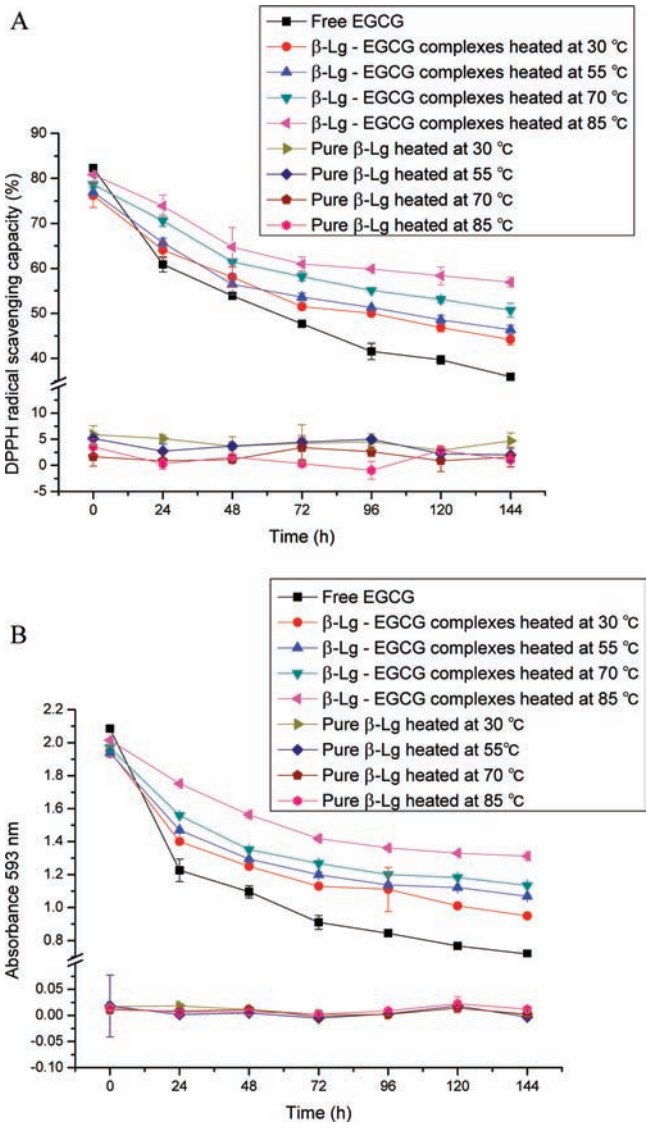


**Figure 2.** Antioxidant activities of $\beta$-Lg-EGCG complexes and pure $\beta$-Lg prepared at various heating temperatures and free EGCG as assessed by DPPH assay. (A) DPPH assay; (B) FRAP assay. Error bars represent standard deviation (pH 7.0; molar ratio of $\beta$-Lg to EGCG, 1:2).

according to the previous reports:[43−45] bands at 1621.4, 1628.6, and 1639.4 were assigned to $\beta$-sheets, at 1646.6 to random coils, at 1657.4 to the $\alpha$-helix, at 1664.6 to turns, and at 1675.5, 1686.3, and 1693.5 to antiparallel $\beta$-sheets. A smaller component arises at 1610.6, assigned to side-chain vibrations, which has no contribution to the secondary structure. These bands were clearly identified by the valleys in the second derivative. Table 5 shows the proportions of different secondary structure elements of free $\beta$-Lg and $\beta$-Lg−EGCG complexes prepared at representative temperatures. Although an occasional significant difference ($p < 0.05$) in the proportions of the secondary structure elements of the two samples was observed, it was considered that all the samples had similar secondary structure, since these differences were independent of treatment. The secondary structure of samples was composed of ~55% of $\beta$-strands ($\beta$-sheet and antiparallel $\beta$-sheet), ~16% of $\alpha$-helix, ~15% of random coils, and ~12% of turns. These data are consistent with spectroscopic studies of $\beta$-Lg previously reported.[46,47]

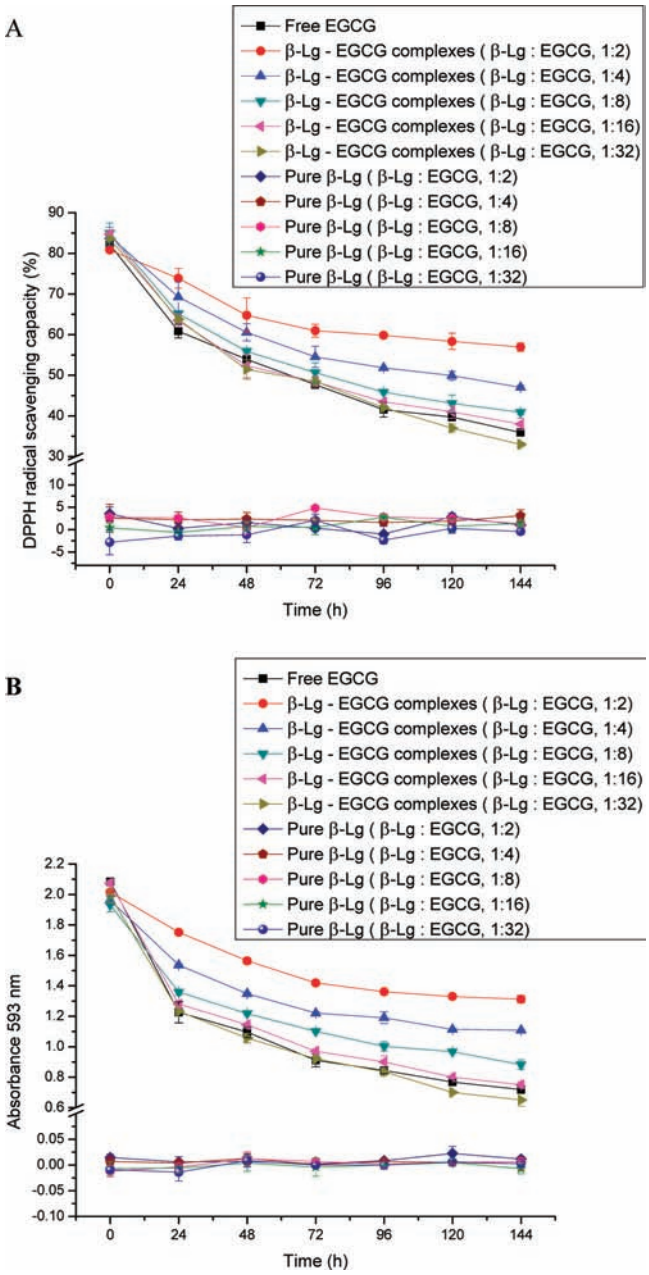


**Figure 3.** Antioxidant activities of $\beta$-Lg-EGCG complexes and pure $\beta$-Lg prepared at various molar ratios of $\beta$-Lg to EGCG and free EGCG as assessed by DPPH assay: (A) DPPH assay; (B) FRAP assay. Error bars represent standard deviation (pH 7.0; heating temperature, 85 °C).

It has been reported that all the $\alpha$-helixes and up to a fifth of the $\beta$-sheets have been lost during heating between 60 and 70 °C.[46] However, our results showed heat treatment up to 85 °C for 20 min, and the addition of EGCG did not cause any significant alteration in the secondary structure of $\beta$-Lg. The heat-induced conformational change of $\beta$-Lg is reversible, and the level is dependent on protein concentration, pH, and screening effects of surface charges by ionic salts and also on the temperature and length of exposure.[48] Previous studies on globular proteins from Soya showed that thermally unfolded proteins refold during the cooling step.[49] In our work, the fast cooling step after the mixture of EGCG and heat treated $\beta$-Lg may reverse the change of secondary structure. However, the


dx.doi.org/10.1021/jf300307t | J. Agric. Food Chem. 2012, 60, 3477−3484

Table 5. Proportions (%) of Different Secondary Structure Elements of β-Lg–EGCG Complexes and Pure β-Lg Prepared at Representative Temperatures (pH 7.0; Molar Ratio of β-Lg to EGCG, 1:2; β-Lg Concentration, 10 mg/mL)[a]

| | | preheating temperature (°C) | | | |
|---|---|---|---|---|---|
| | secondary structure | 30 | 55 | 70 | 85 |
| β-Lg–EGCG complex | α-helix (1650–1660 $cm^{-1}$) | 16.83 ± 0.37 A | 16.36 ± 0.42 A | 16.12 ± 1.39 A | 16.28 ± 0.99 A |
| | β-sheet (1620–1640 $cm^{-1}$) | 36.04 ± 0.41 B | 33.14 ± 2.55 B | 38.13 ± 0.46 A | 38.13 ± 0.46 A |
| | turn (1660–1670 $cm^{-1}$) | 12.63 ± 0.72 AB | 13.43 ± 1.22 A | 11.62 ± 0.33 B | 11.86 ± 0.82 AB |
| | random coil (1640–1650 $cm^{-1}$) | 16.12 ± 0.49 A | 15.98 ± 0.74 A | 15.58 ± 0.91 A | 15.38 ± 0.97 A |
| | antiparallel β-sheet (1670–1695 $cm^{-1}$) | 16.76 ± 1.32 A | 19.44 ± 1.82 A | 16.53 ± 0.83 A | 16.53 ± 0.95 A |
| pure β-Lg | α-helix (1650–1660 $cm^{-1}$) | 16.20 ± 0.93 AB | 17.42 ± 0.53 A | 15.27 ± 0.69 B | 16.36 ± 1.77 AB |
| | β-sheet (1620–1640 $cm^{-1}$) | 36.22 ± 1.54 A | 38.69 ± 2.03 A | 37.97 ± 0.85 A | 38.98 ± 1.37 A |
| | turn (1660–1670 $cm^{-1}$) | 13.07 ± 1.20 A | 10.55 ± 0.99 A | 12.59 ± 1.37 A | 11.04 ± 2.61 A |
| | random coil (1640–1650 $cm^{-1}$) | 16.79 ± 0.74 A | 14.70 ± 1.32 A | 16.26 ± 0.94 A | 15.02 ± 1.97 A |
| | antiparallel β-sheet (1670–1695 $cm^{-1}$) | 16.19 ± 0.57 A | 17.13 ± 0.64 A | 16.00 ± 1.52 A | 16.32 ± 0.83 A |

[a]Values in a row followed by different letters are significantly different ($p < 0.05$).

tertiary structure of heat treated β-Lg would not be restored by the cooling step, since the larger size of β-Lg–EGCG particles due to aggregation at higher temperature was observed.

In conclusion, β-Lg–EGCG nanoparticles were successfully prepared for preservation of EGCG antioxidant activity. Four factors (pH, heating temperature of β-Lg, molar ratio of β-Lg to EGCG, and β-Lg concentration) significantly influence the particle size, ζ-potential, entrapment efficiency of EGCG, and EGCG loading in β-Lg particles. A stable and clear solution system could be obtained at pH 6.4–7.0. The best protection for EGCG antioxidant activity was obtained with β-Lg heated at 85 °C and the molar ratio of 1:2 (β-Lg/EGCG). β-Lg–EGCG complexes had the same secondary structure as native β-Lg. The fast cooling step may reverse the change of secondary structure induced by heat. These results were helpful to the utilization of β-Lg as a protective vehicle for EGCG and other polyphenols in food and drinks.

## ■ ASSOCIATED CONTENT

### Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION


### Corresponding Author

*Tel/fax: +86 571 88071024 8575. E-mail: qizhendu@126.com.



### Funding

This research was supported by the National Key Technology R&D Program (2012BAD36B06) and the Specialized Research Fund for the Doctoral Program of Higher Education (20113326110001), China.


### Notes

The authors declare no competing financial interest.

## ■ REFERENCES


(1) Yilmaz, Y. Novel uses of catechins in foods. *Trends Food Sci. Technol.* **2006**, *17*, 64–71.

(2) Nagle, D. G.; Ferreira, D.; Zhou, Y. D. Epigallocatechin-3-gallate (EGCG): Chemical and biomedical perspectives. *Phytochemistry* **2006**, *67*, 1849–1855.

(3) Higdon, J. V.; Frei, B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit. Rev. Food Sci. Nutr.* **2003**, *43*, 89–143.

(4) Khan, N.; Mukhtar, H. Tea polyphenols for health promotion. *Life Sci.* **2007**, *81*, 519–533.

(5) Kumudavally, K. V.; Phanindrakumar, H. S.; Tabassum, A.; Radhakrishna, K.; Bawa, A. S. Green tea—A potential preservative for extending the shelf life of fresh mutton at ambient temperature (25 ± 2 °C). *Food Chem.* **2008**, *107*, 426–433.

(6) Su, Y. L.; Leung, L. K.; Huang, Y.; Chen, Z. Y. Stability of tea theaflavins and catechins. *Food Chem.* **2003**, *83*, 189–195.

(7) Wang, R.; Zhou, W.; Wen, R. H. Kinetic study of the thermal stability of tea catechins in aqueous system using a microwave reactor. *J. Agric. Food Chem.* **2006**, *54*, 5924–5932.

(8) Mizooku, Y.; Yoshikawa, M.; Tsuneyoshi, T.; Arakawa, R. Analysis of oxidized epigallocatechin gallate by liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1915–1918.

(9) Tanaka, T.; Kounok, I. Oxidation of tea catechins: Chemical structures and reaction mechanism. *Food Sci. Technol. Res.* **2003**, *9*, 128–133.

(10) Fang, Z.; Bhandari, B. Encapsulation of polyphenols—a review. *Trends Food Sci. Technol.* **2010**, *21*, 510–523.

(11) Edwards, P. J. B.; Jameson, G. B.; Palmano, K. P.; Creamer, L. K. Heat-resistant structural features of bovine β-lactoglobulin A revealed by NMR H/D exchange observations. *Int. Dairy J.* **2002**, *12*, 331–344.

(12) Ponniah, K.; Loo, T. S.; Edwards, P. J. B.; Pascal, S. M.; Jameson, G. B.; Norris, G. E. The production of soluble and correctly folded recombinant bovine β-lactoglobulin variants A and B in *Escherichia coli* for NMR studies. *Protein Expression Purif.* **2010**, *70*, 283–289.

(13) Sawyer, L.; Kontopidis, G. The core lipocalin, bovine β-lactoglobulin. *Biochim. Biophys. Acta* **2000**, *1482*, 136–148.

(14) de Wit, J. N. Thermal behaviour of bovine β-lactoglobulin at temperatures up to 150 °C. A review. *Trends Food Sci. Technol.* **2009**, *20*, 27–34.

(15) Livney, Y. D. Milk proteins as vehicles for bioactives. *Curr. Opin. Colloid Interface Sci.* **2010**, *15*, 73–83.

(16) Liang, L.; Tajmir-Riahi, H. A.; Subirade, M. Interaction of beta-lactoglobulin with resveratrol and its biological implications. *Biomacromolecules* **2008**, *9*, 50–56.

(17) Sneharani, A. H.; Karakkat, J. V.; Singh, S. A.; Rao, A. G. A. Interaction of curcumin with β-lactoglobulin stability, spectroscopic analysis, and molecular modeling of the complex. *J. Agric. Food Chem.* **2010**, *58*, 11130–11139.

(18) Zimet, P.; Livney, Y. D. Beta-lactoglobulin and its nanocomplexes with pectin as vehicles for [omega]-3 polyunsaturated fatty acids. *Food Hydrocolloids* **2009**, *23*, 1120–1126.

(19) Riihimäki, L. H.; Vainio, M. J.; Heikura, J. M. S.; Valkonen, K. H.; Virtanen, V. T.; Vuorela, P. M. Binding of phenolic compounds and their derivatives to bovine and reindeer β-lactoglobulin. *J. Agric. Food Chem.* **2008**, *56*, 7721–7729.

(20) Tavel, L.; Moreau, C.; Bouhallab, S.; Li-Chan, E. C. Y.; Guichard, E. Interactions between aroma compounds and β-lactoglobulin in the heat-induced molten globule state. *Food Chem.* **2010**, *119*, 1550–1556.

(21) Shpigelman, A.; Israeli, G.; Livney, Y. D. Thermally-induced protein–polyphenol co-assemblies: beta lactoglobulin-based nanocomplexes as protective nanovehicles for EGCG. *Food Hydrocolloids* **2010**, *24*, 735–743.
(22) Zorilla, R.; Liang, L.; Remondetto, G.; Subirade, M. Interaction of epigallocatechin-3-gallate with β-lactoglobulin: molecular characterization and biological implication. *Dairy Sci. Technol.* **2011**, *91*, 629–644.
(23) Long, T.; Ramsburg, C. A. Encapsulation of nZVI particles using a Gum Arabic stabilized oil-in-water emulsion. *J. Hazard. Mater.* **2011**, *189*, 801–808.
(24) Hu, B.; Pan, C.; Sun, Y.; Hou, Z.; Ye, H.; Hu, B.; Zeng, X. Optimization of fabrication parameters to produce chitosan–tripolyphosphate nanoparticles for delivery of tea catechins. *J. Agric. Food Chem.* **2008**, *56*, 7451–7458.
(25) Yong, Y. H.; Foegeding, E. A. Effects of caseins on thermal stability of bovine-lactoglobulin. *J. Agric. Food Chem.* **2008**, *56*, 10352–10358.
(26) Kumari, A.; Yadav, S. K.; Pakade, Y. B.; Singh, B.; Yadav, S. C. Development of biodegradable nanoparticles for delivery of quercetin. *Colloids Surf., B: Biointerfaces* **2010**, *80*, 184–192.
(27) Shi, J.; Gong, J.; Liu, J.; Wu, X.; Zhang, Y. Antioxidant capacity of extract from edible flowers of *Prunus mume* in China and its active components. *LWT—Food Sci. Technol.* **2009**, *42*, 477–482.
(28) Schokker, E. P.; Singh, H.; Pinder, D. N.; Creamer, L. K. Heat-induced aggregation of β-lactoglobulin AB at pH 2.5 as influenced by ionic strength and protein concentration. *Int. Dairy J.* **2000**, *10*, 233–240.
(29) Kumari, A.; Yadav, S. K.; Pakade, Y. B.; Kumar, V.; Singh, B.; Chaudhary, A.; Yadav, S. C. Nanoencapsulation and characterization of *Albizia chinensis* isolated antioxidant quercitrin on PLA nanoparticles. *Colloid Surf., B: Biointerfaces* **2011**, *82*, 224–232.
(30) Schmitt, C.; Bovay, C.; Vuilliomenet, A. M.; Rouvet, M.; Bovetto, L.; Barbar, R.; Sanchez, C. Multiscale characterization of individualized β-lactoglobulin microgels formed upon heat treatment under narrow pH range conditions. *Langmuir* **2009**, *25*, 7899–7909.
(31) Jung, J. M.; Savin, G.; Pouzot, M.; Schmitt, C.; Mezzenga, R. Structure of heat-induced β-lactoglobulin aggregates and their complexes with sodium-dodecyl sulfate. *Biomacromolecules* **2008**, *9*, 2477–2486.
(32) Mohanty, C.; Sahoo, S. K. The in vitro stability and in vivo pharmacokinetics of curcumin prepared as an aqueous nanoparticulate formulation. *Biomaterials* **2010**, *31*, 6597–6611.
(33) Staszewski, M.; Jagus, R. J.; Pilosof, A. M. R. Influence of green tea polyphenols on the colloidal stability and gelation of WPC. *Food Hydrocolloids* **2011**, *25*, 1077–1084.
(34) Müller, R. H.; Jacobs, C.; Kayser, O. Nanosuspensions as particulate drug formulations in therapy rationale for development and what we can expect for the future. *Adv. Drug Delivery Rev.* **2001**, *47*, 3–19.
(35) Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, H. M.; Baker, E. N.; Jameson, G. B. Structural basis of the Tanford transition of bovine β-lactoglobulin. *Biochemistry* **1998**, *37*, 14014–14023.
(36) Sharma, A.; Zhou, W. A stability study of green tea catechins during the biscuit making process. *Food Chem.* **2011**, *126*, 568–573.
(37) Byun, M. W.; Jo, C.; Lee, J. W.; Jo, S. K.; Kim, K. S. Application of radiation technology to develop green tea leaf as a natural resource for the cosmetic industry. *Radiat. Phys. Chem.* **2004**, *71*, 485–487.
(38) Hans, R. K.; Agrawal, N.; Verma, K.; Misra, R. B.; Ray, R. S.; Farooq, M. Assessment of the phototoxic potential of cosmetic products. *Food Chem. Toxicol.* **2008**, *46*, 1653–1658.
(39) Sawyer, L. β-Lactoglobulin. In *Advanced Dairy Chemistry: Vol. 1: Proteins, Parts A*, 3rd ed.; Fox, P. F., McSweeney, P., Eds.; Kluwer Academic/Plenum Publishers: New York, 2003; Vol. *1*, pp 319–386.
(40) Wu, X.; Wu, H.; Liu, M.; Liu, Z.; Xu, H.; Lai, F. Analysis of binding interaction between (−)-epigallocatechin (EGC) and β-lactoglobulin by multi-spectroscopic method. *Spectrochim. Acta, Part A: Molec. Biomolec. Spectrosc.* **2011**, *82*, 164–168.
(41) Staszewski, M.; Jagus, R. J.; Pilosof, A. M. R. Influence of green tea polyphenols on the colloidal stability and gelation of WPC. *Food Hydrocolloids* **2011**, *25*, 1077–1084.
(42) Muyonga, J. H.; Cole, C. G. B.; Duodu, K. G. Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (*Lates niloticus*). *Food Chem.* **2004**, *86*, 325–332.
(43) Byler, D. M.; Susi, H. Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers* **1986**, *25*, 469–487.
(44) Lefèvre, T.; Subirade, M. Interaction of β-lactoglobulin with phospholipid bilayers: a molecular level elucidation as revealed by infrared spectroscopy. *Int. J. Biol. Macromol.* **2000**, *28*, 59–67.
(45) Xie, J.; Huang, H. Time-dependent adsorption behavior of β-lactoglobulin on ZnSe crystal surface studied by 2D correlation ATR/FTIR spectroscopy. *Colloids Surf., B: Biointerfaces* **2011**, *85*, 97–102.
(46) Qi, X. L.; Holt, C.; Mcnulty, D.; Clarke, D. T.; Brownlow, S.; Jones, G. R. Effect of temperature on the secondary structure of β-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: a test of the molten globule hypothesis. *Biochem. J.* **1997**, *324*, 341–346.
(47) Vetri, V.; Militello, V. Thermal induced conformational changes involved in the aggregation pathways of beta-lactoglobulin. *Biophys. Chem.* **2005**, *113*, 83–91.
(48) Relkin, P. Reversibility of heat-induced conformational changes and surface exposed hydrophobic clusters of β-lactoglobulin: their role in heat-induced sol–gel state transition. *Int. J. Biol. Macromol.* **1998**, *22*, 59–66.
(49) Damodaran, S. Refolding of thermally unfolded soy proteins during the cooling regime of the gelation process: effect on gelation. *J. Agric. Food Chem.* **1988**, *36*, 262–269.