Synthesis and controlled-release properties of chitosan/ β -Lactoglobulin nanoparticles as carriers for oral administration of epigallocatechin gallate

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Abstract A nano-sized double-walled carrier composed of chitosan and β -lactoglobulin (β -Lg) for oral administration of epigallocatechin gallate (EGCG) was developed to achieve a prolonged release of EGCG in the gastrointestinal tract. Carboxymethyl chitosan (CMC) solution was added dropwise to chitosan hydrochloride (CHC) containing EGCG to form a primary coating by ionic complexation. Subsequently, β -Lg was added to create a secondary layer by ionic gelation. The obtained EGCG-loaded chitosan/ β -Lg nanoparticles had sizes between 100 and 500 nm and zeta potentials ranging from 10 to 35 mV. FT-IR spectroscopy revealed a high number of hydrogen-bonding sites in the nanoparticles, which could incorporate EGCG, resulting in high encapsulation efficiency. EGCG incorporated in the primary coating was released slowly over time by diffusion from the swollen CMC-CHC matrix after the outer layer of β -Lg was degraded in the intestinal fluid. The sustained-release property makes chitosan/ β -Lg nanoparticles an attractive candidate for effective delivery of EGCG.

Keywords: epigallocatechin gallate, chitosan, β -lactoglobulin, double wall formation, controlled release

Introduction

Epigallocatechin gallate (EGCG) is the major catechin component in green tea, with strong antioxidant, antitumor, and other biological activities (1); EGCG has received extensive attention in the functional foods field because of its health benefits. However, the bioavailability of EGCG after oral administration is low because of its poor stability in the gastrointestinal (GI) tract. Thus, a suitable carrier is needed to enhance the stability and intestinal bioavailability of EGCG (2). Some bioactives such as tea polyphenols have to be released in the GI tract slowly over time in order to enhance their bioavailability. Recently, sustained-release nano-scale carriers have contributed to the improved stability, cellular uptake, and prolonged clearance time of the enclosed bioactives (3).

Chitosan has been extensively used as a sustained-release carrier for delivery of bioactives, such as catechins, because of its mucoadhesive property in the GI tract (4). Chitosan-based carriers can adhere to the intestinal membrane, and therefore, prolong residence time in the GI tract (2). However, the poor solubility of chitosan at neutral pH conditions and its low delivery efficiency for several orally administered bioactives have limited its applications in the food industry. Therefore, it is necessary to design a new type of

chitosan-based sustained-release nano-scale carrier with enhanced aqueous solubility and resistance to the harsh conditions of the GI tract. We recently developed chitosan nanoparticles by using two different water-soluble chitosans, carboxymethyl chitosan (CMC) and chitosan hydrochloride (CHC), to improve the bioavailability of tea polyphenols (5,6). These chitosans form nano-sized particles by ionic gelation between carboxyl methyl and amino groups of CMC and CHC, respectively. The water-soluble CMC-CHC nanoparticles are suitable for the sustained release of their core material (7). However, compared with some complex nanoparticles prepared by using CMC or CHC materials, the orally administered CMC-CHC nanoparticles were not stable at low pH in the gastric fluid and underwent rapid acid-induced dissociation and degradation, which could lead to a quick release of their core materials in the stomach (8).

In this study, we hypothesized that the limitations of CMC-CHC nanoparticles can be overcome by an additional protective coating against the acidic conditions of the gastric fluid. Therefore, we designed a novel EGCG nano-carrier with a secondary coating resistant to low pH, layered on the primary EGCG-loaded CMC-CHC nanoparticles, to provide additional protection against the acidic gastric environment. This secondary coating is removed in the intestinal fluid, and subsequently, the exposed EGCG-loaded CMC-

CHC nanoparticles with mucoadhesive properties can achieve a prolonged release of EGCG in the intestinal tract. Recently published studies have shown that double-walled chitosan nanoparticles coated with several food proteins or bioactive peptides, such as soy protein (9), caseinophosphopeptides (10), could prevent rapid degradation of bioactives in GI conditions and improve their gastric stability. Therefore, our second hypothesis was that a biopolymer for the secondary coating of the primary EGCG-loaded CMC-CHC nanoparticles would be resistant to peptic digestion and acidic hydrolysis in the stomach, but would still be enzymatically degradable in the small intestine.

Beta-lactoglobulin (β -Lg), comprising 50 to 60% of the total whey protein in cow milk, is resistant to degradation by pepsin hydrolysis in the stomach (11). Another important property of β -Lg is its ability to form stable structures with chitosan by ionic gelation (12). We previously reported the physicochemical and prolonged release properties of chitosan/ β -Lg microcapsules, formed through a simple ionic gelation process with chitosan and denatured β -Lg (12). Other studies suggested that chitosan/ β -Lg nanoparticles can be used as carriers for oral administration of nutraceuticals such as quercetin and vitamin D (9,13).

The main objective of this study was to develop EGCG-loaded chitosan/ β -Lg nanoparticles as a novel carrier to achieve a prolonged release of orally administered EGCG in the GI tract. Furthermore, the physicochemical properties and prolonged release performance of EGCG-loaded chitosan/ β -Lg nanoparticles were investigated during simulated GI digestion. Finally, we propose formation and release mechanisms for the EGCG-loaded chitosan/ β -Lg nanoparticles.

Materials and Methods

Materials EGCG (purity >95%) was provided by Hefei Shangu Chemical Reagent Company (Hefei, Anhui, China). Two types of chitosan materials, N-carboxymethyl chitosan (molecular weight 61 kDa and deacetylation degree 83%) and chitosan hydrochloride (molecular weight 90 kDa and deacetylation degree 85%) were purchased from Haidebei Marine Bioengineering Company (Jinan, Shandong, China). β -Lg (93% purity) was obtained from Davisco Foods International, Inc. (Le Sueur, MN, USA). All other reagents were of analytical grade.

Preparation of EGCG-loaded chitosan/ β -Lg nanoparticles The EGCG-loaded chitosan/ β -Lg nanoparticles with core-shell double-walled structures were prepared by ionic gelation using CMC, CHC and β -Lg as wall materials and EGCG as the core material. Different amounts of EGCG (0, 10, 20, 30, and 40 mg) were added to 30 mL of CHC 1.0 mg/mL aqueous solution while stirring at 25°C as described previously (5,6). Next, 15 mL of CMC 2.0 mg/mL aqueous solution was added dropwisely to the CHC solution containing EGCG while

stirring at 500 rpm at 25°C. EGCG-loaded CMC-CHC (simply called chitosan) nanoparticles were obtained through this ionic gelation step.

β -Lg aqueous solutions with different concentrations (0, 10, 20, 30, and 40 mg/mL) were heated at 85°C for 1.5 h to obtain a denatured β -Lg solution as described previously (12). Finally, the EGCG-loaded chitosan/ β -Lg double-walled nanoparticles were formed by adding the denatured β -Lg solution to the EGCG-loaded CMC-CHC nanoparticle solution. The solution containing EGCG-loaded chitosan/ β -Lg double-walled nanoparticles was centrifuged at 30,000 $\times g$ for 20 min; the pellet was then collected and freeze-dried. The EGCG-loaded CMC-CHC nanoparticles without β -Lg were used as a control.

Particle size and zeta potential measurement Size and zeta potential of the EGCG-loaded chitosan/ β -Lg nanoparticles prepared were determined by a commercial particle size and zeta potential analyzer (Delsa Nano C, Beckman Coulter, Inc., Fullerton, CA, USA). For size and zeta potential measurements, the EGCG-loaded chitosan/ β -Lg nanoparticles prepared were dispersed in distilled water, and then measured with a scattering angle of 165° at 25°C. The measurement was repeated three times for every sample.

FT-IR study CMC, CHC, β -Lg, EGCG, and EGCG-loaded chitosan/ β -Lg nanoparticles were analyzed by FT-IR spectroscopy. Each sample was diluted to a suitable concentration and analyzed with a Spectrum 100 FT-IR spectrometer. The infrared transmittance was acquired at a wave number from 800 to 4,000 cm^{-1} , and then analyzed using the OMNIC software.

Encapsulation efficiency The encapsulation efficiency of EGCG-loaded chitosan/ β -Lg nanoparticles was calculated as reported previously (14). Briefly, the EGCG-loaded chitosan/ β -Lg nanoparticles were filtered through an ultracel membrane. After centrifugation at 4,000 $\times g$ for 30 min, free EGCG that penetrated through the membrane was measured using an HPLC-based detection method (15). An Agilent 1200 series HPLC equipped with a DAD analysis system was used to quantify EGCG. The mobile phase was composed of (A) formic acid at pH 2.5 and (B) methanol. The gradient elution was carried out on an Agilent Eclipse XDB-C18 column (4.6 mm $\times$ 150 mm, 5 μm) at a flow rate of 1.0 mL/min with a linear gradient as follows: 0–15 min, A from 82 to 40%, and B from 18 to 60%. The sample (10 μL) was filtered through a 0.45- μm cellulose filter prior to injection. The column was maintained at 40°C, and the detection wavelength was set at 280 nm. A calibration curve was obtained by analyzing five concentrations of EGCG standard (1.0–1,000 $\mu\text{g/mL}$) vs. the peak area of the eluted peak. The linear regression equation was $Y = 11.238X - 882.3$ ($R^2 = 0.9992$), where X is the concentration and Y is the peak area. The EE of EGCG entrapped in double-walled chitosan/ β -Lg nanoparticles was calculated using the following equation:

$$EE (\%) = \frac{(\text{Total amount of EGCG} - \text{amount of EGCG in ultrafiltrate})}{\text{Total amount of EGCG}} \times 100$$

Release studies in simulated GI conditions The release characteristics of EGCG-loaded chitosan/ β -Lg nanoparticles in simulated GI tract conditions were investigated (12). The EGCG-loaded chitosan/ β -Lg nanoparticles were added to 25 mL of 20 mM phosphate buffer, pH 6.8 in a 250 mL flask. After adjusting the pH to 2.0 with a 1 M HCl solution, 1 mL of porcine pepsin (0.4%, porcine gastric mucosa with 800-2,000 unit/mg protein, Sigma-Aldrich, St. Louis, MO, USA) was added and the flasks were incubated at 37°C in a shaking water bath at 150 rpm. Samples were collected at 0, 5, 10, 20, 30, 60, 90, and 120 min to analyze the release characteristics of EGCG from the chitosan/ β -Lg nanoparticles.

Digesta samples with EGCG-loaded chitosan/ β -Lg nanoparticles treated in simulated gastric conditions were used for the release study in simulated small intestine conditions. The pH of the gastric digesta was adjusted to 5.3 by adding 25 mM sodium bicarbonate solution. Next, 1.5 mL of 20 mM phosphate buffer containing 0.2 mg of lipase, 0.4 mg of pancreatin and 2.4 mg of bile extract from porcine pancreas was added to the gastric digesta, and subsequently the pH was adjusted to 6.8 with 1 M NaOH solution. Finally, the flasks containing the digesta samples with EGCG-loaded chitosan/ β -Lg nanoparticles were incubated at 37°C in a shaking water bath at 150 rpm. Samples were collected at 0, 10, 20, 30, 60, 120, 180, and 240 min to determine the release characteristics of EGCG from the chitosan/ β -Lg nanoparticles. The amounts of EGCG released from the chitosan/ β -Lg nanoparticles at given time intervals were determined by the same HPLC method used for EE measurement. EGCG-loaded chitosan nanoparticles without β -Lg were used as control.

Statistical analysis Statistical analysis was performed using SPSS software (SPSS ver. 17.0, SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) with a post-hoc Tukey test was used to determine statistical significance ($p < 0.05$).

Results and Discussion

Formation of EGCG-loaded chitosan/ β -Lg nanoparticle EGCG-loaded chitosan/ β -Lg nanoparticles with a core-shell double-walled structure were synthesized using chitosan (CMC and CHC) and denatured β -Lg for encapsulating EGCG. The formation of the inner core structure was mainly because of CMC-CHC ionotropic interactions

through the opposite charges on their surfaces. EGCG in solution had a negative charge because of many hydroxyl groups in its structure. The CMC-CHC ionotropic reaction could make the interior structure compact and tight. After formation of EGCG-loaded CMC-CHC nanoparticles, their surface structure also had the ability to form ionotropic linkages with other molecules, such as β -Lg.

Denatured β -Lg formed the outer layer of the EGCG-loaded chitosan nanoparticles. β -Lg generally has a negative charge in aqueous solution at pH > 5.2, and a positive charge at pH < 5.2 (16). In this study, β -Lg molecules were negatively charged since the pH of the aqueous solution containing EGCG-loaded CMC-CHC nanoparticles was about 6.0-7.0. As a result, the negatively charged denatured β -Lg molecules formed an external coating on the positively charged EGCG-loaded CMC-CHC nanoparticles through electrostatic interactions. In conclusion, the formation of double-walled chitosan/ β -Lg nanoparticles was mainly because of ionotropic interactions between CMC and CHC molecules for the primary coating, and electrostatic interactions between the primary CMC-CHC nanoparticles and denatured β -Lg molecules for the secondary coating.

Morphology of EGCG-loaded CMC-CHC nanoparticles and EGCG-loaded chitosan/ β -Lg nanoparticles are shown in Fig. 1A and 1B, respectively. The formation mechanism affected the particle size and zeta potential of EGCG-loaded CMC-CHC nanoparticles (Table 1). Generally, the size of primary nanoparticles should increase after the addition of a secondary coating. Interestingly, the average particle size of EGCG-loaded chitosan/ β -Lg nanoparticles was smaller than that of the primary CMC-CHC nanoparticles. This could be due to primary CMC-CHC nanoparticles linked together to form loose structures (6) by external extension of chitosan chains on the surface. After β -Lg coating, the primary CMC-CHC nanoparticles had a single charge on their surfaces, which could reduce the space occupied by the molecules in the particles due to the formation of a tight bond structure, thereby resulting in a relatively small average particle size. Moreover, Cho *et al.* (17) also published similar report that the particle sizes of N-acyl chitosan nanoparticles were reduced as vitamin C was loaded.

The mechanism for size reduction through the secondary outer β -Lg coating is supported by the polydispersity index of the nanoparticles. The polydispersity index value of the primary CMC-CHC nanoparticles was relatively large indicating a less uniform size distribution, due to a high degree of particle aggregation (Table 1). The primary CMC-CHC nanoparticles showed a relatively high degree of inter-particle aggregation as compared to the EGCG-loaded chitosan/ β -Lg nanoparticles due to the different surface characteristics of the β -Lg-containing outer layer. In conclusion, β -Lg allowed the primary CMC-CHC

Table 1. The particle size, polydispersity index and zeta potential of EGCG-loaded chitosan/ β -Lg nanoparticles

Sample	Mean size (nm)	Polydispersity index	Zeta potential (mV)
EGCG-loaded CMC-CHC nanoparticles	289.20 $\pm$ 3.62 ^{a1)}	0.21 $\pm$ 0.01 ^a	28.32 $\pm$ 1.05 ^a
EGCG-loaded chitosan/ β -Lg nanoparticles	193.80 $\pm$ 5.82 ^b	0.13 $\pm$ 0.01 ^b	24.94 $\pm$ 0.26 ^b

¹⁾Different letters in the columns indicate a significant difference ($p < 0.05$).

Fig. 1. TEM images of (A) EGCG-loaded CMC-CHC nanoparticles and (B) EGCG-loaded chitosan/ β -Lg nanoparticles.

nanoparticles to be relatively monodisperse under the preparation conditions, and subsequently formed a secondary coating on their surface.

The shape of EGCG-loaded CMC-CHC nanoparticles was relatively irregular and inconsistent due to inter-particle aggregation, whereas EGCG-loaded chitosan/ β -Lg nanoparticles were mostly spherical in shape with a relatively uniform size (Fig. 1). The β -Lg molecules changed the surface characteristics of EGCG-loaded chitosan/ β -Lg nanoparticles. The unfolded β -Lg molecules reduced inter-particle aggregation among the primary CMC-CHC nanoparticles. Moreover, the addition of the secondary β -Lg layer reduced the mean size of EGCG-loaded chitosan/ β -Lg nanoparticles as compared to the primary CMC-CHC nanoparticles. In addition, the interaction between β -Lg and chitosan molecules improved EE through their stronger protein-polysaccharide electrostatic interactions.

FT-IR spectroscopy analysis of EGCG-loaded chitosan/ β -Lg nanoparticles The EGCG-loaded chitosan/ β -Lg nanoparticles were analyzed by FT-IR spectroscopy to assess the binding interactions

Fig. 2. FT-TR spectra of (A) CMC, (B) CHC, (C) β -Lg, (D) EGCG and (E) EGCG-loaded chitosan/ β -Lg nanoparticles.

forming the double-walled structure. The FT-IR spectra of pure raw materials are shown in Fig. 2. A major peak at 3,329, 3,308, and 3,337 cm^{-1} was observed in the spectra of chitosan (CMC and CHC), β -Lg, and EGCG, respectively. These peaks represented the stretching of -NH and -OH groups. The peaks at 2,800–3,000 cm^{-1} were observed for C-H stretching, at 1,633–1,638 cm^{-1} in the spectra of CMC, CHC, β -Lg, and EGCG was assigned to the stretching of the -NH group. However, after loading of EGCG, the peak was shifted to the lower wave number at 1,466 cm^{-1} in the FT-IR spectra of EGCG-loaded chitosan/ β -Lg nanoparticles. This indicated that the hydrogen bonding was successfully formed during the synthesis of the EGCG-loaded chitosan/ β -Lg nanoparticles. Hu *et al.* (10) also reported that the absorption peaks at 1,631 and 1,150 cm^{-1} disappeared after the formation of chitosan nanoparticles. Moreover, the peaks around 1,072 and 1,155 cm^{-1} in the CMC and CHC raw materials spectra, which represented C-O-C and C-H stretching (5), also appeared at 1,107 and 1,160 cm^{-1} in the EGCG-loaded chitosan/ β -Lg nanoparticles. The peak at 1,127 cm^{-1} , which was attributed to C-O stretching (18), also appeared at 1,128 cm^{-1} for the EGCG-loaded chitosan/ β -Lg nanoparticles (Fig. 2B and 2E). The peaks around 1,380 and 952 cm^{-1} in the control reflected the stretching vibration of the C-N and CH_3 wagging along chain (19), while two relatively strong absorption peaks appeared at 1,379 and 954 cm^{-1} in the nanoparticle group. In particular, the strong and wide O-H stretching changed into a narrow absorption peak at 3,337 cm^{-1} in the EGCG-loaded chitosan/ β -Lg nanoparticles as compared to the control group. Given these results, we could speculate that the ionotropic reactions could be formed between CMC, CHC, and β -Lg, which probably enhanced the encapsulation efficiency of EGCG.

Fig. 3. Effects of (A) β -Lg content, (B) EGCG content, and (C) temperature on the particle size and zeta potential of EGCG-loaded chitosan/ β -Lg nanoparticles (Different prepared condition with a: 10 mg EGCG was added and the molar ratio of CMC and CHC to EGCG is 3:3:1; b: 20 mg β -Lg was added and the molar ratio of CMC and CHC to β -Lg is 3:3:2; c: 20 mg β -Lg and 10 mg EGCG were added and the molar ratio of CMC and CHC to β -Lg is 3:3:2).

Effect of β -Lg content, EGCG content, and temperature on particle size, zeta potential, and EE of EGCG-loaded chitosan/ β -Lg nanoparticles

The mean particle size and zeta potential of EGCG-loaded chitosan/ β -Lg nanoparticles relative to β -Lg content are shown in Fig. 3A. The

Fig. 4. Percent entrapment efficiency of EGCG in EGCG-loaded chitosan/ β -Lg nanoparticles as determined by different addition levels of (A) β -lactoglobulin and (B) EGCG (a: molar ratio of CMC and CHC to EGCG is 3:3:1 and b: molar ratio of CMC and CHC to β -Lg is 3:3:2).

particle size of EGCG-loaded chitosan/ β -Lg nanoparticles was stable at β -Lg content up to 20 mg and then increased with increasing β -Lg. This indicated that a relatively low β -Lg concentration was more conducive to electrostatic reactions on the surface of the primary CMC-CHC nanoparticles to form the double-walled structure. In fact, it was difficult to measure mean particle size when the β -Lg content exceeded 50 mg because of severe aggregation and precipitation of the formed nanoparticles. In addition, the zeta potential of the EGCG-loaded chitosan/ β -Lg nanoparticles gradually decreased with increasing β -Lg (Fig. 3A). Generally, a high zeta potential reflects a stable colloidal aggregation state in solution (20). Therefore, an excess of β -Lg could make the EGCG-loaded chitosan/ β -Lg nanoparticles more unstable in solution.

Figure 3B shows the effect of adding different amounts of EGCG on the particle size and zeta potential of EGCG-loaded chitosan/ β -Lg nanoparticles. Increasing the EGCG content did not affect particle size within the 180 and 220 nm range, while the zeta potential gradually increased. This indicated that the effect EGCG on nanoparticle formation was relatively small, but higher EGCG concentration could enhance the stability of nanoparticles in solution.

Interestingly, the average particle size of EGCG-loaded chitosan/ β -Lg nanoparticles was smaller than that of nanoparticles without EGCG. It is possible that the EGCG molecules containing a number of

Fig. 5. (A) Release mechanism and (B) Release percentage of the EGCG-loaded chitosan/ β -Lg nanoparticles compared to the non-coated chitosan nanoparticles in gastrointestinal conditions.

hydroxyl groups could link electrostatically to the side chains in chitosan and β -Lg molecules, forming tight intermolecular bonds. Thus, relatively small particles could be formed as a result of the electrostatic attraction between EGCG and the wall materials. This result is consistent with that of a previous study in which the particle size of thermally induced β -Lg-EGCG nano-vehicles was smaller than that of the controls without EGCG (21).

The particles size and zeta potential of EGCG-loaded chitosan/ β -Lg nanoparticles were affected by the temperature used for β -Lg heat treatment (Fig. 3C). Both mean particle size and zeta potential increased with temperatures up to 70°C beyond which they decreased due to the severe thermal denaturation of β -Lg (22). β -Lg may undergo a conformational change after heat treatment at temperatures up to 70°C to expose non-polar side-chains usually buried in the interior sites of the molecular structure. The change in β -Lg molecular conformation after the thermal treatment could electrostatically affect the double-walled structure formation mechanism (23). As a result, the heat treatment could affect the formation of the EGCG-

loaded chitosan/ β -Lg nanoparticle secondary coating and thus particle size, although the effect was relatively small in the 200-240 nm range.

Figure 4 shows the EE of EGCG-loaded chitosan/ β -Lg nanoparticles with different levels of β -Lg or EGCG. EE increased from 54.15 to 60.67% as β -Lg increased from 10 to 40 mg (Fig. 4A). EGCG molecules were entrapped primarily by the CMC and CHC matrix and subsequently the remaining EGCG molecules combined with β -Lg molecules. This result is in agreement with that of a previous report in which EE of EGCG-loaded β -Lg nanoparticles increased with an increase in β -Lg concentration (24).

Figure 4B shows that EE increases at low EGCG content up to 30 mg and subsequently decreases at higher EGCG levels. EE reached a maximum value of about 60% when the EGCG content was in the 20 to 30 mg range. The improvement in EE was due to the inter- and intra-molecular electrostatic interactions between EGCG molecules and wall materials (CMC, CHC, and β -Lg).

Prolonged release of EGCG from EGCG-loaded chitosan/ β -Lg nanoparticles in simulated GI conditions The release properties of double-walled nanoparticles in simulated GI tract conditions were investigated by changing the pH and adding different digestive enzymes. Swelling properties and degradation behavior during the prolonged release process could also be estimated using control chitosan nanoparticles without β -Lg. A schematic diagram of the release process for double-walled nanoparticles as compared with the control is shown in Fig. 5A. The controlled release mechanism of the EGCG-loaded chitosan/ β -Lg nanoparticles maybe involve several different stages in the stomach (pepsin digestion and partial release) and the small intestine (swelling, enzymatic degradation, and full release).

Figure 5B shows the release profiles of EGCG from the EGCG-loaded chitosan/ β -Lg nanoparticles in simulated GI conditions. The relative release rate of the EGCG-loaded chitosan/ β -Lg nanoparticles was determined by analyzing the release concentration of EGCG in solution. Compared to the control group the EGCG-loaded chitosan/ β -Lg nanoparticles had a relatively slow release rate in simulated stomach conditions. It suggested that the β -Lg secondary coating improved the controlled release of EGCG. Moreover, the double-walled nanoparticles also had a relatively low release rate as compared to the control in simulated small intestine conditions. The controlled release may be due to low external release (Fig. 5A). The negatively charged β -Lg layered on the outer surface of the primary CMC-CHC nanoparticles might bind free EGCG resulting in enhanced EE. Another reason for the sustained release properties of the EGCG-loaded chitosan/ β -Lg nanoparticles could be that the β -Lg outer layer was pepsin-resistant, which contributed to the relatively slow release of EGCG in simulated stomach conditions. This is consistent with our results that the EGCG release rate in the EGCG-loaded chitosan/ β -Lg nanoparticles at 20 min was comparable to that in controls at 5 min (Fig. 5B). The release rate and degradation of EGCG-loaded chitosan/ β -Lg nanoparticles (20 and 60 min, respectively) in simulated stomach conditions were slower than those of the control particles (5 and 30 min, respectively) as shown in Fig. 5B. These results suggested that the CMC-CHC matrix coated with β -Lg could effectively prevent the degradation of EGCG in the simulated gastric fluids.

Under simulated intestinal conditions, the β -Lg coating on the EGCG-loaded chitosan/ β -Lg nanoparticles was broken down into small protein molecules with the change of PH value or peptides chains by swelling and pancreatic enzymes, resulting in disintegration of EGCG from the wall structure (8,12). Figure 5B clearly shows that the EGCG amount released from EGCG-loaded chitosan/ β -Lg nanoparticles in the simulated small intestinal fluid is larger than that released in the simulated stomach conditions. Due to the slower degradation of the chitosan/ β -Lg double-walled structure, the EGCG-loaded chitosan/ β -Lg nanoparticles maintained their prolonged release characteristics as compared to the controls. Especially, the β -lactoglobulin could be easily hydrolyzed by trypsin in neutral aqueous solution (pH 7.7) (25). It may be the digestion of β -Lg

located in the outer of nanocarrier leading to its quick release in the small intestine condition. In addition, after the external wall degradation, the exposed chitosan chains can adhere to the intestinal wall, thereby increasing residence time in the intestinal tract, resulting in improved bioavailability of the core materials (12). The prolonged release capabilities and excellent adhesion properties of EGCG-loaded chitosan/ β -Lg nanoparticles could enhance the effective absorption of EGCG in the human intestine. These results suggest that the chitosan/ β -Lg nanoparticles could be an attractive carrier for the encapsulation and oral administration of EGCG.

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