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ARTICLE

Novel Technology for the Preparation of Self-Assembled Catechin/Gelatin Nanoparticles and Their Characterization

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In this study, self-assembled tea catechin/gelatin nanoparticles were prepared by directly mixing the catechins and gelatin solutions. The mean particle sizes were almost less than 200 nm, and the zeta potential values were negatively charged. FT-IR spectral analysis indicated that hydrogen bonding between aliphatic and aromatic hydroxyl groups, repectively, on gelatin and catechins is responsible for the self-assembly of nanoparticles. Free radical (DPPH• and ABTS•$^{+}$) scavenging assays showed that tea catechins could be protected by the nanoparticles and that the antioxidant activity of tea catechins was almost retained after three weeks of storage. The tea catechin/gelatin nanoparticles exhibited 28−41% inhibition to trypsin against the degradation of gelatin. This result suggested that the tea catechin/gelatin nanoparticles might be a useful antioxidant carrier because catechins and gelatin were, respectively, protected from oxidation and enzymatic digestion.

KEYWORDS: Tea catechins; gelatin; nanoparticles; radical scavenging; enzymatic digestion

INTRODUCTION

Catechin, belonging to a group of bioflavonoids, can act as an antioxidant either through the chelation of metals with redox properties or by acting as a scavenger of free radicals (*1*, *2*). Tea catechins exhibit antioxidant and neuroprotection activity, inhibit tumor angiogenesis, prevent atherosclerosis, and modulate cholesterol metabolism (*3*−*7*). Tea catechins also protect against the oxidative cellular damage of UV radiation and prevent photocarcinogenesis in mice through DNA repair (*8*). EGCG, the major constituent of catechin-based flavonoids in green tea, has been demonstrated to inhibit matrix metalloproteinase, playing an important role in tumor invasion and metastases (*9*−*11*).

Gelatin is a protein produced by partial hydrolysis of collagen, the main protein component in skins, bones, hides, and other animal body tissues. Gelatin is traditionally used in food industries as a stabilizer, thickener, or texturizer in foods such as jams, yoghurt, cream cheese, and margarine. Gelatin is primarily used as a gelling agent, forming transparent elastic thermoreversible gelatins on cooling below about 35 °C, which constitutes the shells of pharmaceutical capsules. Accordingly, gelatin has been developed in the pharmaceutical and food processing industry to produce microcapsules or nanocarriers for specific drug delivery and flavor release (*12*−*15*). Application of gelatin in agriculture to control the release of chemical compounds and pheromones for pest management was also investigated (*16*, *17*).

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Microencapsulation techniques have widely been used for a variety of food applications such as taste and odor masking, protection of food ingredients against oxidation, isomerization and degradation during storage, and release of flavoring ingredients or functional components of healthy foods (*18*−*20*). Over recent years, advancement in nanotechnology has attracted increasing interest in food and pharmaceutical applications to produce nanosized carriers for delivering drugs. Nanoparticles prepared from biodegradable and biocompatible polymers are good candidates for drug carriers to perform the same functions as those of microcapsules but additionally provide targeted delivery of drugs in a cell- or tissue-specific manner (*21*). Unlike microcapsules or microparticles, nanoparticles have a long shelf life and can enhance the crossing of drugs or functional components across the intestinal epithelium (*22*).

In recent years, nanotechnology has been used for preparing carriers for protecting or delivering several natural products such as quercetin, glycyrrhizinate, tea catechins, and ellagic acid (*23*−*27*). The aim of this study was to develop a novel self-assembly nanotechnology to prepare nanoparticles comprising tea catechins and gelatin by directly mixing these two components. Because tea polyphenols (TP), in particular, the catechins can inhibit the activity of several enzymes (*28*), protection of gelatin against trypsin digestion by the catechin/gelatin self-assembled nanoparticles was investigated in this study. Additionally, the antioxidant activity of the catechin/gelatin nanoparticles was determined by $ABTS^{+}$ and DPPH radical-scavenging analysis. The factors that influence their antioxidant activity and enzymatic digestion were all determined. The physical and chemical

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properties of the nanoparticles such as the catechin/gelatin interactions, mean particle sizes, and zeta potential values were examined by Fourier transform infrared (FT-IR) and dynamic light scattering (DLS).

MATERIALS AND METHODS

Materials. Tea catechin (EGCG > 65%) was kindly gifted by Universe-Medical Biotechnology Co. Ltd. (Taiwan). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammoniumsalt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), *N*-α-benzoyl-L-arginine ethylester (BAEE), gelatin (type B), and trypsin were purchased from Sigma-Aldrich Company Ltd. (Louis, MO USA). Methanol (MeOH) (HPLC grade) and acetonitrile (ACN) (HPLC grade) were obtained from Mallinckrodt Baker (Phillipsburg, NJ, U.S.A.). Trifluoroacetic acid (TFA) (99%) was obtained from Aldrich (Milwaukee, WI, U.S.A.).

Self-Assembly of Catechin/Gelatin Nanoparticles. Catechins/gelatin nanoparticles were prepared using a simple, self-assembly method under magnetic stirring at room temperature. Briefly, aqueous gelatin [2.0, 1.0, 0.5, and 0.25 mg/mL, 1 mL, in deionized (DI) water] was added by flush mixing with a pipet tip into aqueous catechins (2.0, 1.0, 0.5, and 0.25 mg/mL, 1 mL, in DI water) under magnetic stirring at room temperature and the mixed solution was stirred for 10 min. The self-assembled nanoparticles were collected by centrifugation at 12000 rpm for 20 min, freeze-dried, and storage at room temperature.

The mean particle sizes and zeta potential values of nanoparticles were measured using a Zetasizer (3000HS, Malvern Instruments Ltd., Worcestershire, UK). The chemical structures of catechins, gelatin, and the catechin/gelatin assembled nanoparticles were analyzed by FT-IR (Perkin-Elmer Spectrum RX1 FT-IR System, Buckinghamshire, England). The morphology of the nanoparticles was examined by transmission electron microscopy (TEM). The TEM sample was prepared by placing a drop of the nanoparticle suspension onto a 400 mesh, carbon-coated copper grid. About 2 min after deposition, the grid was tapped with a filter paper to remove surface water, followed by air-drying. The dried samples were observed by TEM (Hitachi H-600, Japan).

Catechin Composition and Loading. To determine the catechin composition and total catechin loading efficiency, the catechin/gelatin nanoparticles were collected by centrifugation at 12000 rpm, 4 °C for 20 min, and the catechin concentration in the supernatant was assayed by determining catechin concentrations by high-performance liquid chromatography (HPLC) assay, with reference to calibration curves (EGCG or catechin concentration: 100 ppm, 75 ppm, 25 ppm, and 10 ppm) (*29*). A PerkinElmer HPLC system (600 Series LINK) equipped with a UV detector (PerkinElmer Series 200 UV/vis) and a C-18 reverse phase column (pak C_{18}, ODS1 4.6 mm × 150 mm) with a guard column packed with the same stationary phase were utilized. Injection volume was 10 μL. System flow rate was 1.2 mL/min. Typical system operating pressure range was 10−15 MPa, and operating temperature was 25 °C. Phase A was ddH_2O, ACN, and TFA (919/80/1, v/v), and phase B was ddH_2O, ACN, MeOH, and TFA (699/270/30/1, v/v). Prior to each injection, the system was equilibrated to 95/5 (A/B). Following sample injection, the phase composition changed according to the following gradient: 95/5 at 0 min, 30/70 at 1.5 min (convex), 1/99 at 3 min (convex), and 95/5 from 3 to 5 min (step, immediate) for a total chromatographic run time of 9.5 min. Individual catechin quantification was accomplished by construction of multilevel calibration curves from response at 280 nm resulting from an injection of authentic standards of EGCG. Total catechins were calculated as the sum of all measured individual catechins.

Measurement of Radical Scavenging Activity by DPPH. The free radical scavenging activity was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) following the protocols of Zhao et al. (*30*). Then, 0.1 mL of each nanoparticle-containing medium and 3.9 mL of reacting solution (100 mM DPPH in methanol) were mixed. The mixtures were left for 30 min at room temperature in the dark. Absorbances were measured with a UV−vis spectrophotometer (Hitachi, Tokyo, Japan) at 517 nm. DPPH radical scavenging activity was calculated as an inhibition percentage based on the following equation:

$$\text{free radical scavenging activity (\%)} = (1 - \text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100$$

Measurement of Radical Scavenging Activity by ABTS•$^+$. The radical scavenging activity of catechin/gelatin nanoparticles against the ABTS•$^+$ radical cation was measured using the method of Zhao et al. (*30*) with some modifications. ABTS•$^+$ was dissolved in water to a 7 mM concentration. The ABTS•$^+$ radical cation was produced by reacting ABTS•$^+$ stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. The ABTS•$^+$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C. An aliquot of each nanoparticle-containing medium (0.1 mL) was mixed with 3.9 mL of diluted ABTS•$^+$ solution. After reaction at 30 °C for 20 min, the absorbance at 734 nm was measured. ABTS•$^+$ radical scavenging activity was calculated as an inhibition percentage based on the above-mentioned equation.

Interactions of Catechins with Enzyme. Enzyme activity and its inhibition by tea catechins were measured as described previously (*28*, *31*). Briefly, trypsin was assayed in a reaction medium (100 μL) containing 40 mM Tris (pH 7.9), 7.0 mM NaCl, 0.38 mg/mL *N*-α-benzoyl-L-arginine ethylester (BAEE), and 0.1 mg/mL trypsin. All of the assays were conducted in the presence or absence of catechins. As a control, equal volumes of distilled water without catechins were added. To determine the enzyme activity in the mixture, diluted tea catechins (2 mg/mL, 1 mg/mL, 0.5 mg/mL, and 0.25 mg/mL) were mixed with trypsin and incubated at 37 °C for 10 min, and the reaction was terminated by quickly decreasing the temperature to 4 °C. All samples were analyzed in triplicate. The inhibition of enzyme activity in the presence of catechins is calculated as follows:

$$\text{inhibition (\%)} = [1 - (\text{activity test}/\text{activity control})] \times 100$$

Enzymatic Digestion Properties of Nanoparticles. The catechin/gelatin nanoparticles were incubated in trypsin-containing medium (0.1 mg/mL), and the dispersive stabilities of the nanoparticle were evaluated by turbidity measurements using a UV−vis spectrophotometer (Uvikon923, Kontron Instruments, Italy) at 500 nm (*32*). An increase of light transmittance represents the disintegration or instability of nanoparticles.

The effect of catechins on the protection of nanoparticles from enzyme digestion was also examined by monitoring the release of hydroxyproline from insoluble catechin/gelatin nanoparticles. The nanoparticles were incubated in 500 μL of trypsin-containing medium (0.1 mg/mL) in a sealed microtube and incubated in a 37 °C shaking water bath at 20 rpm for 24 h. After centrifugation, 250 μL of supernatant was withdrawn, hydrolyzed in 6 N HCl at 110 °C for 20 h, and hydroxyproline content determined using a colorimetric method for hydroxyproline quantification (*33*). Hydroxyproline is a unique amino acid for gelatin, and it offers itself as a useful marker for identifying gelatin. The method of determining hydroxyproline involves the oxidation of hydroxyproline to pyrrole-2-carboxylic acid, which complexes with *p*-dimethylaminobenzaldehyde exhibiting maximum absorbance at 557 nm. The concentration of the hydroxyproline released from the nanoparticles was determined at 557 nm using pure hydroxyproline as a standard.

$$\text{\% gelatin} = \text{\% hydroxyproline} \times 7.4$$

On the basis of the digested gelatin content in the supernatant solution of the trypsin treated catechin/gelatin nanoparticles, the % degradation of gelatin for catechin/gelatin nanoparticles is calculated as

$$\text{\% gelatin degradation} = 100 - [(\text{initial gelatin} - \text{soluble gelatin})/\text{initial gelatin}] \times 100$$

Statistical Analysis. Statistical analysis for the determination of differences in the measured properties between groups was accomplished using one-way analysis of variance and determination of confidence intervals, performed with a computer statistical program (Statistical Analysis System, Version 6.08, SAS Institute Inc., Cary, NC). All data are presented as a mean value with its standard deviation indicated (mean ± SD).

RESULTS AND DISCUSSION

Characterization of Chemical Compositions and Catechin/Gelatin Interactions. A chromatograph profile of the tea catechins

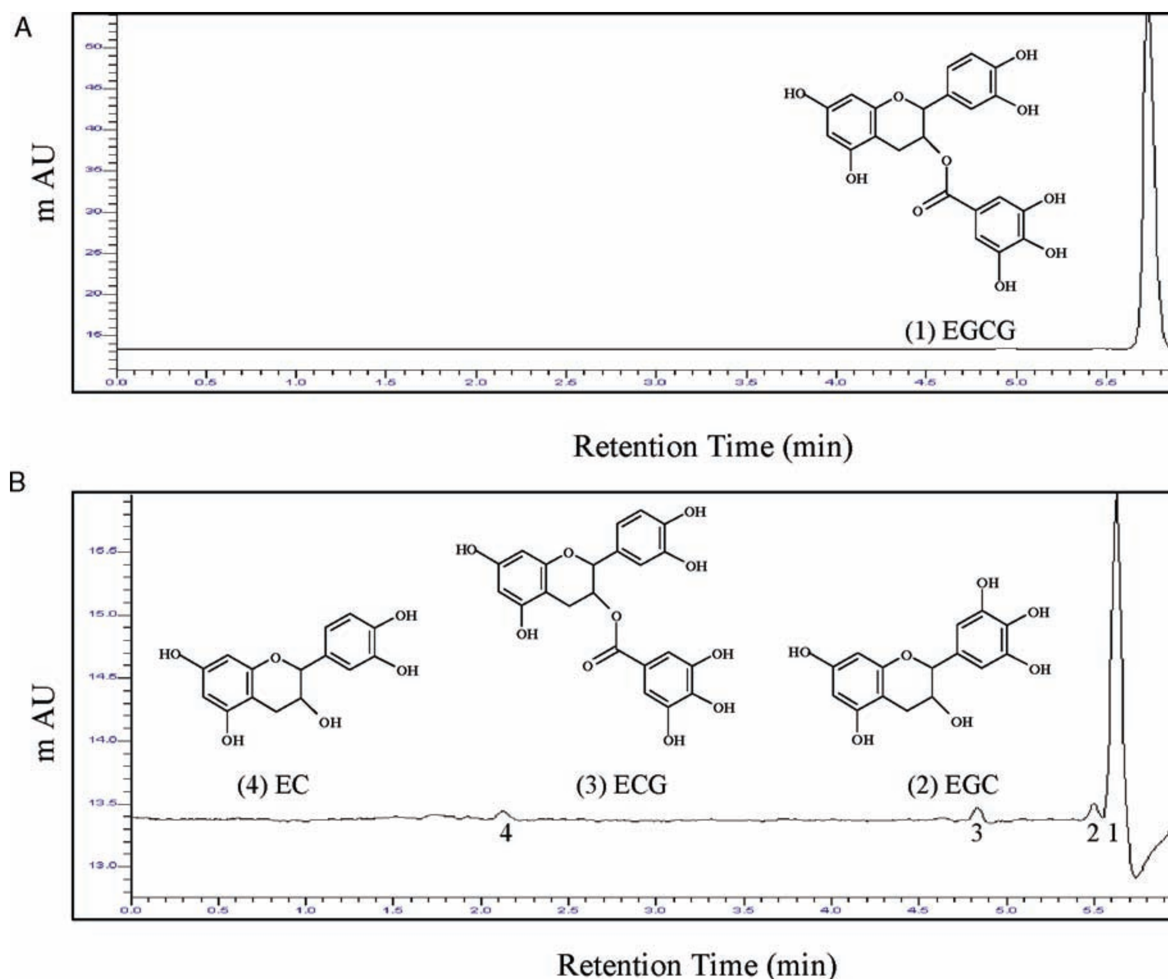

Figure 1. HPLC chromatograms: (**A**) EGCG standard and (**B**) tea catechins containing EC, ECG, EGC, and EGCG.

analyzed by HPLC is shown in **Figure 1**. It clearly shows four components including (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC) in the catechins. The tea catechins contain more than 65% of (−)-EGCG, which is the most abundant component among the catechins.

In our previous studies, chitosan/poly(γ-glutamic acid) (γ-PGA) self-assembled nanoparticles have been prepared for oral delivery of peptide drugs (*33*−*35*). In this study, novel catechin/gelatin nanoparticles with the characteristics of protected antioxidant activity of catechins and inhibited proteolytic activity of trypsin toward gelatin were prepared. The selection of gelatin and catechins as nanoparticle components was based on their biocompatibilty and their safe use in food application and health care. Self-assembled nanoparticles were prepared by adding a catechin aqueous solution into a gelatin aqueous solution under magnetic stirring at room temperature. Specific interactions between catechins and gelatin were responsible for the self-assembly of catechin/gelatin nanoparticles. **Figure 2** shows the FT-IR spectra of the original catechin and gelatin powders as well as the catechin/gelatin assembled nanoparticles. Catechins demonstrating absorption bands at around 3358 cm^{-1} are due to the vibration of the O−H linkage of phenolic hydroxyl groups (*36*). Gelatin demonstrating absorption bands at 1651 cm^{-1} and 1538 cm^{-1} is assigned to C=O (amide I) and N−H (amide II) bending, respectively (*37*). Hydrogen bonding between alphatic and aromatic O−H groups, repectively, on gelatin and catechins is observed for O−H stretching since this peak shifted from 3426 cm^{-1} for gelatin toward 3388 cm^{-1} and became broad for

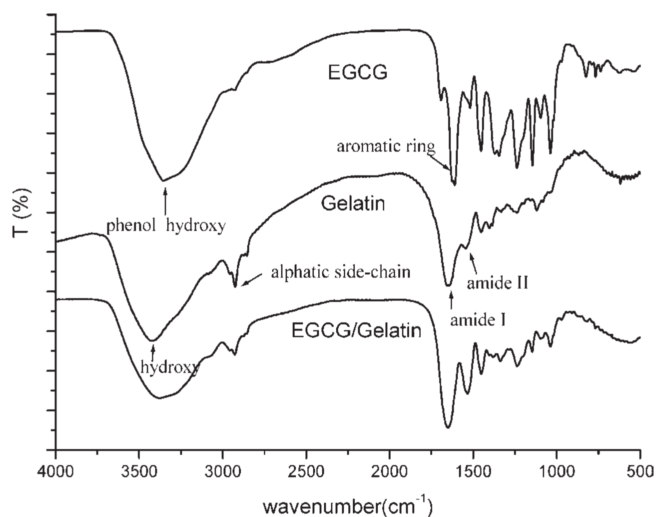

Figure 2. FT-IR spectra of catechins, gelatin, and catechin/gelatin self-assembled nanoparticles.

the catechin/gelatin nanoparticles. Other changes are also observed in the characteristic peaks such as 3358 cm^{-1} and 2840−2990 cm^{-1}, respectively, assigned to the phenolic hydroxyl of catechin and the alphatic side chain of gelatin (*38*). These results suggest that hydrophilic/hydrophobic interactions are taking place and may be contributing to the self-assembly of nanoparticles.

Properties of Self-Assembled Catechin/Gelatin Nanoparticles. The particle sizes, scattering light intensity, and zeta potential

Table 1

(A) Mean Particle Sizes and Scattering Light Intensity of Catechins/Gelatin Nanoparticles (n = 5 Batches)

gelatin (mg/mL)	catechin (mg/mL) (mean particle sizes/scattering light intensity)		
	2	1	0.5
2	140.5 ± 9.5/255.4 ± 13.6	160.4 ± 6.3/68.9 ± 5.7	208.6 ± 8.1/48.6 ± 3.1
1	122.5 ± 6.8/203.6 ± 9.6	152.1 ± 8.7/50.1 ± 4.8	207.2 ± 7.5/23.8 [a]
0.5	161.3 ± 7.2/116.9 ± 6.6	124.0 ± 11.6/16.5[a]	162.5 ± 4.7/7.02[a]
0.25	362.5 ± 15.6/37.02[a]	62.5 ± 7.9/7.02[a]	1.1/3.05[a]

(B) Zeta Potentials of Catechins/Gelatin Nanoparticles (n = 5 Batches)

gelatin (mg/mL)	catechin (mg/mL)		
	2	1	0.5
2	0.2 ± 0.1	−11.9 ± 2.1	−18.5 ± 15
1	−7.6 ± 0.5	−17.1 ± 1.7	−14.7[a]
0.5	−14.2 ± 1.8	−19.2[a]	−3.3[a]
0.25	−8.7[a]	−6.9[a]	−5.4[a]

[a] Data is not reliable because scattering light intensity is too low.

values of catechin/gelatin nanoparticles, prepared at varying concentrations of catechins and gelatin, were determined, and the results are shown in **Table 1**. The particle size and the zeta potential values of the prepared nanoparticles were mainly determined by the relative amount of the local concentration of catechins in the added solution to the surrounding concentration of gelatin in the sink solution. At a fixed concentration of catechins (2 mg/mL or 1 mg/mL), a decrease in the gelatin concentration allowed catechin molecules interacting with less gelatin molecules to form fewer nanoparticles. Therefore, the nanoparticle-containing medium displayed a lower scattering light intensity and decreased mean particle size. In contrast, a decrease in the catechin concentration allowed gelatin molecules to interact with fewer catechin molecules and thus formed a lower scattering light intensity of nanoparticle-containing medium (**Table 1**, section A).

Especially, catechins with a concentration less than 1 mg/mL are unable to form enough nanoparticles (scattering light intensity < 50 unit). The results suggest that catechins play an important role in binding with gelatin in the intermolecular assembly of nanoparticles. The mean particle size approximately increased with the decrease of catechin concentration. When the amount of gelatin molecules exceeded that of local catechin molecules, some of the excessive gelatin molecules could also bind to catechins and were entangled onto the surfaces of catechin/gelatin nanoparticles. Thus, the resulting nanoparticles may display a larger mean particle size. While, in contrast, scattering light intensity of the nanoparticles decreased due to the formation of fewer nanoparticles.

It is known that the phenol hydroxyl group (Ar−OH) of catechins could be slightly deprotonated ($Ar-O^-$) in DI water because the pK_a values of EC, EGC, ECG, and EGCG were in the range of 7.73−8.72 (*39*). Most of the nanoparticles had negative charge of zeta potentials, suggesting that the resulting nanoparticles may be surrounded by a negatively charged layer ensuring colloidal stabilization. (**Table 1**, section B). The zeta potential becomes relatively less negative (0.2 and −7.6 mV) for the nanoparticles prepared from higher concentrations of catechins (2 mg/mL) and gelatin (2 mg/mL or 1 mg/mL). Because the dissociated H^+ from the catechins can help the protonation of some amino-containing groups on gelatin, the nanoparticles prepared from high concentration of catechins may display a less negatively charged gelatin shell. The morphology of the nanoparticles obtained by the TEM examination showed that the prepared nanoparticles were spherical in shape with a smooth surface (**Figure 3**). The loading efficiency of total catechins in self-assembled nanoparticles prepared from 2 mg/mL catechins and 2 mg/mL gelatin were higher than 96%, suggesting that catechins were almost incorporated with gelatin to be assembled into nanoparticles. As shown in **Table 2**, the catechin loading efficiency decreases with the decrease of mixed gelatin concentration (2, 1, 0.5, and 0.25 mg/mL) for preparing the nanoparticles, at a fixed catechin concentration (2 mg/mL). In contrast, the loading content approximately increases with the decrease of mixed gelatin concentration because a lower yield of nanoparticles was obtained from those prepared from lower gelatin concentration.

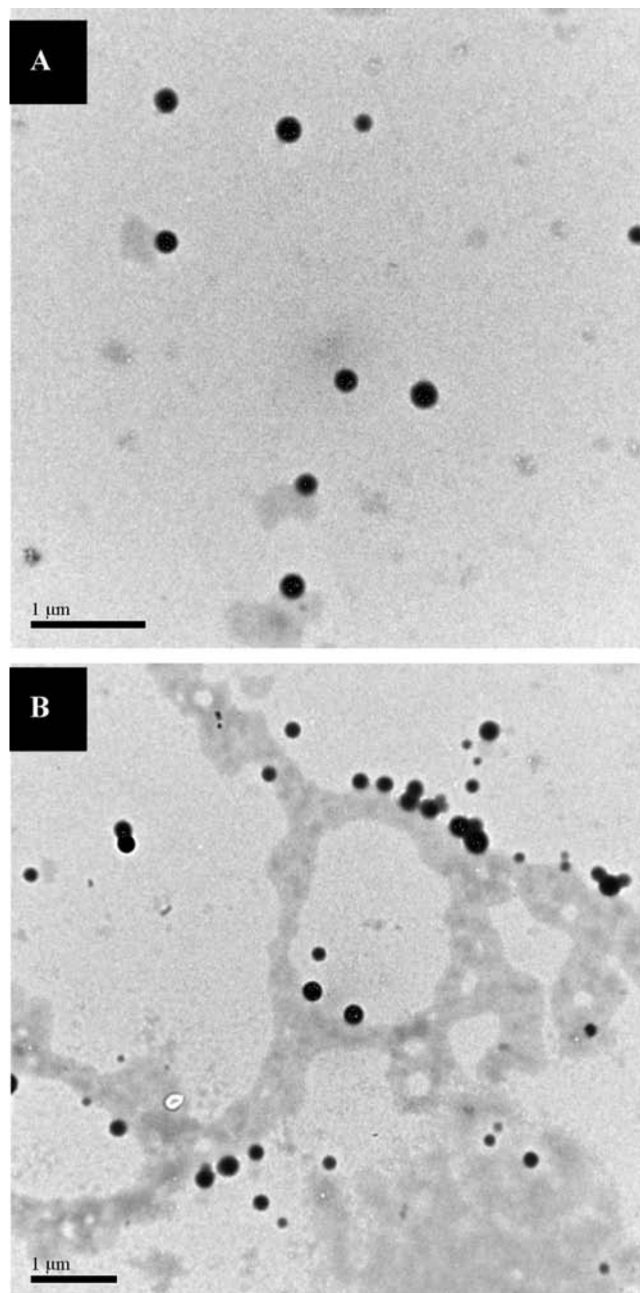

Figure 3. TEM micrography of catechin/gelatin self-assembled nanoparticles: (**A**) 2 mg/mL catechin/0.5 mg/mL gelatin and (**B**) 2 mg/mL catechin/1 mg/mL gelatin.

DPPH• and ABTS• Scavenging Activity. The free radical scavenging activity of prepared nanoparticles was assessed by the DPPH• and ABTS•$^+$ cation method. **Figure 4A** shows that

Table 2. Loading Efficiency and Loading Contents of Catechins/Gelatin Nanoparticles (n = 5 Batches)[a]

gelatin (mg/mL)	catechins lost[b] (μg/mL)	loading efficiency[c] (%)	NPs yield[d] (mg/mL)	loading content[e] (mg/mg)
2	34.2 ± 2.1	96.6 ± 2.2	1.85 ± 0.35	0.52 ± 0.02
1	97.3 ± 2.7	90.3 ± 4.5	1.33 ± 0.42	0.68 ± 0.03
0.5	155.8 ± 8.3	86.4 ± 2.9	0.93 ± 0.26	0.91 ± 0.05
0.25	866.5 ± 26.4	13.3 ± 0.5	0.23 ± 0.03	0.58 ± 0.03

[a] Nanoparticles were prepared with the same catechin concentration (1 mL, 2 mg/mL catechins) and different concentrations of gelatin (1 mL, 0.25−2 mg/mL gelatins). [b] Catechin concentration in the supernatant after the centrifugation of nanoparticles. [c] he weight percentage of incorporated catechins divided by the total used catechin. [d] The yield of nanoparticles (NPs) after centrifugation. [e] The weight of incorporated catechins divided by the weght of nanoparticles.

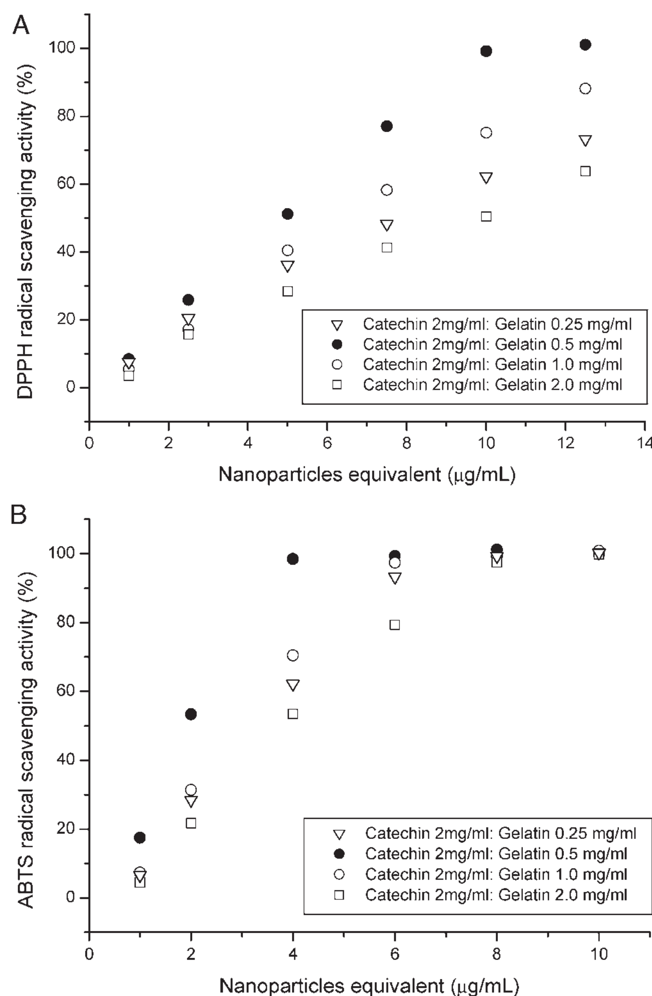

Figure 4. Free radical scavenging activity of catechin/gelatin nanoparticles: (**A**) DPPH radical and (**B**) ABTS radical scavenging activity.

catechin/gelatin nanoparticles have concentration-dependent antioxidant activity toward DPPH•, whereas their potency is dependent on the catechin/gelatin ratios for preparing the nanoparticles. A higher catechin loading content is associated with a stronger DPPH• scavenging activity. **Figure 4B** also showed the concentration-dependent free radical scavenging response to ABTS•$^+$ of the catechin/gelatin nanoparticles. As can be seen, the nanoparticle with a higher loading content of catechins (nanoparticles prepared from 2 mg/mL catechins and 0.5 mg/mL gelatin) shows the best ABTS•$^+$ scavenging activity. This work therefore demonstrates that the antioxidant powers of the nanoparticles against DPPH• and ABTS•$^+$ approximately increased with the increase of the catechin/gelatin ratio for preparing the nanoparticles.

Table 3. EC_{50} Values (DPPH and ABTS Radical Scavenging Activity) of Catechins/Gelatin Nanoparticles[a]

gelatin (mg/mL)	DPPH (μg/mL)	ABTS (μg/mL)
2	9.6 ± 0.6	4.1 ± 0.2
1	6.8 ± 0.3	3.2 ± 0.1
0.5	5.2 ± 0.3	2.1 ± 0.1
0.25	7.9 ± 0.4	3.4 ± 0.2

[a] Nanoparticles were prepared with the same catechin concentration (1 mL, 2 mg/mL catechins) and different concentrations of gelatin (1 mL, 0.25−2 mg/mL gelatins).

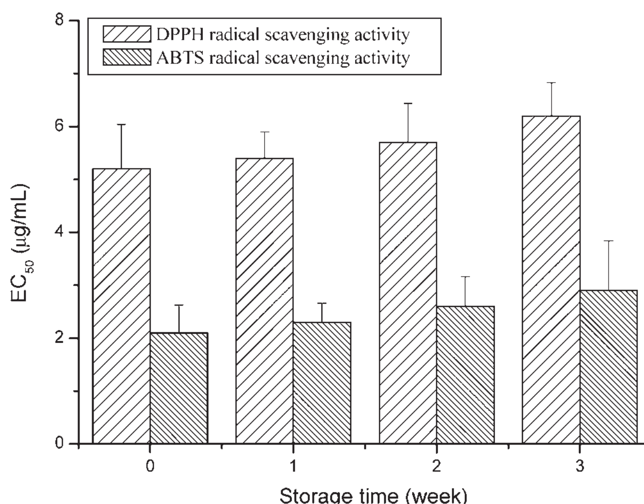

Figure 5. Effect of storage time on EC_{50} values of the prepared nanoparticles (2 mg/mL catechin/0.5 mg/mL gelatin) for scavenging DPPH or ABTS•$^+$ radicals.

The required catechin/gelatin nanoparticles equivalent to quench DPPH or ABTS radicals by 50% were expressed as EC_{50} values to examine the free radical scavenging activity. The EC_{50} values were 5.2 and 2.1 μg/mL for the nanoparticles prepared from 2 mg/mL catechins and 0.5 mg/mL gelatin, respectively, against DPPH and ABTS•$^+$ (**Table 3**). The EC_{50} values approximately decrease with the decrease of catechin to gelatin ratios for preparing the nanoparticles. This result indicated that the nanoparticles with a high loading content of catechins exhibited potent DPPH and ABTS•$^+$ radical scavenging activity, and those prepared with lower catechins to gelatin ratios had lower activity. **Figure 5** also showed the free radical scavenging responses to DPPH• and ABTS•$^+$ of the catechin/gelatin nanoparticles (2 mg/mL catechins/0.5 mg/mL gelatin) stored for a predetermined time. The EC_{50} values did not significantly increase after three weeks of storage at room temperature. The result suggested that catechins could be protected by the nanoparticles and that the antioxidant activity of catechins was almost completely retained.

Interactions of Catechins with Enzyme. As shown in **Figure 6**, the inhibition ratio of trypsin was 26.4%, when the catechin concentration was 0.5 mg/mL. Tea catechins contain polar, hydrophilic hydroxyl groups and hydrophobic galloyl groups in their molecular structure (*40*). The phenolic hydroxyl groups can form hydrogen bonds with the polar groups of protein while the hydrophobic amino acids present in enzyme protein, such as proline, phenylalanine, and tyrosine, can be strongly bound to the galloyl groups in catechins through hydrophobic association.

Therefore, the composition and quantity of the polar/hydrophobic groups in enzyme proteins will affect the formation and

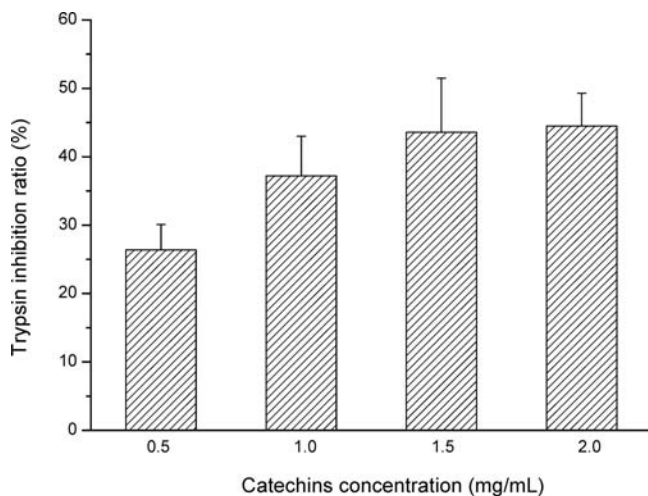

Figure 6. Inhibition ratios of trypsin's activity by different concentrations of catechins. The catechin concentrations are 0.5, 1.0, 1.5, and 2.0 mg/mL.

stability of hydrogen bonds and hydrophobic association between catechins and different enzymes. The cooperative effects of hydrophobic association and hydrogen bond formation between catechins and the trypsin are able to change the enzyme molecular configuration, resulting in a significant inhibitory effect on this digestive enzyme. The inhibition ratio of trypsin increased from 26.4% to 46.5%, when the catechin concentration increased from 0.5 mg/mL to 2.0 mg/mL (**Figure 6**). These results suggested that the inhibitory effect increased nonlinearly with the increase of catechin concentration.

Enzyme-Responsive Characteristics of Nanoparticles. Polyphenols are known to bind with collagen via noncovalent interactions. The side chain hydroxyl group of the amino acids serine and hydroxyproline, the carboxyl group of aspartic acid, the amino group of lysine, and the amide group of aspargine are considered as the potential interacting sites for the formation of hydrogen bonds with catechins and therefore inhibit the enzyme's activity toward collagen (*41*). Similarly, catechins can interact with gelatin through the formation of the same hydrogen bond. **Figure 7A** shows the variation of light transmittance T_{trans} (λ = 500 nm) of the catechin/gelatin nanoparticles in trypsin-free or trypsin-containing medium. The turbidity change took place in the nanoparticle-containing medium, and the decrease in stability or disintegration of nanoparticles could be defined as the increase in light transmittance (T_{trans}) of the solutions. In the trypsin-free medium, the low T_{trans} values suggested that the catechin/gelatin nanoparticles were stable. In contrast, in the trypsin-containing medium, the light transmittance increased, suggesting the disintegration of nanoparticles due to the instability of nanoparticles. The instability of nanoparticles was possibly due to the high pH value (pH 7.9) of the trypsin buffer solution because the pK_a values of EC, EGC, ECG, and EGCG were in the range of 7.73−8.72.

Therefore, the stability of nanoparticles in the trypsin-containing medium was additionally examined by determining the hydroxyproline release from digested gelatin. The catechin/gelatin nanoparticles exhibited catechin-dose dependent inhibition of trypsin-digestive activities against gelatin. The nanoparticles with higher catechin loading content showed better stability to release less hydroxyproline in trypsin-containing medium (**Figure 7B**). The stability of the catechin/gelatin nanoparticles against trypsin could be due to the effectiveness of the catechins in exhibiting specific interaction with gelatin that protects the active sites in gelatin (through interaction with polyphenols) recognized by trypsin. Additionally, the inhibitory effect on trypsin's activity

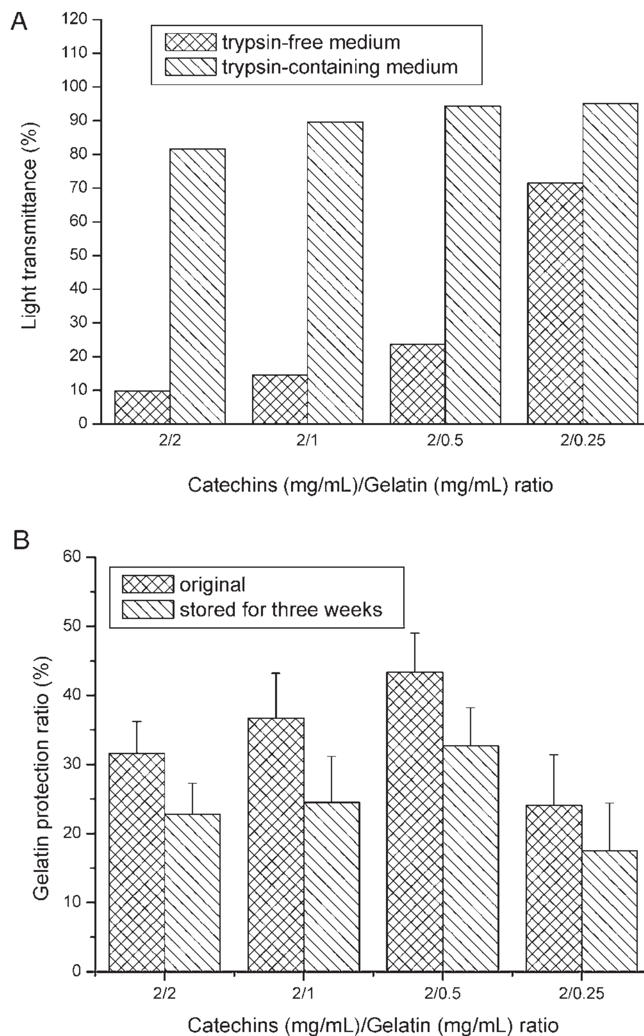

Figure 7. Enzyme-responsive characteristics of catechin/gelatin nanoparticles: (**A**) light transmittance T_{trans} (λ = 500 nm) of the catechin/gelatin nanoparticles in trypsin-free or trypsin-containing medium and (**B**) gelatin protection ratios of the catechin/gelatin nanoparticles against trypsin digestion.

increased with the increase of catechin loading content. The nanoparticles prepared from 2 mg/mL catechins and 2 mg/mL gelatin exhibited 31% inhibition of trypsin against the degradation of gelatin. The inhibition increased with an increase in the catechin to gelatin ratios of the nanoparticles, and 43% inhibition has been observed from the nanoparticles prepared from 2 mg/mL catechins and 0.5 mg/mL gelatin.

Previous studies employed traditional methods such as polymerization, cross-linking, ionic gelation, or a polyelectrolyte complex to prepare nanoparticles for protecting or delivering natural antioxidants such as quercetin, glycyrrhizinate, tea catechins, and ellagic acid (*23−27*). Our study provides a novel self-assembly nanotechnology to prepare nanoparticles comprising tea catechins and gelatin by directly mixing these two components.

LITERATURE CITED

(1) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933–956.

(2) Dugas, A. J., Jr.; Castaneda-Acosta, J.; Bonin, G. C.; Price, K. L.; Fischer, N. H.; Winston, G. W. Evaluation of the total peroxyl radicalscavenging capacity of flavonoids: structure−activity relationships. *J. Nat. Prod.* **2000**, *63*, 327–331.

(3) Levites, Y.; Amit, T.; Mandel, S.; Youdim, M. B. H. Neuroprotection and neurorescue against A beta toxicity and PKCdependent release of non-amyloidogenic soluble precursor protein by green tea polyphenol (−)-epigallocatechin-3-gallate. *FASEB J.* **2003**, *17*, 952–954.
(4) Fassina, G.; Vene, R.; Morini, M.; Minghelli, S.; Benelli, R.; Noonan, D. M.; Albini, A. Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallocatechin-3-gallate. *Clin. Cancer Res.* **2004**, *10*, 4865–4873.
(5) Terao, J.; Piskula, M.; Yao, Q. Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipids bilayers. *Arch. Biochem. Biophys.* **1994**, *308*, 278–284.
(6) Bursill, C. A.; Roach, P. D. Modulation of cholesterol metabolism by the green tea polyphenol (−)-epigallocatechin gallate in cultured human liver (HepG2) cells. *J. Agric. Food Chem.* **2006**, *54*, 1621–1626.
(7) Miura, Y.; Chiba, T.; Tomita, I.; Koizumi, H.; Miura, S.; Umegaki, K.; Hara, Y.; Ikeda, M.; Tomita, T. Tea catechins prevent the development of atherosclerosis in apoprotein E-deficient mice. *J. Nutr.* **2001**, *131*, 27–32.
(8) Meeran, S. M.; Mantena, S. K.; Elmets, C. A.; Katiyar, S. K. (-)-Epigallocatechin-3-gallate prevents photocarcinogenesis in mice through interleukin-12-dependent DNA repair. *Cancer Res.* **2006**, *66*, 512–520.
(9) Ahmed, S.; Wang, N.; Lalonde, M.; Goldberg, V. M.; Haqqi, T. M. Green tea polyphenol epigallocatechin-3-gallate (EGCG) differentially inhibits interleukin-1β-induced expression of matrix metalloproteinases-1 and -13 in human chondrocytes. *J. Pharmacol Exp Ther* **2004**, *308*, 767–773.
(10) Garbisa, S.; Sartor, L.; Biggin, S.; Salvato, B.; Benelli, R.; Albini, A. Tumor gelatinases and invasion inhibited by the green tea flavanol epigallocatechin-3-gallate. *Cancer* **2001**, *91*, 822–832.
(11) Bae, J. Y.; Choi, J. S.; Choi, Y. J.; Shin, S. Y.; Kang, S. W.; Han, S. J.; Kang, Y. H. (−)Epigallocatechin gallate hampers collagen destruction and collagenase activation in ultraviolet-B-irradiated human dermal fibroblasts: Involvement of mitogen-activated protein kinase. *Food Chem. Toxicol.* **2008**, *46*, 1298–1307.
(12) Junyaprasert, V. B.; Mitrevej, A.; Sinchaipanid, N.; Boonme, P.; Wurster, D. E. Effect of process variables on the microencapsulation of vitamin A palmitate by gelatin-acacia coacervation. *Drug Dev. Ind. Pharm.* **2001**, *27*, 561–566.
(13) Yeo, Y.; Bellas, E.; Firestone, W.; Langer, R.; Kohane, D. S. Complex coacervates for thermally sensitive controlled release of flavor compounds. *J. Agric. Food Chem.* **2005**, *53*, 7518–7525.
(14) Leclercq, S.; Milo, C.; Reineccius, G. A. Effects of cross-linking, capsule wall thickness, and compound hydrophobicity on aroma release from complex coacervate microcapsules. *J. Agric. Food Chem.* **2009**, *57*, 1426–1432.
(15) Linforth, R. S. T.; Pearson, K. S. K.; Taylor, A. J. In vivo flavor release from gelatin-sucrose gels containing droplets of flavor compounds. *J. Agric. Food Chem.* **2007**, *55*, 7859–7863.
(16) Yosha, I.; Shani, A.; Magdassi, S. Slow release of pheromones to the atmosphere from gelatin-alginate beads. *J. Agric. Food Chem.* **2008**, *56*, 8045–8049.
(17) Wang, Q.; Song, Z.; Tang, J.; Yan, D.; Wang, F.; Zhang, H.; Guo, M.; Cao, A. Efficacy of 1,3-dichloropropene gelatin capsule formulation for the control of soilborne pests. *J. Agric. Food Chem.* **2009**, *57*, 8414–8420.
(18) Madene, A.; Jacquot, M.; Scher, J.; Desobry, S. Flavour encapsulation and controlled releases: A review. *Int. J. Food Sci. Technol.* **2006**, *41*, 1–21.
(19) Chen, X. G.; Lee, C. M.; Park, H. J. O/W emulsification for the self-aggregation and nanoparticle formation of linoleic acids modified chitosan in the aqueous system. *J. Agric. Food Chem.* **2003**, *51*, 3135–3139.
(20) Chiu, Y. T.; Chiu, C. P.; Chien, J. T.; Ho, G. H.; Yang, J.; Chen, B. H. Encapsulation of lycopene extract from tomato pulp waste with gelatin and poly(γ-glutamic acid) as carrier. *J. Agric. Food Chem.* **2007**, *55*, 5123–5130.
(21) Hamidi, M.; Azadi, A.; Rafiei, P. Hydrogel nanoparticles in drug delivery. *Adv. Drug Delivery Rev.* **2008**, *60*, 1638–1649.
(22) des Rieux, A.; Fievez, V.; Theate, I.; Mast, J.; Preat, V.; Schneider, Y. J.; Artursson, P. Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium. *Eur. J. Pharm. Sci.* **2005**, *25*, 455–465.
(23) Wu, Y.; Yang, W.; Wang, C.; Hu, J.; Fu, S. Chitosan nanoparticles as a novel delivery system for ammonium glycyrrhizinate. *Int. J. Pharm.* **2005**, *295*, 235–245.
(24) Hu, B.; Pan, C.; Sun, Y.; Hou, Z.; Ye, H.; 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) Wu, T. H.; Yen, F. L.; Lin, L. T.; Tsai, T. R.; Lin, C. C.; Cham, T. M. Preparation, physicochemical characterization, and antioxidant effects of quercetin nanoparticles. *Int. J. Pharm.* **2008**, *346*, 160–168.
(26) Sharma, G.; Italia, J. L.; Sonaje, K.; Tikoo, K.; Ravi Kumar, M. N. V. Biodegradable in situ gelling system for subcutaneous administration of ellagic acid and ellagic acid loaded nanoparticles: evaluation of their antioxidant potential against cyclosporine induced nephrotoxicity in rats. *J. Controlled Release* **2007**, *118*, 27–37.
(27) Li, H.; Zhao, X.; Ma, Y.; Zhai, G.; Li, L.; Lou, H. Enhancement of gastrointestinal absorption of quercetin by solid lipid nanoparticles. *J. Controlled Release* **2008**, *346*, 160–168.
(28) He, Q.; Lv, Y.; Yao, K. Effects of tea polyphenols on the activities of α-amylase, pepsin, trypsin and lipase. *Food Chem.* **2006**, *101*, 1178–1182.
(29) Neilson, A. P.; Green, R. J.; Wood, K. V.; Ferruzzi, M. G. High-throughput analysis of catechins and theaflavins by high performance liquid chromatography with diode array detection. *J. Chromatogr., A* **2006**, *1132*, 132–140.
(30) Zhao, H.; Dong, J.; Lu, J.; Chen, J.; Li, Y.; Shan, L.; Lin, Y.; Fan, W.; Gu, G. Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley (*Hordeum vulgare* L.). *J. Agric. Food Chem.* **2006**, *54*, 7277–7286.
(31) Kasai, N.; Nakatsubo, G. Size-exclusion chromatography of tea tannins and intercepting potentials of peptides for the inhibition of trypsin-caseinolytic activity by tea tannins. *J. Agric. Food Chem.* **2006**, *54*, 5149–5156.
(32) Tachibana, Y; Kurisawa, M; Uyama, H; Kobayashi, S. Thermo- and pH-responsive biodegradable poly(alpha-N-substituted gamma-glutamine)s. *Biomacromolecules* **2003**, *4*, 1132–1134.
(33) Mi, F. L.; Wu, Y. Y.; Lin, Y. H.; Sonaje, K.; Ho, Y. C.; Chen, C. T.; Juang, J. H.; Sung, H. W. Oral delivery of peptide drugs using nanoparticles self-assembled by poly(gamma-glutamic acid) and a chitosan derivative functionalized by trimethylation. *Bioconjugate Chem.* **2008**, *19*, 1248–1255.
(34) Lin, Y. H.; Mi, F. L.; Chen, C. T.; Chang, W. C.; Peng, S. F.; Liang, H. F.; Sung, H. W. Preparation and characterization of nanoparticles shelled with chitosan for oral insulin delivery. *Biomacromolecules* **2007**, *8*, 146–152.
(35) Lin, Y. H.; Sonaje, K.; Lin, K. M.; Juang, J. H.; Mi, F. L.; Yang, H. W.; Sung, H. W. Multi-ion-crosslinked nanoparticles with pH-responsive characteristics for oral delivery of protein drugs. *J. Controlled Release* **2008**, *133*, 141–149.
(36) Zhu, B.; Li, J.; He, Y.; Yoshie, N.; Inoue, Y. Hydrogen-bonding interaction and crystalline morphology in the binary blends of poly(ε-caprolactone) and polyphenol catechin. *Macromol. Biosci.* **2003**, *3*, 684–693.
(37) Pinotti, A.; Garcia, M. A.; Martino, M. N.; Zaritzky, N. E. Study on microstructure and physical properties of composite films based on chitosan and gelatin. *Food Hydrocolloids* **2007**, *21*, 66–72.
(38) Zaccaron, C.; Oliveira, R.; Guiotoku, M.; Pires, A.; Soldi, V. Blends of hydroxypropyl gelatin and poly(1-vinylpyrrolidoneco-vinyl acetate): Miscibility and thermal stability. *Polym. Degrad. Stab.* **2005**, *90*, 21–27.
(39) Muzolf, M.; Szymusiak, H.; Gliszczyńska-Świgło, A.; Rietjens, I. M. C. M.; Tyrakowska, B. pH-dependent radical scavenging capacity of green tea catechins. *J. Agric. Food Chem.* **2008**, *56*, 816–823.
(40) He, Q.; Shi, B.; Yao, K. Interactions of gallotannins with proteins, amino acids, phospholipids and sugars. *Food Chem.* **2006**, *95*, 250–254.
(41) Madhan, B.; Subramanian, V.; Rao, J. R.; Nair, B. U.; Ramasami, T. Stabilization of collagen using plant. Polyphenol: Role of catechin. *Int. J. Biol. Macromol.* **2005**, *37*, 47–53.

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