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Preparation and Functional Evaluation of Chitosan-EGCG Conjugates

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ABSTRACT: Chitosan, as a novel food additive, is proposed to conjugate with EGCG using a free radical grafting procedure. Chitosan is activated by hydroxyl free radicals produced by the redox reaction of hydrogen peroxide and ascorbic acid and subsequently react with EGCG. Chitosan-EGCG conjugate has demonstrated by UV, FTIR, and ^{1}H-NMR spectroscopy and exhibits a dramatical increase in DPPH free radical scavenging activity. In addition, the LC (low-molecular-weight chitosan)-EGCG conjugate is used to prepare O/W emulsion and then monitor the average droplet size, polydispersity index, zeta-potential and transmission changes during centrifuge process, results show that the conjugate exhibited excellent emulsifying activity and superior emulsifying stability as compared with chitosan. Therefore, LC-EGCG conjugate could be used as an efficient natural food antioxidant and emulsifier. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 39732.

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INTRODUCTION

Chitosan, a deacetylated form of chitin, having a subunit of β-(1-4)-2-amido-2-deoxy-β-D-glucopyranose [Figure 1(a)], is the second most abundant, naturally occurring biopolymer and is found in the exoskeleton of crustaceans, in fungal cell walls, and in other biological material.[1] Chitosan has received increased attention for its commercial applications in food industry.[2] Furthermore, chitosan exhibits the potential to be used as food supplements with antitumor, antiulcer, antiuricemic and hypo cholesterolaemic properties.[3–5]

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions.[1] Studies have shown that chitosan possess antioxidative activity, which was effected by the deacetylation degree, molecular weight and substituted groups of chitosan,[6–8] but the activity was weaker than phenols, which are most often utilized as antioxidants in food, and was limited by its water solubility, hence, chitosan can not be a practical antioxidant. In the literature, many studies reported that the conjugation of phenols, such as gallic acid,[9–11] catechin,[11] caffeic acid,[12] eugenol,[1] rutin,[13] and quercetin,[13] onto chitosan have an appreciable improvement on antioxidative activity compared to native chitosan. But (−)-epigallocatechin-3-gallate [EGCG, Figure 1(b)], which is the most abundant polyphenol in green tea with potent antioxidant and chemopreventive activities,[14] seldom used in reaction with chitosan, except the study of Sousa et al.,[13] who enzymatically functionalized chitosan with EGCG. In this manuscript, another method was utilized for preparing chitosan-EGCG conjugates and the products will be compared with the enzymatic one.

Emulsions are intimate mixtures of two immiscible liquids in which one phase is dispersed throughout the other as small discrete droplets.[15] Two main parameters which usually affect emulsification and stabilization of emulsions are O/W interfacial tension and viscosity of the external phase (i.e., chitosan solution).[16] One of the most important methods of improving the stability of emulsions is to utilize emulsifiers.[17] The present of hydrophilic amino groups and hydrophobic acetamido groups make chitosan could be used as an emulsifier in acidic condition.[18] Payet and Terentjev[16] monitored the surface tension of the water–paraffin interface and shown that although chitosan itself had only a weak surface activity, it enhanced the stability of O/W emulsions by increasing the matrix viscosity and forming a dense polyelectrolitic brush on the water side of oil–water interface. Li and Xia[3] reported that 1% chitosan showed almost the same emulsifying activities as 1% gum Arabic but the emulsifying stabilities of 1% chitosan was much higher than that of 1% gum Arabic.

To improving the emulsifying property of chitosan, its modification via conjugating with proteins was investigated. Lysozyme-chitosan conjugate was prepared through Maillard reaction and its emulsifying properties, especially emulsion

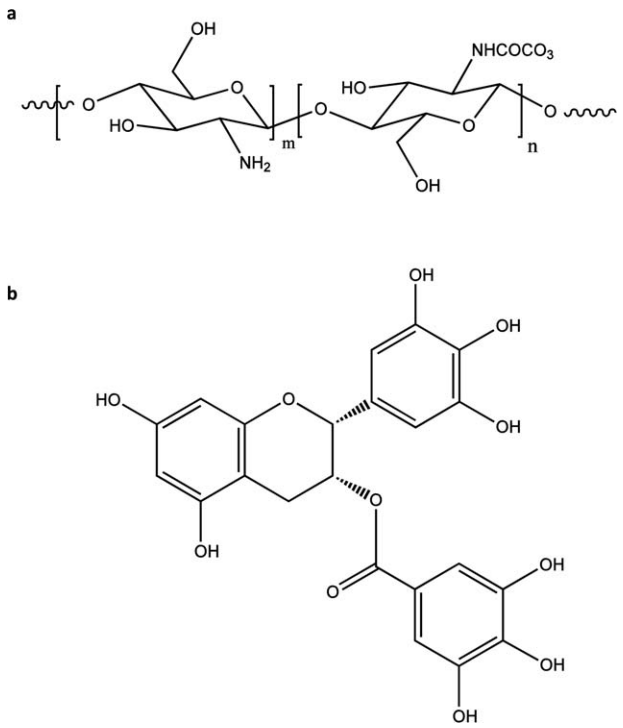

Figure 1. Chemical structures of chitosan (a) and EGCG (b).

stability, were improved by conjugation[19]; Whey protein isolate and chitosan were conjugated through Maillard reaction and the product formed after 1 day of reaction between WPI and chitosan at a mass ratio of 1 : 4 at 50°C showed a 10-fold increase in emulsion stability compared to their physical mixture.[20] Although chitosan-polyphenol conjugates has been studied in same literatures, but few data are available about their emulsifying properties changes.

Our work is similar to the study of Curcio et al.[11] in that we are using a free radical grafting procedure. An ascorbic acid/hydrogen peroxide pair was chosen as redox initiator system. The interaction mechanism between the two components of the redox pair involves the oxidation of ascorbic acid by H_2O_2 with the formation of hydroxyl radicals, which initiate the reaction via attacking the reactive amino group, H-atoms in α-methylene (CH_2) or hydroxyl groups (OH) of the hydroxylmethylene group of the chitosan, at those sites, the insertion of phenolics may occur.[11,21]

In this article, we focus our attention on the development of a biopolymer based-antioxidant and emulsifier for use as a commodity polymer food additive. Here, we consider chitosan as a biopolymer back bone, of which the antioxidant property and the emulsifying property have to be developed.

This work is thus based on a molecular design of chitosan as a novel biopolymer-based antioxidant and emulsifier by demonstrating the preparation steps of conjugating with EGCG to obtain chitosan—EGCG. The work also studies the use of chitosan—EGCG as an antioxidant additive by showing the antioxidative potential of chitosan—EGCG via a DPPH free radical model and the use as an emulsifier additive by measuring the physical characteristics of chitosan—EGCG conjugates stabilized emulsions.

EXPERIMENTAL

Materials and Chemicals

(−)-Epigallocatechin gallate (EGCG, ≥95%, from green tea), three commercial chitosan products with different molecular weights (low-molecular-weight chitosan, 50–190 kDa; medium-molecular-weight chitosan, 190–310 kDa; high-molecular-weight chitosan, 310–375 kDa, all with deacetylation degree of 75–85%), Folin-Ciocalteu reagent and 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH) were all purchased from Sigma-Aldrich (St Louis, MO). Medium chain triglyceride (MCT) oil was obtained from Lonza (NJ). Dialysis bag (MWCO 12,000–14,000 Da) was provided by Biodee Biotechnology (Beijing, China). All other chemicals used were of analytical grade, unless otherwise stated.

Synthesis of Chitosan-EGCG Conjugates

The synthesis of EGCG-chitosan conjugates was performed according to the methods of Curcio et al.[11] with some modifications. The use of H_2O_2/ascorbic acid redox system allowed the chemical functionalization of chitosan to be performed in mild reaction condition and without the generation of toxic compounds.[11]

Chitosan was dissolved in acetic acid solution (2%, v/v) and stirring overnight to ensure complete dispersion and dissolution. The volume of 100 mL chitosan solution (0.5%, w/v) was added into 150 mL glass flask and adjusted pH to 3.5 with 1 *M* HCl, 1 mL of 0.5 *M* H_2O_2 containing 0.025 g of ascorbic acid was added. The glass flask was shaken for 1 h in water bath at 40°C, then EGCG was introduced into the reaction flask and the mixture was maintained at 40°C with continuously shaking for 12 h.

The obtained polymer solution was introduced into dialysis bag and dipped into a glass vessel containing distilled water at room temperature with several changes of water until no free EGCG existed in the system, which was determined by UV absorption spectra analysis. The resulting solution was frozen and dried with a freezing-drying apparatus to afford vaporous solids.

Native chitosan was prepared according to mentioned above procedure but in the absence of redox pair and EGCG. Control chitosan was prepared according to mentioned above procedure but without adding EGCG.

Characterization of Chitosan-EGCG Conjugate

The samples were dissolved in 2% (v/v) acetic acid with magnetic stirring until complete dispersion and dissolution to obtain a certain concentration solutions. UV spectroscopy was recorded using a UV-1800 spectrophotometer (Shimadzu, Japan) in the wavelength range of 200–400 nm.

FT-IR spectrum of sample was obtained using a Spectrum 100 FT-IR spectrophotometer (Perkin-Elmer) in the range 4000–450 cm^{-1} by the KBr method.

The samples were dissolved in D_2O. Proton nuclear magnetic resonance (^{1}H-NMR) spectrum were recorded at room temperature using a Bruker UXNMR/XWIN-NMR Avance DPX400.

Determination of EGCG Content in Chitosan-EGCG Conjugate

The content of EGCG was determined using Folin-Ciocalteu reagent procedure, according to the literature[22] with some modifications. Adding 0.5 mL chitosan-EGCG conjugate solution (dissolved in 10 mL 2%, v/v acetic acid) and 2.5 mL 0.2 *N* Folin-Ciocalteu reagent into a 10 mL centrifuge tube, mixed thoroughly. After 5 min, 2 mL of Na_2CO_3 (7.5%, w/v) was added, then the mixture was allowed to stand for 2 h. The absorbance of conjugate was measured at 760 nm against control chitosan solution. The EGCG equivalent concentration of sample was obtained by using the equations obtained from the calibration curves of EGCG. These were recorded by employing five different EGCG standard solutions. The final results were expressed as mg EGCG equivalent / g sample.

Determination of Antioxidative Activity

The antioxidant activity of native chitosan, control chitosan and chitosan-EGCG conjugates was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, which was measured by using the method reported by Gong et al.[23] with some modification. The DPPH solution was freshly prepared in ethanol at a concentration of 1.75×10^{-4} mol/L. Taking 100 μL sample solutions (0.5 mg/mL, dissolved in of 2%, v/v acetic acid) into 10 mL centrifuge tube and adding 1.9 mL distilled water and 2 mL DPPH solution, vibrated for 20 s at room temperature. The absorbance of the mix at 517 nm was recorded after reacting for 1 h in the dark. A control, in which the sample was replaced by ethanol, was measured in the same way. The scavenging activities of samples were measured as the decrease in absorbance of the DPPH, and it was expressed as percent scavenging of DPPH radicals calculated according to the following eq. (1),

$$\text{Scavenging rate}\,(\%) = \left(1 - \frac{A}{A_0}\right) \times 100 \qquad (1)$$

where A_0 is the absorbance of a standard that was prepared in the same conditions, but without any samples, and A is the absorbance of sample.

Emulsion Preparation

Emulsions were prepared with the methods as previously described with some modification.[24,25] The sample (native chitosan and control chitosan, LC-EGCG conjugates) solutions were prepared at a concentration of 0.5% (w/v) in acetate buffer of pH 3, stirred overnight at a speed of approximately 200 rpm to ensure complete dispersion and dissolution. Oil-in-water emulsions were prepared with 2% (v/v) MCT oil as the dispersed phase and sample solutions as the continuous phase. Briefly, the two phases were mixed at a speed of 10,000 rpm for 10 min with Ultra-Turrax T25 high- speed blender (IKA, Staufen, Germany) to form coarse emulsions, which were further homogenized using a Niro-Soavi Panda two-stage valve homogenizer (Parma, Italy) for three cycles at 60 Mpa. Emulsions exiting from the homogenizer were immediately cooled down to room temperature.

Determination of Droplet Size and Size Distribution

Droplet size and size distribution of emulsions were determined by dynamic light scattering using a Zetasizer Nano-ZS90 (Malvern Instruments, Worcestershire, UK) at a fixed angle of 90°. Emulsions were diluted with distilled water prior to each measurement to minimize multiple scattering effects. Results were described as cumulants mean diameter (size, nm) for droplet size, polydispersity index (PdI) for size distribution.[26]

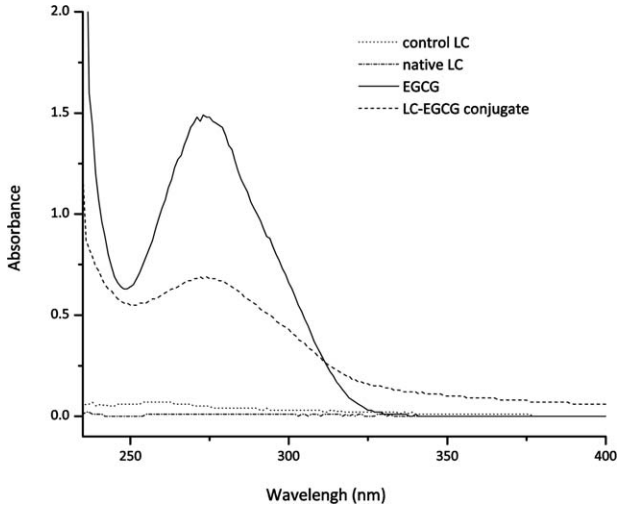

Figure 2. UV spectrum of native LC, control LC, LC-EGCG conjugate (at 2 : 1 molar ratio) and EGCG at a same concentration.

ζ-Potential Measurements

Diluted emulsions were injected directly into the chamber of a particle electrophoresis instrument (Nano-ZS90, Malvern Instruments, Worcestershire, UK). The ζ-potential was determined by measuring the direction and velocity of droplet movement in a well-defined electric field.[25]

Measurement of Emulsion Stability

The stability of the emulsions was measured by the LUMi-Sizer (L.U.M. GmbH, Germany), an instrument employing centrifugal sedimentation to accelerate the occurrence of instability phenomena such as sedimentation, flocculation, or creaming. Emulsion samples were subjected to centrifugal force, while near-infrared light illuminated the entire sample cell to measure the intensity of transmitted light as a function of time and position over the entire sample length simultaneously. Emulsion stability was shown as a space- and time-related transmission profile over the sample length. The instrumental parameters used for the measurement were as follows: volume, 1.8 mL of dispersion; 4000 rpm, $time_{Exp}$, 7650 s; time interval, 30 s; temperature, 25°C.[27]

Statistical Analysis

All experiments were conducted in duplicate. Data were subjected to analysis of variance (ANOVA) using the software package SPSS 18.0 for Windows (SPSS, Chicago). Differences between means were evaluated by Duncan methods at the 5% significance level.

RESULTS AND DISCUSSION

Characterization of Chitosan-EGCG conjugates

As depicted in Figure 2, UV spectra of native LC (low-molecular-weight chitosan) and control LC almost had no absorption from 200 to 400 nm. For the spectrum of EGCG, λ_{max} value

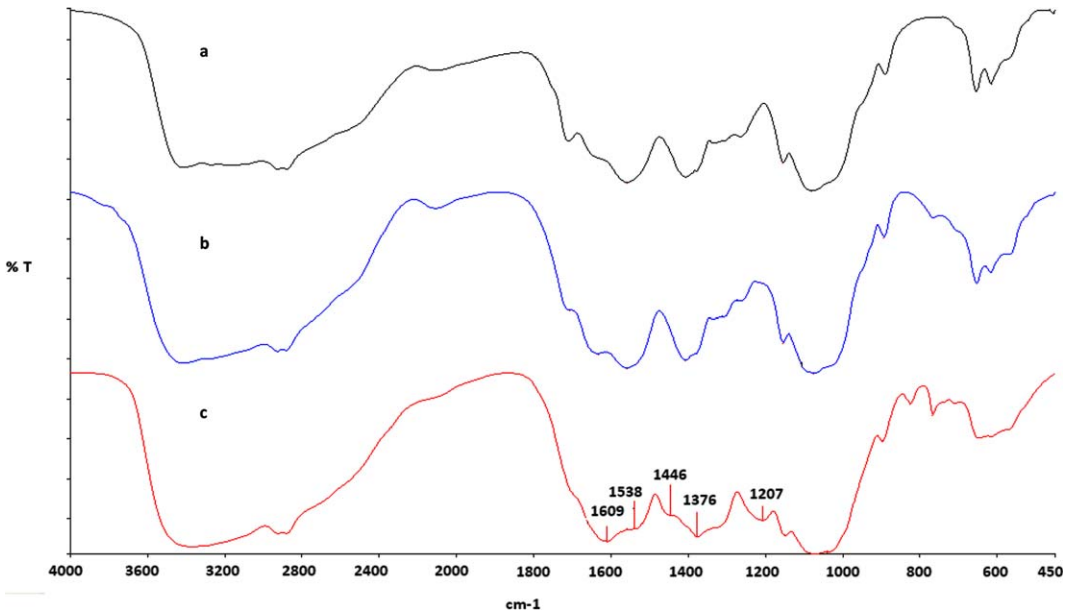

Figure 3. FTIR spectrum of native LC (a), control LC (b) and LC-EGCG conjugate (c, at 1 : 1 molar ratio). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

was found at 273 nm (π–π^* transition of the phenolic group).[28] This typical peak was identified in the spectrum of conjugate, which indicated that EGCG molecules inserted into chitosan when redox couple was added. To confirm these EGCG were covalent binding with chitosan, we performed FT-IR analysis.

The FT-IR spectrum of LC-EGCG conjugate was compared with the spectra of native LC and control LC. From the spectrum of native LC [Figure 3(a)] and control LC [Figure 3(b)], the strong absorption bands at 1638, 1560, and 1407 cm^{-1} were assigned to the amide bands I (C═O stretching vibration), II (N—H bending vibration and III (C—N stretching vibration) respectively.[29] It should be noted that the absorption band of control LC at 1638 cm^{-1} (N—H deformation vibration in amino groups) became stronger and the peak at 1711 cm^{-1} (carbonyl stretching vibration) became weak when compared with native LC, which might referred to the deacetylation of activation process. The absorption bands at 1154 cm^{-1} (anti-symetric stretching of C—O—C bridge) and 1080 cm^{-1} (skeletal vibration involving the C—O stretching) were characteristics of saccharide structure.[13]

The formation of new peaks at 1609, 1538, and 1446 cm^{-1} observed in the spectrum of LC-EGCG conjugate [Figure 3(c)] could be attributed to the C═C skeletal vibration in aromatic ring.[11] Besides, the multiple tiny absorption peaks of control LC in 1400–1200 cm^{-1} band were covered with wide and strong peaks when compared with the spectrum of conjugate, which was similar to the spectrum of EGCG (spectrum not shown). Moreover, peak at 1560 and 1407 cm^{-1} nearly disappeared in conjugate and the characteristic peaks of saccharide structure at 1154 and 1080 cm^{-1} had a red shift. These changes indicated that hydroxyl radical catalyzed the conjugation of EGCG onto chitosan.[13]

In addition, ^{1}H-NMR was performed to verify the conjugation. Spectrum a in Figure 4 is for control LC. The characteristic peaks for glucosamine residues were at 4.8 ppm for the anomeric proton on C-1 and at 3.0 ppm for the proton on C-2. The peak for the methyl protons of the *N*-acetylglucosamine residues was at 1.9 ppm and the integration of this peak indicated the degree of deacetylation. The peaks between 3.5 and 4.0 were for the protons on C-3, C-4, C-5, and C-6.[30] There was no clearly difference between the spectra of control LC and native LC (spectrum not shown). Figure 4(b) showed the spectrum of the LC-EGCG conjugate. Considerable similarities between spectrum b and a demonstrated that the conjugate was a derivative of chitosan. A new peak at 7.0 ppm was visible in the region for aromatic protons (between 6.0 and 7.5 ppm), which was a characteristic peak of EGCG, and this peak became more notable as the combined EGCG equivalents increasing. These results proved the conjugation of EGCG, while the weak downfield signals indicated that the degree of modification by EGCG-derived moieties was low.[10,29,30]

Effects of Molecular-Weight of Chitosan on Conjugation

Bioactivity of chitosan is strongly dependent on inter- and intra- molecular hydrogen bonds.[31] Chitosan is a polymer and has relatively longer molecular chain, which would tangle or aggregation in solution and produce strong hydrogen bond, bring about low solubility and high viscosity,[12] consequently affecting the biological activity of chitosan.

To investigate the effects of chitosan's molecular-weight on the conjugation, chitosans with different molecular-weights were performed at a molar ratio of 7 : 1 (chitosan/EGCG) and the results were illustrated in Figure 5. The conjugated EGCG equivalent of LC achieved 110.35 ± 2.78 mg/g and was significantly higher than that of MC and HC ($P < 0.05$), which can be attributed to less effect of H bonds in LC molecules improved reaction activities, where the equivalents of MC and LC had no significant difference ($P > 0.05$). Similarly, Aytekin et al.[12]

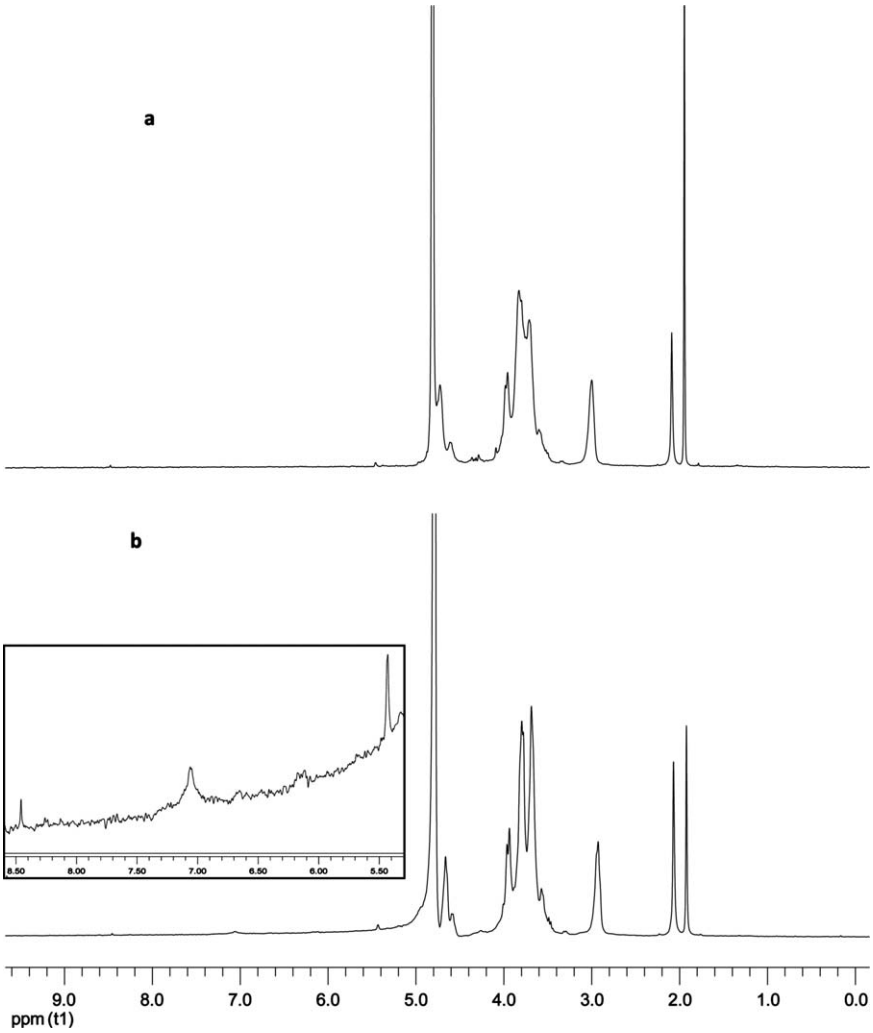

Figure 4. ^{1}H-NMR spectrum of control LC (a) and LC-EGCG conjugate (b, at 1 : 1 molar ratio).

studied the grafting of caffeic acid with different molecular-weight chitosans (HC, 544 kDa; MC, 198 kDa; LC, 47 kDa) and found LC had the highest grafting ratio. Aytekin et al.[12] also found a decreasing trend of grafting ratio as molecular-weight increasing, which was somewhat different from our study, the reason can be the amounts of H bonds in MC and HC molecules had no significant difference because of insufficient molecular-weights difference between MC and HC (190–310 and 310–375 kDa, respectively) in our study.

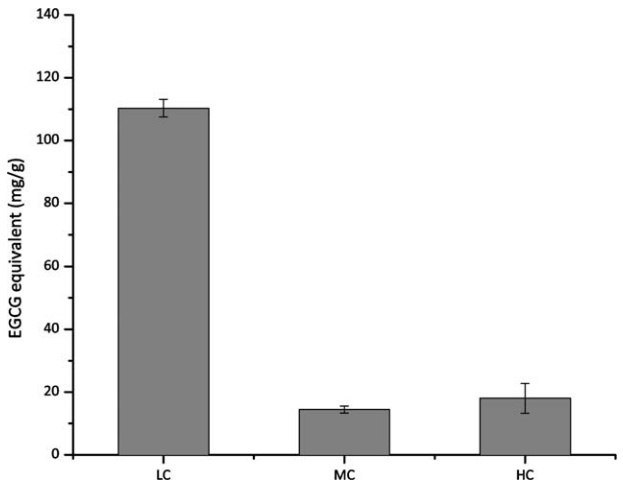

Figure 5. Conjugated EGCG equivalent of chitosan-EGCG conjugate prepared with different molecular-weight chitosan.

Effects of LC/EGCG Initial Molar Ratio on Conjugation

The effects of initial LC/EGCG molar ratio on the conjugation were shown in Figure 6. When calculating the molar ratio, molar concentration of LC was expressed as molar equivalents of the sugar residues assuming complete deacetylation.[29] As expected, conjugated EGCG equivalents increased significantly (from 85.56 ± 7.79 mg/g to 212.59 ± 3.82 mg/g) as the LC/EGCG molar ratio decreased from 10 : 1 to 1 : 1 ($P < 0.05$). Aytekin et al.[12] reported the similar results in the grafting of caffeic acid into chitosan: as the molar ratio of chitosan (47 kDa, 10 m*M*)/caffeic acid decreased from 10 : 1 to 1 : 1, the caffeic acid contents in products increased from 1.3 to 15.0%. Comparing the initial EGCG contents, the conjugation contents were dramatically lower. EGCG molecules in the majority in reaction system might undergo dimerization processes with the

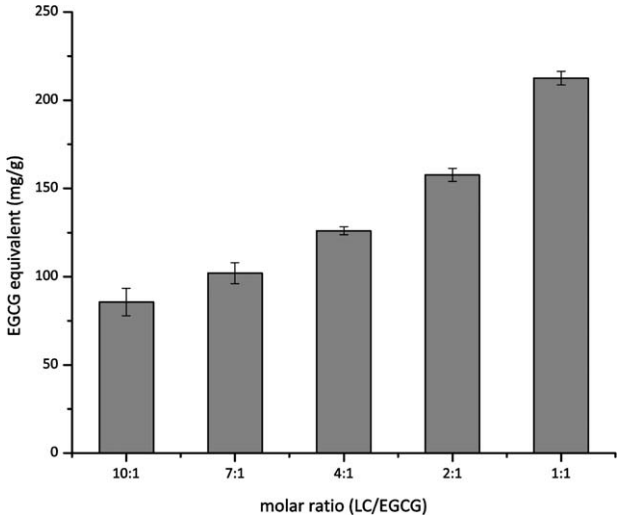

Figure 6. Conjugated EGCG equivalent of LC-EGCG conjugate prepared with different initial molar ratio (LC/EGCG).

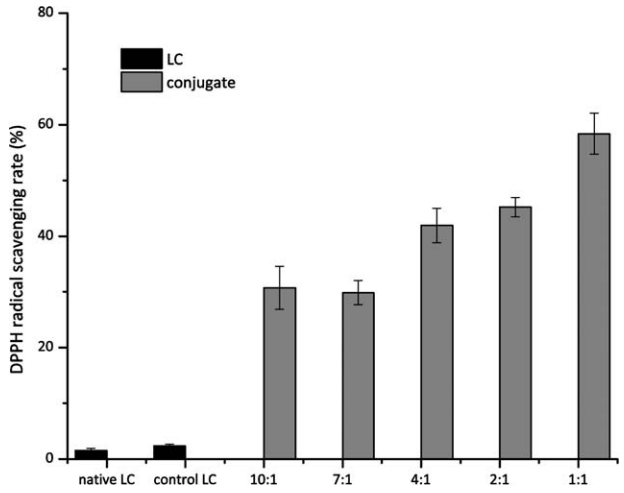

Figure 7. DPPH radical scavenging rate of LC-EGCG conjugate prepared with different initial molar ratio, compared with native LC and control LC.

reaction between the hydroxyl radical and aromatic ring in the ortho or para position relative to the hydroxyl group[32] rather than conjugating with chitosan.

Evaluation of Antioxidative Activity

DPPH radical scavenging capacities of the conjugates with different molar ratios were compared with native LC and control LC. The results depicted in Figure 7 showed that the scavenging rates of native LC and control LC were very low (less than 5%) and had no significant difference in our operating conditions ($P > 0.05$), while the scavenging rates of conjugates were dramatically higher as a consequence of the H-donating hydroxyl groups of EGCG in the polymer backbone. The percentage of increase of the DPPH radical scavenging rate of LC-EGCG conjugates at 1 : 1 molar ratios relative to native LC was the highest and reached about 57%. Similarly, the DPPH radical scavenging rate of chitosan-EGCG conjugate prepared by Sousa et al.[13] via enzymatic synthesis increased about 54%. Curcio et al.[11] used the same method as this article to prepare gallic acid, catechin and chitosan conjugates and study revealed that the DPPH radical scavenging rate of two compounds increased 78 and 84% respectively. Jung et al.,[1] Pasanphan and Chirachanchai,[10] and Božič et al.[33] also found there was a general enhancement of DPPH free-radical scavenging activities of phenol grafted chitosan.

As the molar ratio decreased from 10 : 1 to 1 : 1, the scavenging rates of conjugates were ascended gradually and nearly reached 60% at 1:1 molar ratio. These results shown radical scavenging activities were proportional to the conjugated EGCG contents, which was similar with literatures. Jung et al.[1] found the DPPH radical scavenging activity of chitosan-eugenol derivatives increased from about 60% to nearly 100% with the graft yield of eugenol increased from 10.1 to 21.7%. Aytekin et al.[12] also found as caffeic acid concentration in the low-molecular-weight chitosan and caffeic acid conjugates increased from 1.2 g/100 g to 15 g/100 g, the half-inhibition concentration (IC_{50}) of DPPH radical scavenging activity decreased sharply from about 0.7 mg/mL to less than 0.1 mg/mL.

Droplet Characteristics of Emulsions Stabilized by LC-EGCG Conjugate

The mean droplet size (diameter), PdI and zeta-potential (ZP) of different samples stabilized emulsions were summarized in Table I. Particle size measurement of the emulsion by using Laser particle size analyzer is a scientific and objective method to evaluate the emulsifying activity. According to the formula to

Table I. The Mean Particle Size (Diameter), PdI and ζ-Potential (ZP) of Emulsions Stabilized by Native LC, Control LC, and LC-EGCG Conjugates

Emulsion prepared by	Size (d.nm)	PdI	ZP (mV)
Native LC	653.0 ± 15.63^{b}	$0.618 \pm 0.028^{b'}$	$56.4 \pm 2.40^{e''}$
Control LC	1376 ± 89.10^{c}	$1^{c'}$	$18.9 \pm 1.06^{a''}$
10 : 1 conjugate	238.7 ± 2.55^{a}	$0.131 \pm 0.024^{a'}$	$44.0 \pm 1.06^{cd''}$
7 : 1 conjugate	225.5 ± 1.84^{a}	$0.130 \pm 0.011^{a'}$	$41.3 \pm 3.18^{c''}$
4 : 1 conjugate	225.8 ± 1.20^{a}	$0.133 \pm 0.034^{a'}$	$31.3 \pm 3.11^{b''}$
2 : 1 conjugate	231.3 ± 3.04^{a}	$0.123 \pm 0.028^{a'}$	$46.8 \pm 3.46^{cd''}$
1 : 1 conjugate	254.4 ± 3.75^{a}	$0.133 \pm 0.025^{a'}$	$48.5 \pm 0.21^{d''}$

Different superscript letters (a–c, a′–c′,a″–e″) in each column mean significant difference.

calculate emulsifying activity index (EAI) ($EAI = 3\varphi/Rm$, φ was the oil volume fraction in the emulsion, m was the emulsifiers mass in the aqueous phase, and R was average drop radius), we can concluded that there was a negative correlation between the average drop radius and emulsifying activity.[34] The mean droplet size of native LC stabilized emulsions was about 650 nm while control LC was more than 1 μm, which meant when LC was activated by hydroxyl radical, it could not adsorbed at oil-water interface effectively as a consequence of the destruction of hydrophobic acetyl amino, thus making its emulsifying activity decreased dramatically. The conjugation of EGCG onto LC resulted in marked reduction in droplet size and the size had no significant change as the molar ratio reduced from 10 : 1 to 1 : 1 ($P > 0.05$). The PdI of all the conjugates stabilized emulsions were around 0.13 and significantly smaller than the other two emulsions ($P < 0.05$). From the PdI results, we can conclude that the emulsions stabilized by conjugates had a relatively uniform droplet distribution. Overall, it appears that the conjugation of EGCG had significantly improved the emulsifying activity of LC. EGCG molecule has both hydrophobic benzene ring and hydrophilic phenolic hydroxyl groups. Therefore, the presence of EGCG linked covalently to LC could enhance the amphipathy of LC and make it better adsorbed at water–oil interface, so as to package oil droplets more effectively and improve the emulsifying activity of LC.

Chitosan is a positively charged polysaccharide and the charge derived from the protonated amino. ZP indicates the strength of the exclusion between chitosan molecules adsorbed at oil–water surface, which reflects charged state of chitosan molecules in a certain extent. The greater the ZP, more charged the molecules. As can be seen from Table I, all the emulsions were positive charged. The net charge on control LC coated droplets (18.9 ± 1.06 mV) was the least and significantly less positive than native LC coated droplets (56.4 ± 2.40 mV) ($P < 0.05$), which might be due to the less absorption of control LC onto the droplets surface since its decreased emulsifying activity after activation process. The net charge on conjugates coated droplets were more positive than control LC coated droplets, owing to the covalent insertion of EGCG into LC. Besides, molar ratio had no significant effects on the net charge of conjugates coated droplets.

Emulsion Stability

The stabilities of emulsions were shown as a space- and time-related transmission profile over the sample length. At the start of the measurement, the emulsion was intact, and very little light was transmitted at any point along the sample cell. As the sample was centrifuged, the heavier and more transparent aqueous phase moved to the bottom, which gave higher transmission; the lighter and less transparent oil phase moved to the top, while a cream layer was shown as a trough due to its low transparency.[35] The more change of the transmission with the centrifugation meant the less stability of the emulsion. Furthermore, the creaming was reflected quantitatively by plotting the integrated transmission profiles against the measuring time (Figure 8), and the slope of the resultant curve was inversely related to the stability.[27]

The stability of emulsion formed with control LC was the poorest, a clear cream layer and highest slope value were evident;

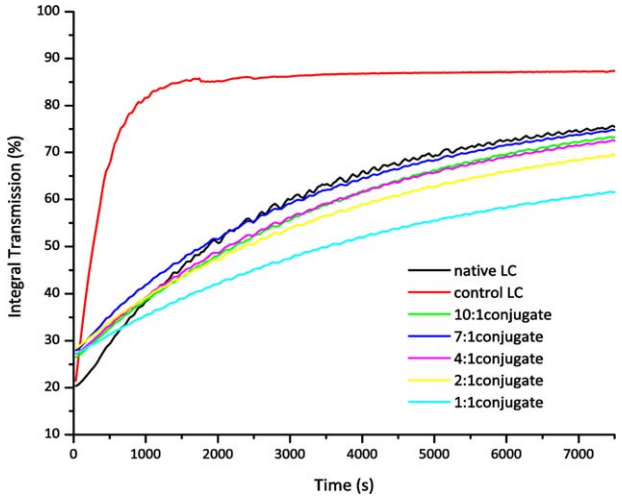

Figure 8. The stability of emulsions prepared with LC-EGCG conjugates of different molar ratios, expressed as integrated transmission-time plots obtained by the LUMisizer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

emulsion formed with native LC had a greater stability than control LC. The following three reasons may contribute to the result: first, lower emulsifying activity made control LC could not adsorbed at interface effectively, so as to promote droplets coalescence and the emulsion became unstable[3]; secondly, the native LC formed emulsion had a higher apparent viscosity (data not shown), according to Stokes' law, the greater the viscosity of the emulsion, the slower the settling or floating rate of the emulsion droplets, thus enhanced the physical stability of the emulsion to a certain extent; finally, the droplets in native LC formed emulsion had much more positive charges than control LC, in turn, electrostatic repulsion between the droplets increased and made it hard to aggregate.[34]

As EGCG conjugated with LC, the emulsifying stability of LC improved significantly ($P < 0.05$); the stabilities of emulsions formed with LC-EGCG conjugates were higher than control LC and native LC, which attributed a lot to the conjugates' strong ability to reduce the interfacial tension, the more positive charges of droplets also improved the stability. Comparing the stabilities of emulsions formed with conjugates of different molar ratios, the stability of 1 : 1 conjugate stabilized emulsion was somewhat better than the others.

CONCLUSIONS

A biopolymer—bound natural antioxidant and emulsifier was successfully prepared by conjugating EGCG onto a chitosan chain. The conjugation was performed via a novel free radical grafting procedure and was testified by UV, FT-IR and ^{1}H-NMR spectroscopic. Comparing to native LC and control LC, LC-EGCG conjugates had dramatically higher DPPH free radical scavenging activity and the scavenging ratio were proportional to conjugation ratio. Emulsions formed with LC-EGCG conjugates had significant smaller particle size and PdI than native LC and control LC stabilized emulsions, which meant the conjugation of EGCG improved the emulsifying activity of LC effectively; besides, LUMi-Sizer experiments shown emulsions

formed with conjugates have greater emulsifying stabilities. Preliminary studies about chitosan-polymer conjugates basically focused on the enhancement of antioxidant activity, this article will be a pioneer to study the emulsifying properties of chitosan-polymer conjugates and will contribute to build up a fully functional evaluation system of chitosan-polymer conjugates and the other chitosan derivatives. However, further research needs to be performed for qualitatively and quantitatively identification of the chitosan-EGCG conjugates.

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