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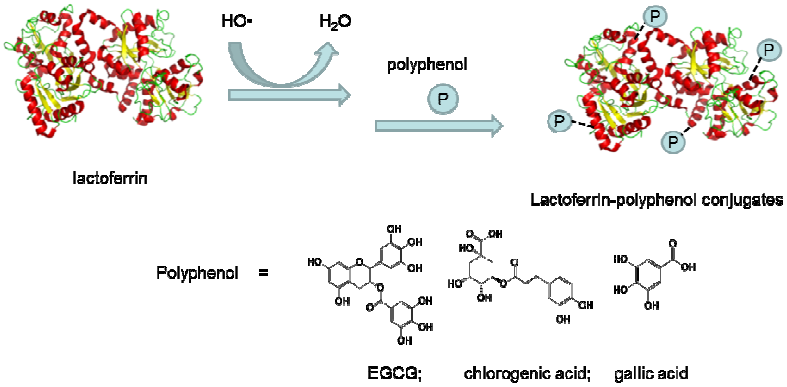
Graphical Abstract



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ARTICLE TYPE

Structural characterization and functional evaluation of lactoferrin-polyphenol conjugates formed by free-radical graft copolymerization

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Covalent modifications of lactoferrin with EGCG, chlorogenic acid and gallic acid were performed by adopting a free-radical grafting procedure in aqueous media. The resulting LF-polyphenol conjugates were characterized in terms of structural and functional properties. Results showed that the covalent binding amount into LF molecule of EGCG, CA and GA was 68, 58 and 17 nmol mg^{-1}, respectively. Covalent insertion of polyphenols into LF molecule was testified by SDS-PAGE and MALDI-TOF-MS analysis, and especially the molecular weight was increased from 84011 Da (LF) to 85906 Da (LF-CA conjugate). The circular dichroismand Fourier transform infrared analyses revealed that the content of α-helix increased and the contents of the remaining structures decreased, while the DSC data indicated that the thermal stability of LF-polyphenol conjugates was enhanced after the modification. In addition, the antioxidant activity of LF-polyphenol conjugates was 0.23- to 2.10-fold (ABTS•$^{+}$scavenging assay), and 0.04- to 2.19-fold (reducing power assay) higher than the control LF. Moreover, the covalent modification obviously changed the solubility and emulsifying properties of LF. The emulsifying property of LF-CA conjugate was better than those of LF-EGCG and LF-GA conjugates.

1. Introduction

Phenolic compounds, widely distributed in plants, are important natural food components with a large range of structures and functions, and generally possessing one or two aromatic rings bearing one or more hydroxy substituents. Currently, they are widely accepted as natural antioxidants, which have numerous bioactivities and can delay or prevent oxidative damage by reactive oxygen species.[1] It has suggested that ingesting polyphenols may be beneficial to human health.[2]

Recent investigations show that interactions between the minor phenolic components in plants with proteins, the main constituents of foods, occur very frequently during harvesting, storage and processing of plant foods. There are two potential types of interactions between phenolics and proteins: non-covalent linkage (hydrogen bonding, π-bonding, hydrophobic effect and ion pairing) and covalent linkage.[3] Depending on the nature of the phenolic compounds and the proteins, the interactions may prevent or enhance enzymatic digestion of proteins and either decrease or increase the antioxidant activity of phenolic compounds.[4-7] The interactions, especially covalent linkage, can lead to changes in structural, physicochemical properties and functionality of proteins.[8-9]

Bovine whey proteins (WP) are valuable food ingredients owing to their aggregation ability, provision of structure to foods, solubility over a wide pH range, and many other functional properties. [10-11]Lactoferrin (LF) as one of the most valuable WP is an active single-chain glycoprotein, and has obtained great interest in food and medicinal researches due to its health benefits.[12-13] The antioxidant properties of LF have been demonstrated in various biochemical environments.[14-15]It was suggested that a combination of LF with polyphenols may have synergistic effects on inhibiting cancer development.[16]

Phenolic compounds can covalently react with proteins via enzymatic or non-enzymatic oxidation.[17]The alkaline method has been studied extensively,[3, 18-21] and the results show that the modification could induce proteins cross-linking and change the isoelectric point of the proteins. However, the covalent protein modification under neutral or mildly acidic condition, which is much more relevant to foods than alkaline condition, have not been investigated in detail. Radical polymerization is a well-known method to improve the properties of natural and synthetic polymers.[22] By using redox initiator system, the single-step reaction between antioxidant molecule and biopolymers has been accomplished.[22-26] This approach is very useful for synthesis of protein- or polysaccharide-antioxidant conjugates at room temperature without the generation of toxic reaction by-products, preventing the antioxidant from degradation.[27]

In this study, three polyphenols including epigallocatechin gallate (EGCG), chlorogenic acid (CA) and gallic acid (GA) were preferred to be covalent with the proteic side chains of LF, using the H_2O_2/ascorbic acid redox pair as an initiator system. EGCG is one abundant bioactive component in green tea and has received increasing attention owing to its various physiological activities, against oxidization, tumors, microbes, and atherosclerosis.[28] CA and GA as important natural antioxidants are found in different vegetable sources and widely used in the food and pharmaceutical industries. They have been used as additives in foods, drugs and cosmetics.

The purpose of the present work was to evaluate how covalent

modification with different phenolic compounds would affect the structural and functional properties of a protein macromolecular system such as LF. First, EGCG, CA and GA were grafted onto LF molecule by a free radical-mediated grafting method, using ascorbic acid/H_2O_2 redox pair system. Then, LF-polyphenol conjugates were characterized by circular dichroism (CD), Fourier transform infrared (FTIR) spectroscopy and intrinsic fluorescence spectroscopy. The thermal behavior of LF-conjugates was investigated by differential scanning calorimetry (DSC). Finally, the antioxidant activity of the conjugates was evaluated *in vitro* via 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH•) and 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical ($ABTS^{•+}$) scavenging as well as reducing power assays. Moreover, to better control the functional properties of proteins in foods, we compared the effects of different polyphenols on the protein solubility and emulsifying properties after the modification. The possibility to graft polyphenols onto a protein represents an interesting innovation that significantly improves the performance of the biomacromolecules, and provides new applications in functional foods and/or the pharmaceutical industry.

2. Materials and methods

2.1. Materials

LF (purity ≥ 92%) from bovine whey was purchased from Westland Milk Products (Hokitika, New Zealand). EGCG (purity ≥ 98%) and CA (purity ≥ 99%) were purchased from BSZH Science Company (Beijing, China). GA, Folin-Ciocalteu's phenol reagent, DPPH, ABTS and 6-hydroxyl-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were obtained from Sigma–Aldrich (Sigma Chemical Co., St. Louis, MO). 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.

2.2. Preparation of LF-polyphenol conjugates

LF-EGCG, LF-CA and LF-GA conjugate polymers by employing ascorbic acid/H_2O_2 redox pair as an initiator system, were prepared according to Spizzirri et al.[23] with slight modification. In a 100-mL glass flask, 0.5 g LF was dissolved in 50 mL of distilled H_2O. A slow stream of nitrogen was flowed over the top of the flask. Then 1.0 mL of 5.0 M H_2O_2 containing 0.25 g of ascorbic acid was added and the mixture was maintained at 25 °C. After 2 h, 0.35 mmol of polyphenols was introduced into the flask. After 24 h, the unreacted polyphenols were removed by dialysis (MWCO: 12000-14000 Da) at room temperature for 48 h with eight changes of water until no free polyphenols existed in the system, which was determined by UV absorption spectra analysis. The resulting solutions were frozen and dried with a freezing-drying apparatus to afford a vaporous solid. Serving as a control, the LF was prepared under the same condition but with the absence of polyphenols.

2.3. Physicochemical characterization of the conjugates

2.3.1 Measurement of contents of free amino, thiol groups and tyrosine residues

The contents of free amino groups in the samples were measured following the ortho-phthaldialdehyde (OPA) method[29] with slight modification. The OPA reagent was prepared daily by mixing the following reagents: 40 mg of OPA (dissolved in 1 mL of methanol), 25 mL of 0.1 M sodium borate buffer (pH 9.85), 100 μL of β-mercaptoethanol, and 2.5 mL of 20% (w/v) sodium dodecyl sulfate (SDS) in deionized water. The mixture was diluted to 50 mL with deionized water. Then 4 mL of OPA reagent and 200 μL of protein solution (4 mg/mL) were mixed thoroughly and then reacted in a 35 °C water bath for 2 min. After that, the absorbance at 340 nm was measured using a double beam spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The content of free amino groups was calculated by using the calibration curve of L-leucine as a standard.

The content of tyrosine in samples was measured according to Hassan[30] as follows: 0.9 mL of a sample (1.0 mg/mL) was mixed with 1 mL of nitric acid (16 mol/L), and then heated in a water bath at 50 °C for 15 min. After cooling to room temperature, 4 mL of ethyl alcohol and 4 mL of NaOH (5 mol/L) were added, and the absorbance at 360 (A_{360nm}) and 430 nm (A_{430nm}) was measured using a double beam spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).The content of tyrosine (Ctyrosine, ng/mL) was determined by the following equation. $Ctyrosine = 0.5357 \times A_{430nm} - 0.3714 \times A_{360nm}$.

The content of free thiol group in samples was measured by the reaction with 5, 5-dithiobis nitro-benzoic acid (DTNB), which reacts with thiol compounds to produce 1 mol of p-nitrothiophenol anion/mol of thiol. The procedure was carried out following Beveridge et al.[31] with some modifications. First, 4 mg of DTNB was dissolved in 1 mL of 50 mMTris/HCl buffer containing 1 mM ethylene diamine tetraacetic acid (EDTA, pH 8.0) to prepare DTNB reagent. Then 15 mg of samples was dissolved in 5 mL of Tris-HCl buffer (50 mM, pH 8) containing 8 M urea. Thereafter, 50 μL of DTNB reagent was added to each of the sample solutions and mixed rapidly. After 1 h of incubation, the absorbance at 412 nm of the samples was measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) before and after the addition of DTNB against a blank. The thiol content was calculated according to the following formula: μM SH/g = $(73.53 \times A_{412})/C$, where A_{412} is the absorbance measured at 412 nm, C is the sample concentration (mg/mL).

2.3.2 Measurement of total phenolic content

The total phenolic content was measured using Folin-Ciocalteu reagent method[32] with some modifications. Briefly, 0.5 mL of a sample (1 mg/mL) was mixed thoroughly with 2.5 mL of freshly prepared Folin-Ciocalteu reagent (10 N). After 3 min, 2 mL of Na_2CO_3 (7.5% w/v) was added, and the mixture was allowed to stand for 2 h in dark with intermittent shaking. The absorbance at 760 nm was measured with the UV-vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) against a control solution prepared using the blank sample under the same condition. The amount of total phenolic groups in each sample was expressed as equivalent contents of EGCG, CA or GA by using the equations obtained from the calibration curves of each polyphenol, and the results were expressed as μmol polyphenol /g sample.

2.3.3 Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was carried out on a 1-mm thickness vertical slab gel (BIO CRAFT model BE-210N, Japan) at a constant voltage of 200 V. The solution of LF or LF-polyphenol conjugates (2 mg/mL) was mixed with the same volume of loading buffer. Then the aliquots (each 5 µL) were loaded on a 5% stacking gel and a 10% polyacrylamide resolving gel in the electrophoresis system according to manufacturer's instruction. The gels were stained with Coomassie Brilliant Blue R250 for protein visualization and were scanned by a HP scan instrument (HP 1000).

2.3.4 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

The lyophilized LF and LF-polyphenol conjugates were subjected to MALDI-TOF-MS experiments as follows: MALDI-TOF-MS experiments of conjugates were performed by dissolving 1 mg of the protein samples in 1 mL of distilled H_2O, then 0.5 µL of these solutions were brought on to the target and covered with 0.5 µL matrix (saturated sinapinic acid in 50% acetonitrile with 0.1% TFA). After crystallization by air-drying, the samples were measured with an Autoflex II TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The mass spectra were recorded in the reflector mode with an acceleration voltage of 20 kV and an effective flight path of 200 cm, and external calibration was obtained using bovine serum albumin.

2.4. Structural characterization of the conjugates

2.4.1 FTIR spectroscopy

The infrared spectra of LF, LF-EGCG, LF-CA and LF-GA conjugates were obtained with the potassium bromide (KBr) pellet method. The dried samples were ground into powder, pressed into pellets and measured by a Spectrum 100 Fourier transform spectrophotometer (Perkin–Elmer, UK) in the range of 400-4000 cm^{-1}, at a resolution of 4 cm^{-1}. KBr was used as a reference. Fourier self-deconvolution and secondary derivative were applied in the range of 1600–1700 cm^{-1} assigned to the amide I band in protein's FTIR spectra, and the major peaks for protein secondary structure were resolved. The above spectral region was deconvoluted by the curve-fitting method with the Levenberg-Marquadt algorithm and the peaks corresponding to α-helix (1658-1650 cm^{-1}), β-sheet (1640-1615 cm^{-1}), β-turn (1700-1660 cm^{-1}), and random coil (1650-1640 cm^{-1}) were adjusted and the area measured with the Gaussian function. The area of all the component bands assigned to a given conformation were then summed up and divided by the total area.[33] The data were analyzed using Origin 8.0 (OriginLab, Northampton, USA).

2.4.2 CD spectra

Far-UV CD spectra of the samples were recorded in the range 190–250 nm with 0.1 mg/mL protein by a Chirascan spectrometer (Applied Photophysics Ltd, UK) using a quartz cylindrical cell in 1 mm path length. Ellipticity was recorded at a speed of 100 nm/min, 0.2 nm resolution, 20 accumulations and 2.0 nm bandwidth. The collected data were analyzed using Dichroweb (Circular Dichroism Website http://dichroweb.cryst.bbk.ac.uk).[34-35] The CD spectra were represented as mean residue ellipticity (mega).

2.4.3 Fluorescence spectroscopy

Fluorescence steady state measurements were performed on a fluorescence spectrophotometer (Varian Instruments, Walnut Creek, CA, USA). Scanning parameters for all measurements were optimized with slit width 5 nm for excitation and 3 nm for emission. The concentration of the samples was 1 mg/mL. The excitation wavelength was set at 295 nm to selectively excite the tryptophan residues and the emission was collected between 300 and 400 nm.

2.4.4 DSC measurement

Calorimetric analyses were performed using a DSC-60 thermal analysis system (Shimadzu, Tokyo, Japan). In a standard procedure, about 5.5 mg of samples were placed inside an aluminum pan and sealed tightly by a perforated aluminum lid, heated from 30 to 180 °C at a constant rate of 10 °C/min with a constant purging of dry nitrogen at a rate of 30 mL/min. An empty aluminum pan was used as a reference. The peak temperature of denaturation was computed using the universal analysis software from each thermal curve.[36]

2.5. Functional evaluation of the conjugates

2.5.1. DPPH• scavenging activity

To evaluate the free radical scavenging properties, the samples were allowed to react with a stable free radical DPPH, according to the method of Gong et al.[37] with slight modification. The 1.75×10^{-4}M DPPH• solution was freshly prepared in MeOH. About 2 mL of a diluted sample (0.5 mg/mL) was mixed with 2 ml of DPPH solution. Then the mixture was stored in the dark for 60 min and the residual DPPH concentration was determined colorimetrically at 517 nm using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The scavenging activity was calculated on basis of the Trolox calibration curve, which was carried out by the method mentioned above and expressed as µmol Trolox equivalents (TE)/ mg sample.

2.5.2. ABTS•$^+$ scavenging activity

The ABTS•$^+$ scavenging activity was evaluated according to Siddhuraju et al.[38]. A stock solution of ABTS•$^+$ (7 mM) was prepared by diluting 10 mg of ABTS with 2.6 ml of potassium persulfate solution (2.45 mM). Then the mixture was kept in the dark for 12-16 h at room temperature before use. Thereafter, the ABTS working solution was diluted with distilled water to an absorbance of 0.70±0.02 at 734 nm. Then 1 mL of a sample (0.5 mg/mL) and 3 mL of ABTS solution were mixed, incubated at room temperature for 1 h, and the absorbance at 734 nm was then measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The ABTS•$^+$ scavenging activity was calculated

on basis of the Trolox calibration curve, which was carried out by the method mentioned above and expressed as μmol Trolox equivalents (TE)/ mg sample.

2.5.3. Reducing power

The ability of samples to reduce iron (III) was determined according to Yildirim et al.[39]. Specifically, 1 mL of the samples was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6), and the reaction was initiated by addition of 1% (w/v) potassium ferricyanide. The mixtures were incubated at 50 °C for 20 min. After that, 2.5 mL of 10% (w/v) trichloroacetic acid was added and the mixture was centrifuged at 3000×g for 10 min. Finally, 2.5 mL of the supernatant was mixed with 0.5 mL of distilled water and 0.1 mL of $FeCl_3$ (0.1%, w/v), followed by measurement of absorbance at 700 nm using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). A higher absorbance indicates higher reducing power. The reducing power was calculated on basis of the Trolox calibration curve, which was carried out by the method mentioned above and expressed as μmol Trolox equivalents (TE)/ mg sample.

2.5.4. Measurement of solubility

The solubility of the protein samples was analyzed according to the method of Klompong et al.[40] with some medications. Protein samples were dispersed in 10 mM buffer solutions with varying pH levels: pH 3.0-5.0, citrate buffer; pH 7.0, phosphate buffer; pH 9.0, borate-boric acid buffer; pH 11.0, carbonate buffer. The dispersions were shaken with a vortex mixer for 30 s at room temperature and centrifuged at 10000×g for 20 min. After that, the protein content in the supernatants was determined by modified Lowry method.[41] Protein solubility was expressed as percentage ratio of supernatant protein content to the total protein content.

2.5.5. Evaluation of emulsifying properties

Emulsifying properties of LF, LF-EGCG, LF-CA and LF-GA were measured according to Pearce and Kinsella[42] with some modifications. Briefly, the samples were firstly dispersed in deionized water at a concentration of 0.3%, and then 95 mL of protein solution and 5 g of MCT oil were mixed at 10000 rpm with a blender to form coarse emulsions. Then the above solutions were homogenized using a Niro-Soavi Panda two-stage valve homogenizer (Parma, Italy) for three cycles at 60 MPa. An aliquot of the emulsion (0.1 mL) was pipetted from the container's bottom at 0 and 30 min separately and 500-fold diluted with 0.1% SDS. The solution was blended thoroughly for 10 s using a vortex mixer. Absorbance at 500 nm was measured using 0.1% SDS solution as the blank with a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The emulsifying activity index (EAI) and emulsifying stability index (ESI) were calculated as follows: EAI (m^2/g) = ($2\times2.303A_0$) DF/ lφC; ESI (min) = $A_0\times\Delta t$ / (A_0-A_{30}), where A_0 and A_{30} are the absorbance at 500 nm after 0 min and 30 min, respectively, DF is the dilution (500), l is path length of the cuvette (0.01m), φ is oil volume fraction and C is protein concentration (g/m^3).

2.6. Statistical analysis

Each measurement was carried out in triplicate. Data were subjected to analysis of variance (ANOVA) using SPSS 18.0 for Windows (SPSS Inc., Chicago, USA). Means of treatments were separated at the 5% significance level using the LSD method.

3. Results and discussion

3.1. Preparation of LF-polyphenol conjugates

The LF-polyphenol conjugates were synthesized by employing the ascorbic acid/H_2O_2 redox pair, a biocompatible and water-soluble radical initiator system. A possible mechanism for polyphenols to bind on proteic side chains was proposed (Fig. 1). Ascorbic acid could react with hydrogen peroxide to form ascorbate and hydroxyl radicals,[43]and then the hydroxyl radicals attacked H-atoms on the side chains of protein molecules to form protein macro-radicals, which further reacted with the phenolic ring of the polyphenols to form a covalent bond.[16] The data in literature[44] suggested that the heteroatom-centered radicals on the side chains of protein preferentially reacted at the *ortho*- and *para*- positions relative to the hydroxyl group on the phenolic ring.

Fig. 1

To remove un-reacted polyphenols, the conjugates were dialysed and the polyphenol in washing media was analyzed by UV absorption spectrometry. The amount of polyphenols bound to LF was determined by Folin-Ciocalteu method. For each grafted polymer, the content of disposable phenolic groups was expressed as equivalent of each polyphenol (μmol) by comparing the obtained data with the relative polyphenol calibration curve (Table 1). The results were 68, 58 and 17 μmol/g in powered LF-EGCG, LF-CA and LF-GA conjugates respectively, which could be due to the presence of free radical reactive sites in the polyphenol molecules.[15]

3.2. Contents of free amino, thiol groups and tyrosine residues

The contents of free amino, thiol groups and tyrosine residues in control LF and LF-polyphenol conjugates are shown in Table 1. The attachment of polyphenol into LF was monitored by the decreased contents of free amino and thiol groups in LF. The reactivity and binding strength with free amino groups were ranked in the order of CA > EGCG > GA. Therefore, we postulated that the reactivity of the phenolic compounds was influenced by the number and the position of hydroxyl groups. Since the number of free amino groups was determined in the presence of 1% SDS (a well known denaturing agent that destroys noncovalent protein interactions), we assumed that the interaction occurred through covalent binding. In addition, the contents of free tyrosine group in LF-EGCG and LF-CA conjugates were lower than that in the control LF ($p < 0.05$). As reported, oxidised phenolic compounds could react with nucleophilic groups, such as amino group, tryptophan, cisterns, methionine, histidine, tyrosine and N-terminal proline in proteins.[8]Prigent et al.[45] studied the covalent interactions between

quinones from caffeoylquinic acid and amino acid side chains with mass spectrometry using N-terminally protected amino acids, and demonstrated that the side chains of lysine and tyrosine were more reactive than those of histidine and tryptophan.

3.3. SDS-PAGE and MALDI-TOF-MS analyses

The formation of covalent conjugates after the incubation of LF with different polyphenols was testified by SDS-PAGE and MALDI-TOF-MS.

The structural changes of LF after its reaction with polyphenols were monitored by SDS-PAGE (Fig. 2(a)). The main band in LF was around 80 kDa, similar to the value cited by Lönnerdal & Iyer [46] and determined using MALDI-TOF-MS (84011 Da, Fig. 2(b)). Compared with the control LF, the molecular composition was not significantly changed in the band of LF-GA conjugate, but slightly migrated up in the LF-EGCG and LF-CA conjugates. These high molecular weight complexes were not separated by the use of SDS or mercaptoethanol, suggesting that the bonds formed between the molecules should be covalent. As the molecular weight of GA is 170 Da, a lack of change in molecular weight of LF-CA conjugate might be due to the low degree of conjugation. These findings were similar to the result of Rohn et al.[47], for the polymerization of protein molecules with phenolic substances under alkaline condition.

The changes in molecular weight of LF-polyphenol conjugates were also evaluated via MALDI-TOF-MS and the results are documented in Fig. 2 (b). The molecular weights of all conjugates were increased as shown in the peaks as compared with control LF alone. The formation of polymerized products, as a result of the reactions of polyphenols with LF, followed the order: CA > EGCG > GA. Generally, the mass spectra showed peaks, which were approximately separated by the increased molecular weight of the reacting molecules (EGCG, CA and GA). The reaction of LF with CA (354.3 Da) delivered the highest molecular weight of 85905.9 Da, which in turn accounted for the insertation of at least five CA molecules to one LF molecule. Similarly, the molecular weights of conjugates with EGCG (458 Da) and GA (170 Da) were 84717.8 Da and 84364.1Da, respectively, accounting for a possible corresponding insertation of one or two molecules in each case. These results further confirmed the possible conjugation of LF with the polyphenols.

Fig. 2

3.4. FTIR analysis

The LF-polyphenol conjugates were characterized by infrared spectroscopy and its derivative methods. FTIR spectroscopy is valuable to monitor the changes in the secondary structure of proteins.[48] The specific stretching and bending vibrations of the peptide backbone in amide I, II, and III bands provide useful information about different secondary structures such as α-helix, β-sheets, turns, and unordered structures (referred to as random coil). The original infrared spectra of the control LF and LF-polyphenol conjugates are shown in Fig. 3. Generally, the spectra of the control LF exhibited major bands at 3302 cm^{-1} (amide A, representative of N-H stretching coupled with hydrogen bonding), 1651 cm^{-1} (amide I, representative of C-O stretching/hydrogen bonding coupled with COO-) and 1533 cm^{-1} (amide II, representative of C-N stretching coupled with NH bending modes). As compared with the control protein, this band in the conjugates was obviously changed in terms of both shapes and peak positions, which implied the changes in the secondary structure of LF in the conjugates.

Fig. 3

In general, FTIR spectra of protein are commonly accompanied by secondary structure changes which are expressed in the amide I (1600–1690 cm^{-1}) and amide II (1480–1575 cm^{-1}) bands of the spectra.[49-50] Since the amide I band was more sensitive to changes in the protein secondary structure than the amide II band, we applied curve fitting method to the original spectra over the region of 1600-1700 cm^{-1}. Figure 4 presents the amide I region fitted with a Gaussian line shape function. In the present study, the control LF contained 11.5% α-helix, 9.5% β-sheet, 54.1% turn and 24.9% random coil, and the LF-EGCG conjugate contained 18.1% α-helix, 15.2% β-sheet, 43.5% turn and 23.1% random coil. Similarly, α-helix content in LF-CA and LF-GA conjugates was significantly increased ($p < 0.05$), and these changes of the secondary structure might result from the covalent interactions between LF and polyphenols. In addition, EGCG, CA and CA caused different changes in the secondary structure distribution of LF, which clearly demonstrated a perturbation that was dependent on phenolic compounds applied.

Fig. 4

3.5. CD analysis

CD spectroscopy is one common used method to study protein conformations in the solution or after the adsorption onto colloidal surfaces. In this study, CD measurements were performed to better understand the conformational behaviors of LF before and after its conjugation with polyphenols. As shown in Fig. 5, the far-UV spectrum of LF exhibited a negative peak in the region of 205-206 nm, which is mainly characteristic of predominantly α-helix proteins. The conjugation caused a change in band intensity at all wavelengths in the far-UV CD with a slight shift of the peaks, negative minimum at 205 nm displaced to a longer wavelength, indicating the obvious change of protein secondary structure after the conjugation. The content of secondary structure was estimated using a DICHROWEB procedure, which was an online server for protein secondary structure analysis using CD spectroscopic data.[35] The fractions of α-helix, β-sheet, turn and unordered coil were estimated by SELCON3 and presented in Fig. 5. The conjugation of LF with polyphenols resulted in an increased fraction in α-helix with a parallel decreased fraction in the random coil structure, indicating the destructuring effect on LF. However, it was reported that the conjugation of CA with BSA would cause a decreased fraction of α–helix together with increased fractions of other structures,[20] which might be attributed to the difference of physical-chemical characterization of LF and BSA as well as the methods to prepare these conjugates.

Fig. 5

However, some differences regarding protein conformation should be noted between FTIR and far-UV CD spectroscopy results. These differences were due to the method of samples preparation, because freeze-dried samples were used for FTIR measurements, whereas aqueous solution for far-UV CD spectroscopy.

3.6. Fluorescence analysis

Fluorescence spectroscopy helps to obtain local information about the conformational and/or dynamic changes of proteins. For proteins with intrinsic fluorescence, more specific local information can be obtained by selectively exciting the tryptophan residues.[51] Therefore, an intrinsic fluorescence measurement was performed to evaluate changes in tertiary structure. As shown in Fig. 6, the maximum emission wavelength for the control LF was 342 nm; generally, the fluorescence intensity was significantly decreased and the maximum emission was red-shifted in LF-EGCG, LF-CA and LF-GA conjugates. The maximum emission was shifted to 351, 345 and 344 nm for the LF-EGCG, LF-CA and LF-GA conjugates, respectively. These differences in fluorescence intensity confirmed that tryptophan was most likely to be involved in the covalent reaction of LF with polyphenols. Commonly, the red shift indicated that tryptophan residues were more exposed to the solvent, or the red shift resulted from the transfer of tryptophan residues into a more hydrophilic environment.[51] From the progressive quenching and the red shift observed in the maximum fluorescence emission of LF-polyphenol conjugates, it could be deduced that the conjugation-induced conformational changes might lead to the unfolding and denaturation of LF.

Fig. 6

3.7. Differential scanning calorimetry (DSC) analysis

The prepared conjugates were also thermally characterized by recording the DSC thermograms of control LF, LF-polyphenol conjugates, and pure polyphenols (Fig. 7). The DSC thermograms of each polyphenol revealed endothermic peaks at around 130.69 °C for EGCG, 86.12 °C for CA, and 106.51 °C for GA, which corresponded to the melting temperature of each polyphenol. The melting endothermic peaks of pure polyphenols disappeared in the DSC thermograms of the conjugates, which could be ascribed to the covalent doping between LF and the polyphenols. The control LF showed a broad endothermic peak at 91.8 °C. Compared with the control LF, the DSC peak temperatures for all the conjugates were severely increased, from 5 to 15.2 °C. This result revealed that the increased denaturation temperature of LF was attributed to the covalent interaction, which was in agreement with a previous report[52] that alkaline-modification by EGCG increased the denaturation temperature of α-lactalbumin.

Fig. 7

3.8. Functional properties

3.8.1. Antioxidant capacities of LF-polyphenol conjugates

To evaluate the effect of conjugation on the antioxidant activity of LF, three antioxidant tests *in vitro* were conducted. First, the antioxidant activity was evaluated by the scavenging ability on DPPH•. The DPPH• is a stable organic free radical with a maximum absorption band around 515-528 nm and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Thus the antioxidant ability is proportional to the disappearance of DPPH in tested samples. Under the experimental operating condition, the DPPH• inhibitory of control LF was 48.51 μmol trolox equivalent (TE)/g sample, but ranged from 59.96 to 88.39 μmol trolox equivalent (TE)/g sample for the LF-polyphenol conjugates (Table 2). According to the aforementioned results, the DPPH• scavenging capacity of the control LF was weak, however, it could be improved with the conjugation of polyphenols, which indicated that polyphenol grafting onto LF by redox pair systems enhanced the antioxidant capacity.

$ABTS^{\bullet+}$ scavenging assay has been widely used to determine the antioxidant capacity as it requires relatively standard equipment and delivers fast and reproducible results. The $ABTS^{\bullet+}$ scavenging activity was generally in accordance with the DPPH• scavenging results (Table2). The LF-polyphenol conjugates had 0.23- to 2.10-fold increase compared to the control LF, and the LF-CA conjugate showed the stronger $ABTS^{\bullet+}$ scavenging activity than others.

The reducing capacity of a tested sample indicates its potential antioxidant activity. Table 2 depicts the reducing power results of the control LF and LF-polyphenol conjugates. Reducing power was observed in the following order: the LF-EGCG conjugate > LF-CA conjugate > LF-GA conjugate. The activities of the conjugates were 0.04- to 2.19-fold greater than the unmodified LF, which was positively correlated with EGCG, CA, and GA contents in LF-polyphenol conjugates. These findings demonstrated that the antioxidant capacity of LF was enhanced by grafting polyphenol onto LF, and revealed that the conjugation of antioxidant polyphenols onto LF was a potential method for the preparation of novel polymeric antioxidants.

3.8.2. Protein solubility

Protein solubility can be considered as a guide to protein functionality because it relates directly to many important properties, such as emulsification and foaming capacities, while proteins with low solubility indices have limited applications. The solubility data of control LF and LF-polyphenol conjugates at different pH are shown in Fig. 8 (a). The minimum solubility of control LF occurred at pH 9.0, which was near the isoelectric point (8.4-9.0) of the protein.[53] After the conjugation with polyphenols, the solubility was changed at pH 5.0-9.0, which was dependent on the phenolic substance applied. The reaction of LF with all three polyphenol led to significant increases ($p < 0.05$) in solubility at pH 7.0, but slight decrease for GA at pH 9.0. Moreover, the solubility of LF-EGCG conjugate was relatively lower at pH 5.0 compared with other samples. The difference in solubility might be due to the changed number of charged groups present in the structure of the conjugates. Rawel et al.[54] revealed that the solubility behavior of myoglobin was significantly

changed by apigenin, kaempferol, quercetin and myricetin, but almost not influenced by flavones.

3.8.3. Emulsifying activity and stability

Proteins are the main emulsifying agents in many foods. EAI and ESI are important parameters generally used to investigate the emulsifying properties of proteins in food emulsion systems. EAI estimates the relative surface coverage of a protein on an oil droplet in a dilute emulsion, whereas ESI estimates its relative stability after a pre-determined time. These parameters between unmodified LF and LF-polyphenol conjugates were also briefly calculated. The EAI and ESI of control LF and LF-polyphenol conjugates are shown in Fig. 8 (b). The EAI was increased for LF-EGCG conjugate, but decreased for LF-GA conjugate compared with the control LF. Nevertheless, EAI of LF-CA conjugate was not significantly changed. On the other hand, ESI of all the conjugates was increased and the emulsifying stability of LF-CA conjugate was much stronger compared with the control LF. Emulsifying activity of a protein emulsifier depends on its ability to form adsorption films around the oil globules and the ability to lower the interfacial tension at the oil-water interface. The improved emulsifying activity might be due to the increased surface hydrophobicity of the modified protein. As reported, the emulsifying activity of soy protein isolates was significantly improved after its covalent modification by EGCG.[55] However, the EAI of gelatin was not changed after the modification with oxidised tannic acid or oxidised ferulic acid, and significantly decreased after the modification with oxidised caffeic acid.[56] These results implied that the covalent modification of LF with polyphenols might affect the emulsifying properties, depending on the types of phenolic compounds.

Fig. 8

4. Conclusions

Covalent conjugates between LF and different polyphenols (EGCG, CA and GA) were successfully synthesized using ascorbic acid/hydrogen peroxide redox pair, a biocompatible and water-soluble radical initiator system. The covalent bond between the amino acid side-chains in LF and the polyphenols was confirmed by SDS-PAGE and MALDI-TOF-MS. Significant structural changes in LF after its reaction with polyphenols were testified by CD, FTIR spectroscopy, DSC and intrinsic fluorescence. The results clearly interpreted the conjugation between LF and polyphenols was an effective method to improve protein functional properties (antioxidant activities, solubility and emusifying properties), depending on the type of phenolic compounds applied. Thus, our results provided a novel and efficient method for the synthesis of protein-polyphenol conjugates, which combine the characteristics of aminoacidic structure (biocompatibility, biodegradability, high molecular weight) and the antioxidant properties of EGCG, CA and GA. This method might be potential for its applications in food, cosmetic and pharmaceutical industries.

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Notes and references

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Table 1

Contents of free amino, thiol and tyrosine groups in control LF and LF–polyphenol conjugates.

Sample	Free amino group (nmol/mg)	Thiol group (nmol/mg)	Tyrosine group (ng/mg)	Polyphones bound (nmol/mg)
Control LF	446.08±1.56^{c}	3.80±0.08^{c}	40.93±2.24^{b}	—
LF-EGCG conjugate	431.83±3.75^{b}	3.53±0.03^{b}	33.11±1.16^{a}	67.76±0.18^{c}
LF-CA conjugate	406.51±1.06^{a}	2.72±0.09^{a}	33.29±1.33^{a}	58.23±3.35^{b}
LF-GA conjugate	408.12±2.45^{a}	3.30±0.15^{b}	39.59±2.77^{b}	16.67±2.27^{a}

Values are means ± SD (n=3). Different superscript letters in the table indicate a significant difference ($p < 0.05$).

Table 2

Antioxidant activities of control LF and LF–polyphenol conjugates.

Sample	DPPH• scavenging activity (μmol Trolox/g sample)	$ABTS^{\bullet+}$ scavenging activity (μmol Trolox/g sample)	Reducing power (μmol Trolox/g sample)
Control LF	48.51±1.77[a]	59.32±8.25[a]	104.05±4.36[a]
LF-EGCG conjugate	81.96±1.44[c]	117.00±7.90[c]	332.07±12.05[d]
LF-CA conjugate	88.39±4.84[d]	138.39±7.76[d]	204.03±8.01[c]
LF-GA conjugate	59.96±1.16[b]	73.25 ±5.04[b]	108.04±5.33[b]

Values are means ± SD (n=3). Different superscript letters in the table indicate a significant difference ($p < 0.05$).

Figure captions

Fig. 1. The proposed mechanism for the formation of LF-polyphenol conjugates by free radical mediated graft copolymerization.

Fig. 2. SDS-PAGE and MALDI-TOF-MS analysis of control LF, LF-EGCG, LF-CA and LF-GA conjugates.

Fig. 3. FTIR spectra of powdered control LF, LF-EGCG, LF-CA and LF-GA conjugates.

Fig. 4. Curve-fitted amide I region (1700-1600 cm^{-1}) with secondary structure determination of control LF(a), LF-EGCG conjugate (b), LF-CA conjugate (c) and LF-GA conjugate (d) in aqueous solution.

Fig. 5. Far-UV CD spectra (a) and secondary structure fractions (b) of control LF, LF-EGCG, LF-CA and LF-GA conjugates.

Fig. 6. Fluorescence of control LF, LF-EGCG, LF-CA, and LF-GA conjugates.

Fig. 7. Calorimetric analyses of LF-EGCG conjugate, control LF, and EGCG (a); LF-CA conjugate, control LF, and CA (b); LF-GA conjugate, control LF, and GA (c).

Fig. 8. The solubility behavior (a) and emulsifying properties (b) of control LF, LF-EGCG, LF-CA and LF-GA conjugates.

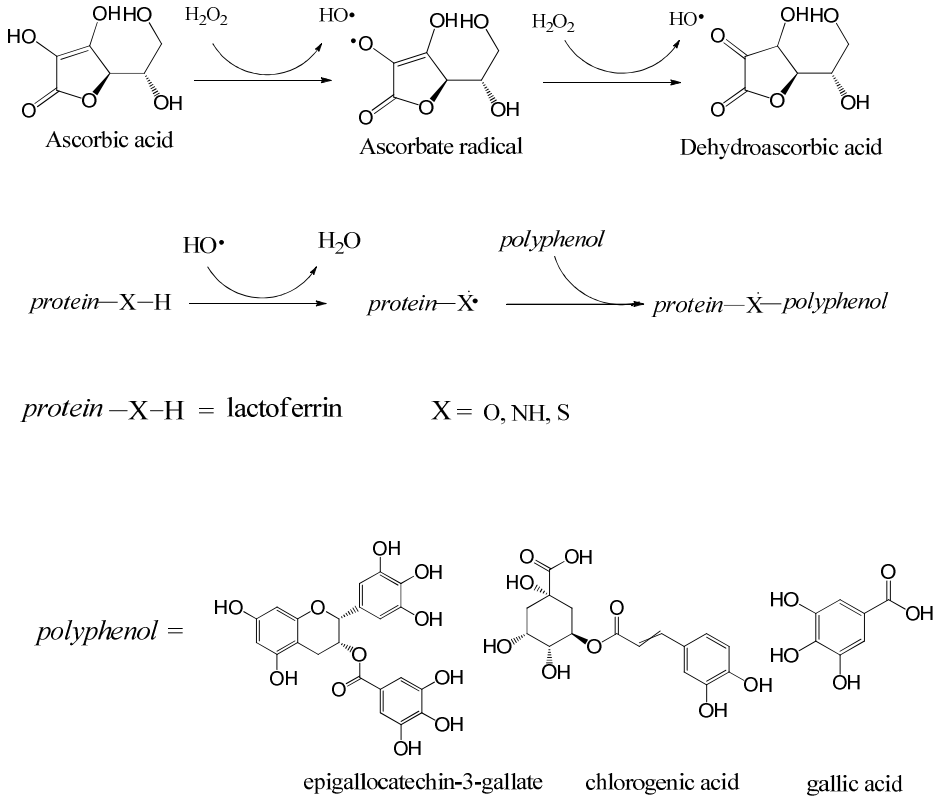
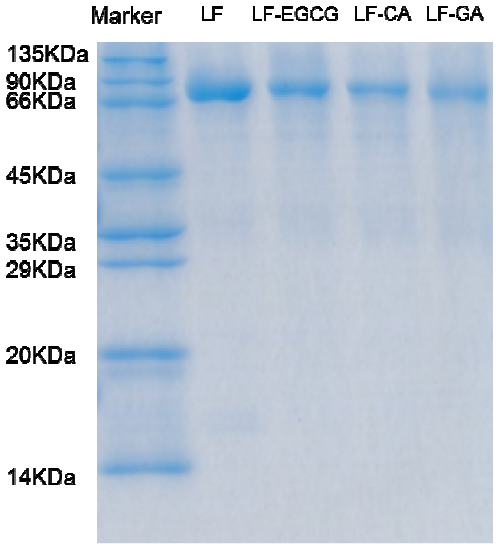


Fig. 1



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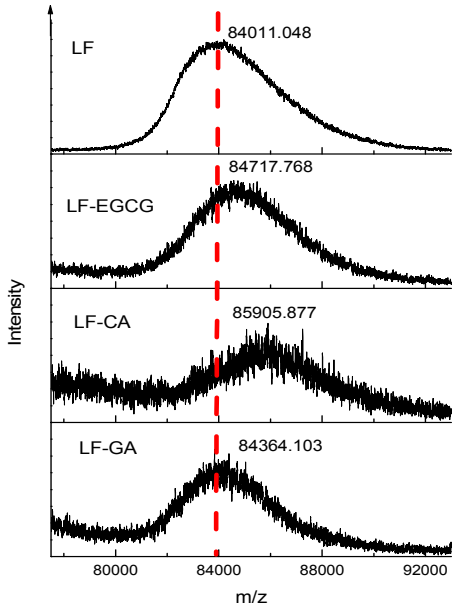


(b)

Fig. 2

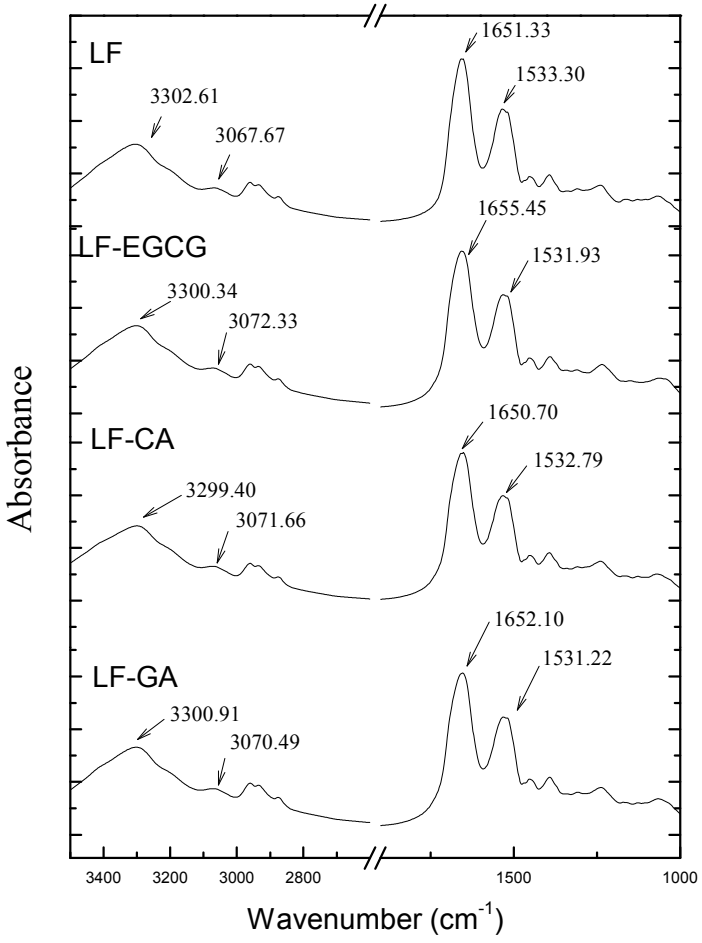
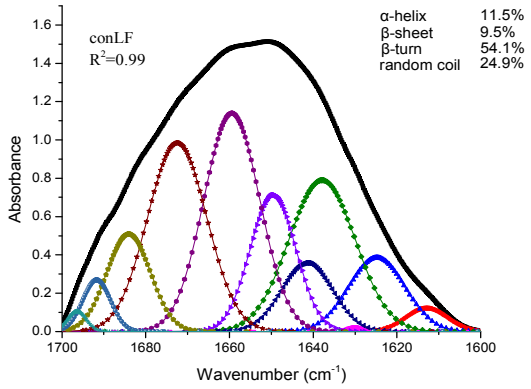
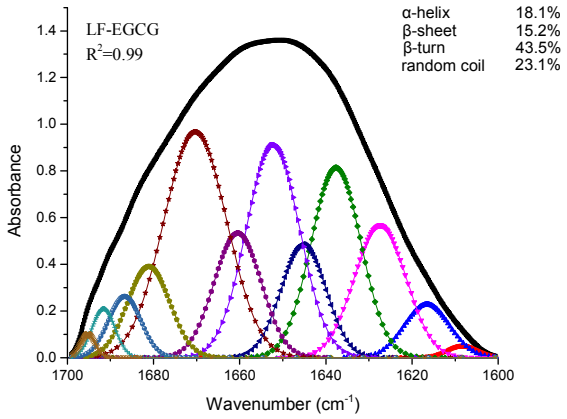


Fig. 3



(a)



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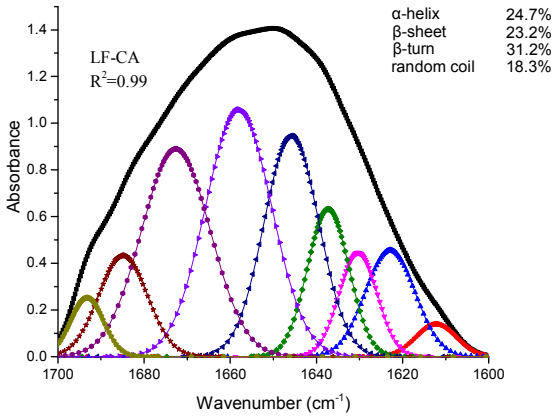
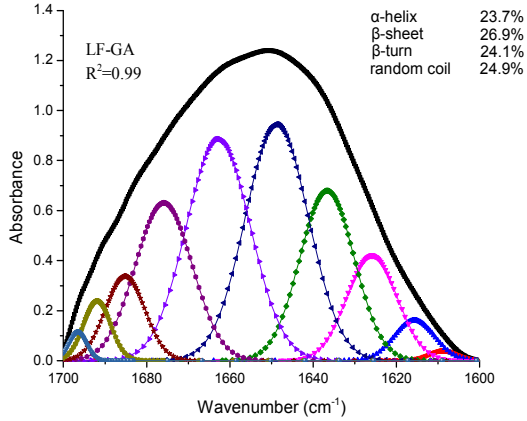
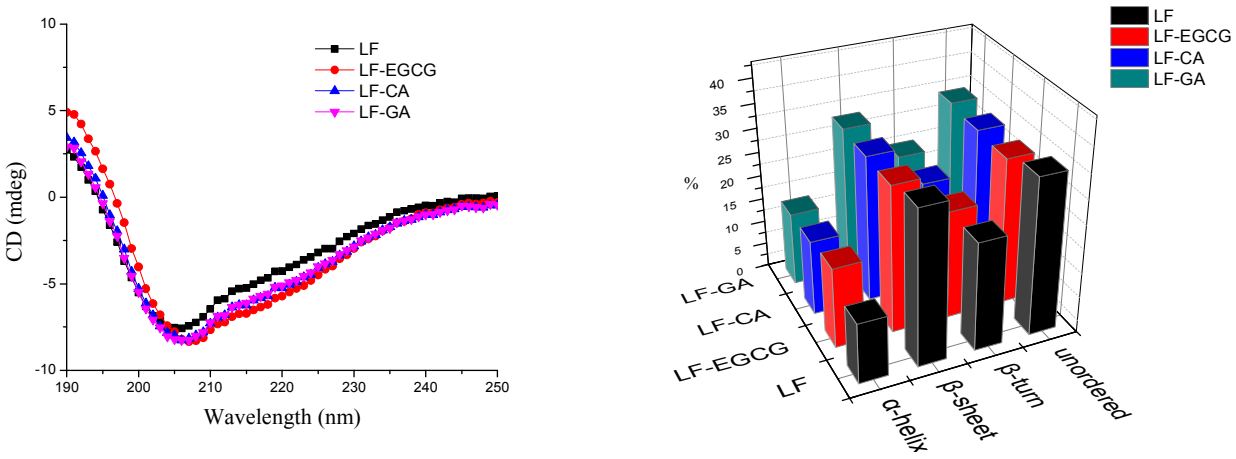


(c)



(d)

Fig. 4



(a) **(b)**

Fig. 5

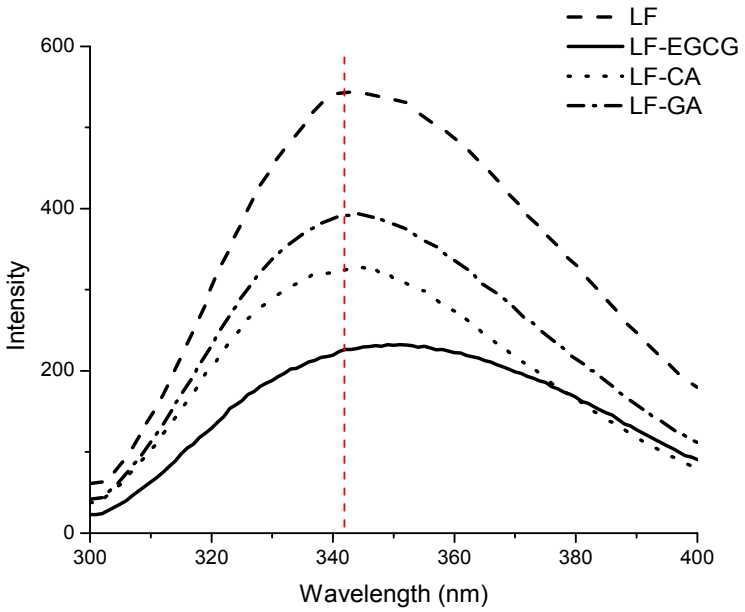
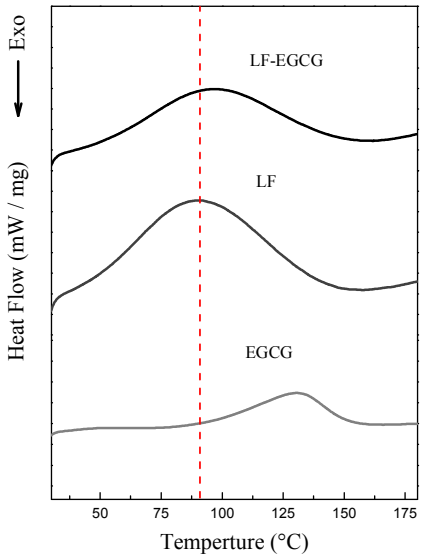
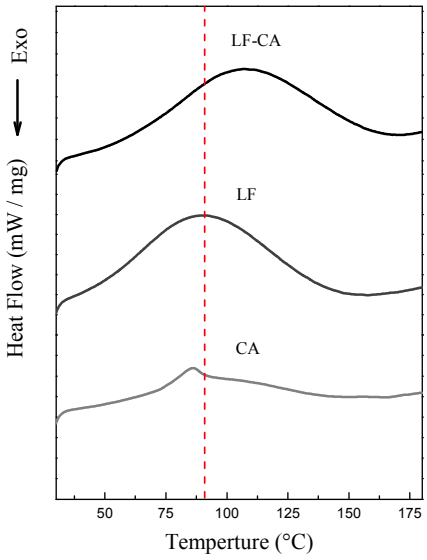


Fig. 6



(a)



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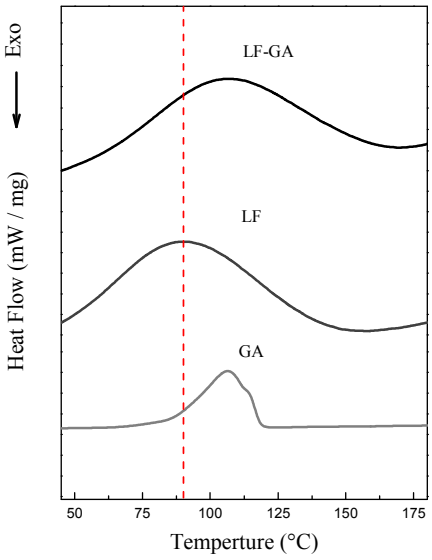
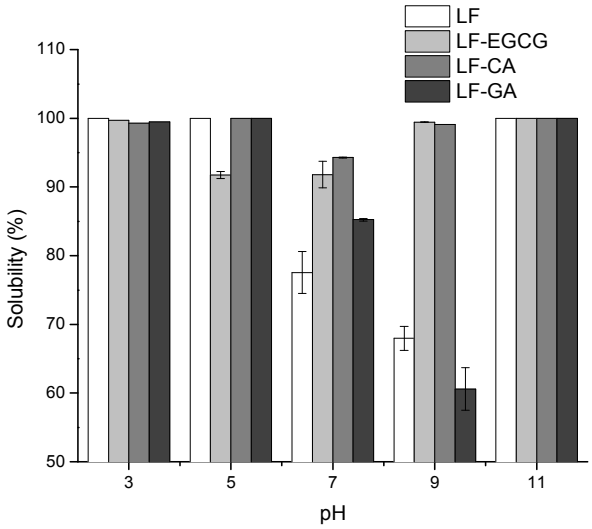


(c)

Fig. 7



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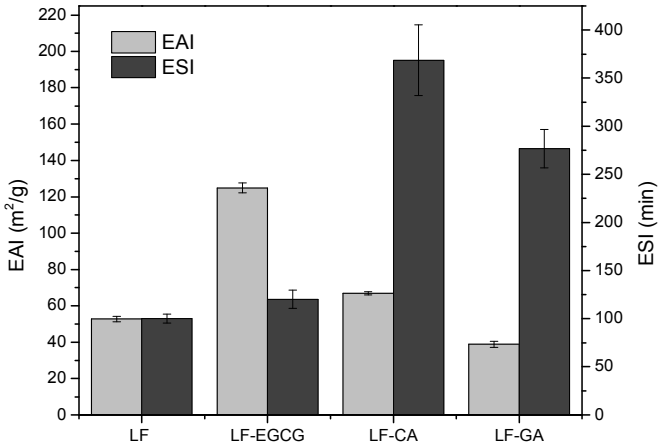

(b)

Fig. 8