Chemical Cross-Linking Gelatin with Natural Phenolic Compounds as Studied by High-Resolution NMR Spectroscopy

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Received February 2, 2010; Revised Manuscript Received February 26, 2010

Cross-linking gelatin with natural phenolic compound caffeic acid (CA) or tannic acid (TA) above pH 9 resulted in formation of insoluble hydrogels. The cross-linking reactivity was controlled by variation of pH, the concentration of the gelatin solution, or the amount of CA or TA used in the reaction. The cross-linking chemistry was studied by high-resolution NMR technique in both solution and solid state via investigation on small molecular model systems or using ^{13}C enriched caffeic acid (LCA) in the reaction with gelatin. Direct evidence was obtained to confirm the chemical reactions occurring between the phenolic reactive sites of the phenolic compounds and the amino groups in gelatin to form C−N covalent bonds as cross-linking linkages in gelatin matrix. The cross-linked network was homogeneous on a scale of 2−3 nm. The cross-linking resulted in a significant decrease in the molecular mobility of the hydrogels, while the modulus of the films remained at high values at high temperatures.

Introduction

Chemical cross-linking of natural proteins has a long history in the development of protein-based polymer materials for food processing, packaging, coating formulations, and pharmaceutical/medical applications. Gelatin, as a water-soluble protein produced from hydrolysis of animal collagen, has been widely used in these applications.[1–6] Because gelatin can be obtained from inexpensive sources derived from waste or byproduct of manufacturing processes comprising tannery, pharmaceutical, and food segments, it can be taken as a renewable and biodegradable material.[7] However, its poor mechanical properties especially when exposing to wet and humid conditions limit its application in many areas. Structure modifications are required to enhance its mechanical strength and water-resistant properties. The traditional aldehyde-based cross-linkers (including formaldehyde, glyoxal, and glutaraldehyde) have shown great efficiency in protein modifications in producing biomaterials,[8–12] the usage of these additives has also caused a series of OH&S issues in product manufacturing. The toxic nature of these compounds during material biodegradation process also prohibits their application in food and medical applications.[13] Alternative cross-linkers such as carbodiimides, epoxy, genipin, oxidized alginate, and transglutaminase have also been studied for protein cross-linking in recent years.[14–18]

Natural phenolic compounds commonly found in plants and fruits[19] as antioxidant reagents have also been widely used in food processing. The study of natural phenol−protein interactions has been carried out for more than half a century. A number of natural phenolic compounds derived from plants have been reported to be interactive or reactive with proteins and resulted in improved gel or film properties for gelatin-based materials.[20–27] It is generally accepted that four potential interactions may be involved, such as hydrogen bonding, ionic, hydrophobic interactions, and covalent bonding.[20,24,28–32] Covalent bonds between phenolic compounds and proteins are more rigid and thermally stable than other interactions. The postulated chemical pathway involves initial oxidization of phenolic structures to form, under alkaline conditions, quinone intermediates that can readily react with nucleophiles from reactive amino acid groups in protein chains such as the sulfuhydryl group of cystine, the amine group of lysine and arginine, the amide group of asparagine and glutamine, the indole ring of tryptophan, and the imidazole ring from histidine.[25,31,33] However, these reactions are complicated, and the interactions between phenolic compounds and proteins in forming cross-linked materials are poorly understood, particularly in regard to the chemical structures of intermediates and the chemistry of covalent cross-linking.

In this paper, gelatin cross-linking reactions were conducted using natural phenolic compounds caffeic acid (CA) and tannic acid (TA) as cross-linking agents. The chemical structure changes occurring during the reaction were examined by high-resolution NMR spectroscopy. Because only a small amount of cross-linking agent is normally used in the reaction and the signals derived from the cross-linker are usually too weak to be observed, a ^{13}C-labeled caffeic acid (LCA) was used in parallel with unlabeled CA to enhance the signals derived from LCA. This makes it possible to detect the behavior of the cross-linker in the cross-linked gelatin network by high-resolution solution- and solid-state NMR. Additionally, small molecular model systems were studied using 2,4-dimethyl phenol (24DMP) and 2,6-dimethyl phenol (26DMP) as model phenolic compounds reacting with lysine as the model amino group in gelatin. Because only a single phenolic reactive site is available in these model compounds (*ortho*-reactive site in 24DMP and *para*-reactive site in 26DMP), no cross-linked materials would be formed in such reactions, allowing product structures to be

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10.1021/bm1001284
Published on Web 03/17/2010

determined by solution NMR. Reactions using CA or TA as the cross-linker were studied systematically by varying pH, gelatin concentration, and the amount of cross-linking agent. The physical properties and phase structures of the modified gelatin materials were also examined using dynamic mechanical analysis (DMA) and solid-state NMR techniques.

Experimental Section

Raw Materials. All raw materials used in this work, including gelatin (type B obtained from bovine skin), caffeic acid (CA), tannic acid (TA), L-lysine (Ly), 2,4 dimethyl phenol (24DMP), and 2,6-dimethyl phenol (26DMP) were obtained from Sigma-Aldrich without further purification. The 97% ^{13}C-labeled caffeic acid (LCA, with all the carbons in the compound were enriched) was synthesized by Sigma-Aldrich as requested. All reactions were conducted in aqueous solution using distilled water.

Oxidation of CA and Ly. CA solution (20 wt %) was prepared by dissolving CA in water under magnetic stirring. The temperature was controlled using water bath set at 50 °C, and the pH was adjusted to a target value (pH 9−10) by the addition of NaOH solution (46 wt %). The solution was bubbled with oxygen during the reaction (50 °C for 1−3 h). Oxidization of Ly was conducted under the same conditions.

Reactions of Small Molecular Model Systems. Reactions in small molecular model systems (24DMP/Ly and 26DMP/Ly) were conducted using sodium periodate ($NaIO_4$) as the oxidizing agent. The molar ratio of Ly to 24DMP or 26DPM was 1:2; the amino functionality of Ly is 2, while the phenolic reactive site in 24DMP or 26DMP is only 1. The reactions were conducted at 50 °C for 2 h. Solution concentration was 20 wt % and the pH was 9, which was adjusted by the addition of NaOH solution (46 wt %).

Reactions of Gelatin and CA or TA. Gelatin (10 wt %) and CA or TA solutions were prepared separately, and the pH of each solution was adjusted to a target value (9−10) by the addition of NaOH (46%) solution. The gelatin solution was heated to 45 °C to ensure complete dissolution. CA or TA solution was then slowly added into the gelatin solution at 58 ± 1 °C under stirring to a predetermined ratio and allowed to react for a varied period of time. Solution pH (9−10) was monitored using a pH meter, and a few drops of NaOH solution (1 M) was required to maintain the desired pH during the reaction. Oxygen was continuously bubbled throughout the reaction at a rate of 47 mL/min. As this study was focused on cross-linking chemistry rather than reaction dynamics, the effects from variations such as the oxygen bubbling rate, bubble size, and stirring rate were not examined.

Gelatin films (thickness of around 0.1−0.2 mm) were produced by solution casting at room temperature for 2 weeks after the reaction without any heating or vacuum. Samples using LCA were prepared under the same conditions. All film samples were stored in a conditioning tank at a relative humidity (RH) of 52% and a temperature of 22 °C for 7 days before characterization. The moisture content of these films was around 17−19%, as determined by drying the samples at 105 °C for 6 h.

Characterization. NMR experiments were conducted at room temperature using a Varian NMR300 System at resonance frequencies of 75 MHz for ^{13}C and 300 MHz for ^{1}H. High-resolution solution NMR spectra were measured using a 10 mm solution probe. High resolution solid-state ^{13}C NMR spectra were observed under cross-polarization, magic angle spinning, and high power dipolar decoupling (CP/MAS/DD) conditions. The 90° pulse was 6.0 μs for ^{1}H and ^{13}C, while the spinning rate of MAS was set at a value in the range of 7−9 kHz. Different CP contact time (either 1 ms or 20 μs) was used to measure the ^{13}C CP/MAS spectra. The chemical shift of ^{13}C CP/MAS spectra was determined by taking the carbonyl carbon of solid glycine (176.3 ppm) as an external reference standard. ^{1}H spin−spin relaxation times (T_2) for the gelatin hydrogels were measured by CPMG pulse sequence[34] using CP/MAS probe without sample spinning. The proton spin−lattice relaxation times in the rotating frame (^{1}H $T_{1\rho}$) were measured through the decay of ^{13}C magnetization prepared by CP after varied ^{1}H spin-locking time, as reported previously.[34,35]

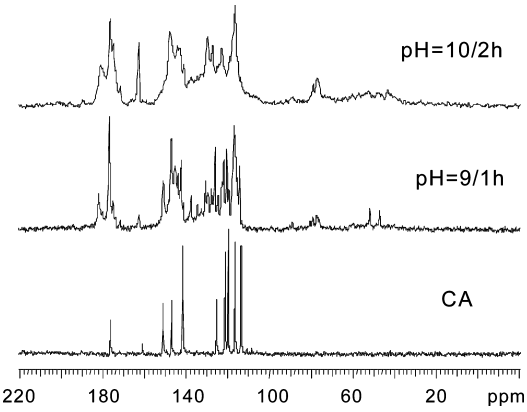


Figure 1. ^{13}C NMR solution spectra of CA before oxidation, oxidized at pH = 9 for 1 h, and at pH = 10 for 2 h in aqueous solution.

Dynamic mechanical analysis (DMA) experiments were operated on a PerkinElmer PYRIS Diamond DMA in tension mode at a frequency of 1 Hz, similar to what reported in literature.[36] The temperature range was set at −100 to 160 °C with a heating rate of 2 °C/min. The storage modulus (E'), the loss modulus (E''), and tan δ were recorded as a function of temperature throughout the experiment.

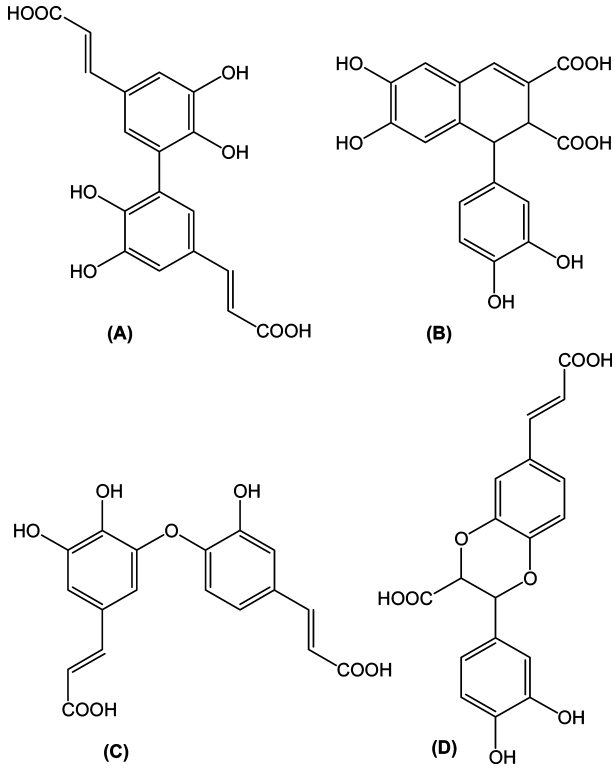
Results and Discussion

Oxidation of CA and Reactions in Model Phenol/Amino Acid Systems. Reactions between natural phenolic compounds and proteins have been postulated to be initiated by oxidation of phenolic structures to form quinone intermediates under alkaline conditions.[37] As quinone structures are very reactive, phenolic oligomers would be expected to form under such conditions. In our studies, the CA solution changed color from pale yellow to dark brown during the oxidation, a color typically associated with phenol oxidation. Figure 1 shows the ^{13}C solution NMR spectra of CA after the oxidation at pH 9 for 1 h and at pH 10 for 2 h. It was noted that oxidation at pH 9/1 h resulted in a series of new resonances. As separation and purification of each product was beyond the scope of the current study, it is not possible to assign all of these new resonances. However, the formation of aliphatic resonances at 45−52 ppm (corresponding to the >CH− and >C< structures) and 75−90 ppm (the >CH−O− structure) are consistent with the formation of the dimeric structures (B) and (D) in Scheme 1 via different mechanisms, as suggested in the literature,[37–39] while resonances around 128−130 ppm are consistent with the formation of structure (A) in Scheme 1. A formic acid signal was also detected at 165 ppm, likely resulting from dissociation of the −COOH groups from CA oligomers and proton transfer during the oxidation.[37] At pH 10/2 h, all of the resonances became broad, indicating formation of polymeric products. No observable oxidation was detected for Ly under the same conditions.

Oxidation of phenolic compounds is prerequisite for their reactive cross-linking with proteins. Such oxidation reactions consume phenolic reactive sites and produce large phenolic molecular species, but a sufficient number of remaining unreacted phenolic sites should still be present and therefore available in these CA oligomers (Scheme 1) for cross-linking reactions with proteins.

The formation of polymeric phenolic products and cross-linked gelatin networks makes it difficult to study the reaction pathway and chemical structures formed during reaction. To overcome this difficulty, small molecular model phenol−amino

Scheme 1. Representative CA Dimeric Structures Proposed in the Literature



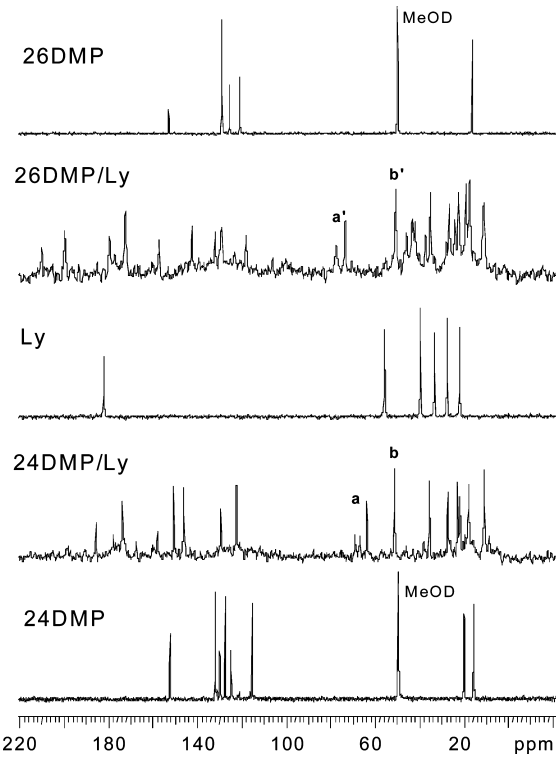


Figure 2. ^{13}C NMR solution spectra of 24DMP, Ly, 26DMP, 24DMP/Ly, and 26DMP/Ly reactions at pH = 9.

acid systems were designed to aid such an investigation. Two compounds, 24DMP and 26DMP, were chosen as model phenols because both compounds have only one (but different) reactive site (*ortho*- or *para*-reactive site for 24DMP or 26DMP, respectively). Ly was chosen as a model amino acid. Although Ly contains two amino groups, 24DMP/Ly and 26DPM/Ly systems are not expected to produce high molecular weight cross-linked products and thus avoid the major difficulties encountered in gelatin systems.

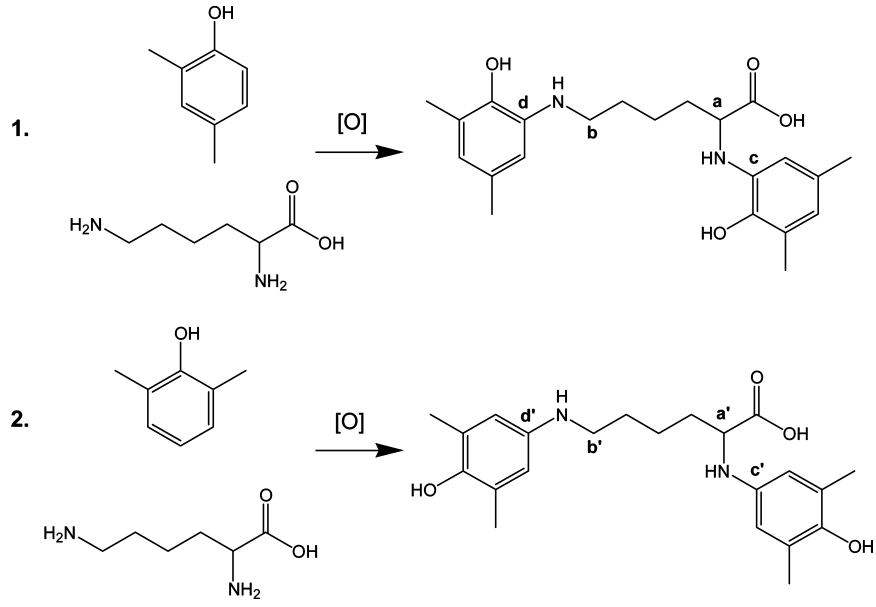
Figure 2 shows ^{13}C solution NMR spectra of 24DMP/Ly and 26DMP/Ly systems after reaction at pH 9. Methanol-d_4 was used as solvent for measuring the spectra of 24- or 26DMP, but the spectra of rest samples were detected in aqueous solution. In the 24DMP/Ly system, a number of new resonances were formed after reaction and the *ortho*-reactive site of 24DMP at 115 ppm disappeared. Two significant peaks were observed at 67 and 54 ppm, corresponding to **a** and **b** carbons in the representative product structure shown in Scheme 2-1 having C−N linkages between 24DMP and Ly. The corresponding phenolic carbon resonances (**c** and **d**) of the structure appeared at 145−147 ppm. Most other resonances also changed their chemical shift due to formation of this 24DMP-Ly product. In the 26DMP/Ly system, the situation was more complicated. However, there was evidence that a similar 26DMP-Ly product was formed from resonances at 77, 54 (**a′** and **b′** carbons), and 146 ppm (**c′** and **d′** carbons), corresponding to the product structure shown in Scheme 2-2. 26DMP dimers and quinone structures (>C═O at 205−215 ppm) were simultaneously formed. Nevertheless, these small molecule model system studies provided direct evidence of chemical reactions between phenolic compounds and protein amino groups to form structures with C−N covalent linkages.

Chemistry and Reactivity of Gelatin Reactions with CA or TA. To enhance the signals derived from CA in the gelatin cross-linking reaction, LCA was used in experiments running in parallel with unlabeled CA. The ^{13}C solution NMR spectra of CA and LCA are shown in Figure 3. A coupling split was observed for all carbon resonances of LCA, because the coupling interaction between ^{1}H and ^{13}C was very strong when all carbons of the compound were ^{13}C-enriched. The minor resonance appearing at 76 ppm indicates the existence, as an impurity, of dimer-D shown in Scheme 1. The ^{13}C CP/MAS spectrum of the solid LCA (top-right) displayed a significant line width broadening due to strong dipolar interactions between ^{1}H and enriched ^{13}C.

The reactions between gelatin and CA at pH 9−10 generated hydrogel materials. This gel formation was a significant phenomenon in the reaction. After a certain period of reaction time, the viscosity of the gelatin solution suddenly started to increase, and the clear solution became a gel material within a few seconds. Figure 4 shows the ^{13}C solution NMR spectra of gelatin, CA, LCA, and gelatin/CA or gelatin/LCA after reaction at pH 9, 58 °C, for 10 min (A), 20 min (B, just before gel formation), and 30 min (C, extra 10 min heating after gel formation). All of these spectra were taken after cooling samples down to room temperature and stabilizing at room temperature for 3−6 h for achieving equilibrium within the systems. The signals derived from unlabeled CA (1.5 wt % to dry gelatin) were barely detectable. But for those using LCA, because of greatly enhanced signals, LCA resonances were observed clearly providing direct information on its structural changes occurred during the reaction.

After 10 min of reaction (A), LCA resonances were effectively unchanged and the system appeared as a homogeneous mixture of pure gelatin and LCA. After 20 min of reaction (B), significant LCA structural changes occurred such that the resonance at 115 ppm (the *ortho*-reactive site of LCA) and 124 ppm (the *para*-reactive site of LCA) disappeared and strong peaks at 130, 138, and 145 ppm were observed. This evidence directly shows that reactions occur at *ortho*- and *para*-unsub-

Scheme 2. Representative Products Formed from Reactions between Ly and 24- or 26DMP



stituted phenolic reactive sites and that the C−N bonds between CA and gelatin, as described in Scheme 3, are indeed formed. As discussed above, CA dimers can also be formed during the reaction. The sharp resonance detected at 165 ppm likely corresponds to the formation of formic acid (HCOOH) through oxidative dissociation from the CA. The double bond of CA (C-7 and C-8 in Figure 3) may also be converted (according to the changes in chemical shift) into other structures as suggested in Scheme 1. The phenolic C−OH resonances (C-1 and C-2 in Figure 3) also shifted to around 148 ppm after the reaction. At longer reaction times, the gelatin solution converted to a gel and the line width of the gel sample (C) significantly broadened due to formation of cross-linked gelatin networks. Interestingly, no new peaks were formed (compare to sample B), but the resonance at 145 ppm (C−N linkages between LCA and gelatin) was enhanced during the extra 10 min reaction.

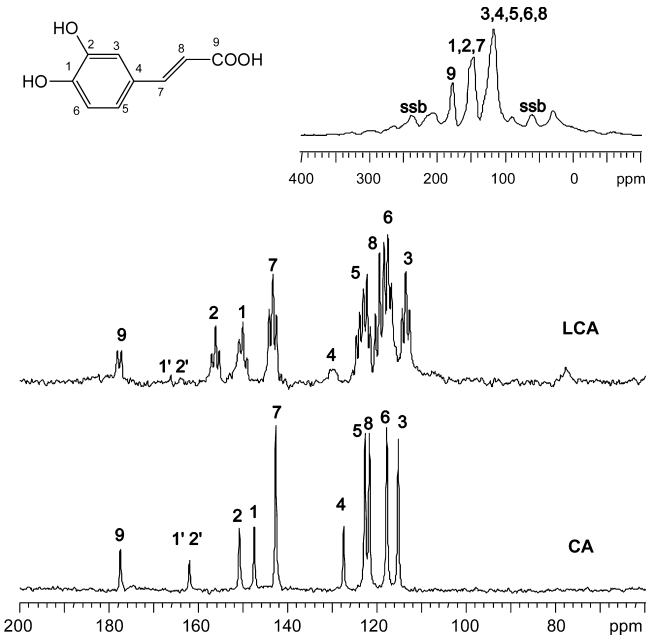


Figure 3. ^{13}C NMR solution spectra of unlabeled CA, 97% ^{13}C-labeled CA (LCA) in aqueous solution and the CP/MAS spectrum of solid LCA (top) where "ssb" represents spinning sideband.

Samples A, B, and C were dried at room temperature for 2 weeks (the same as solution casting) and then conditioned at RH = 52% for 7 days. The ^{13}C CP/MAS NMR spectra of these film samples are shown in Figure 5 together with that of pure gelatin obtained under the same conditions. Although line widths of the spectra became broader for all samples, resonances derived from LCA observed in the range of 115−150 ppm were significant. It is noted that the spectrum pattern of sample A changed on drying and became similar to those obtained at longer reaction times (samples B and C). This suggests that cross-linking reactions also occurred during the drying and conditioning process albeit to a lesser degree. For samples B and C, the resonances at 130 and 145 ppm were much stronger than those for sample A, indicating the number of the C−N linkages in sample A was lower than those in samples B and C.

To implicitly determine whether the formation of C−N covalent linkages between LCA and gelatin had occurred, the carbon resonances of the gelatin/LCA systems were examined by high-resolution solid-state NMR using differing pulse sequences in order to detect different carbon species. Figure 6 shows the ^{13}C CP/MAS NMR spectra of A, B, and C film samples measured by normal CP pulse sequence with a contact time of 1 ms or 20 μs, or by a CP pulse sequence with 10 ms spin-locking time in ^{13}C channel before acquisition.[35] With a CP contact time of 1 ms, most resonances are detected for the gelatin/LCA samples shown in Figures 5 and 6 (A-1, B-1, and C-1). However, only carbons that are directly bonded to hydrogens can be observed at short contact times (20 μs; Figure 6, A-2, B-2, and C-2). Here, the strong C═O peak at 175 ppm as well as resonances at 130, 145−148 either disappeared or their intensities were significantly reduced. This confirms that resonances at 130 and 145 ppm had no bonded hydrogen, the same as the C═O in gelatin and phenolic C−OH carbons of LCA, corresponding to the implicit formation of C−N linkages between LCA and gelatin. In contrast, using 10 ms spin-locking time in ^{13}C channel before acquisition detects only carbons with longer ^{13}C $T_{1\rho}$, that is, normally carbons having no bonded hydrogen(s) in amorphous polymers. Moreover, the spectra shown in Figure 6 (A-3, B-3, and C-3) confirm that the resonances at 130 and 145 ppm have longer ^{13}C $T_{1\rho}$ values and

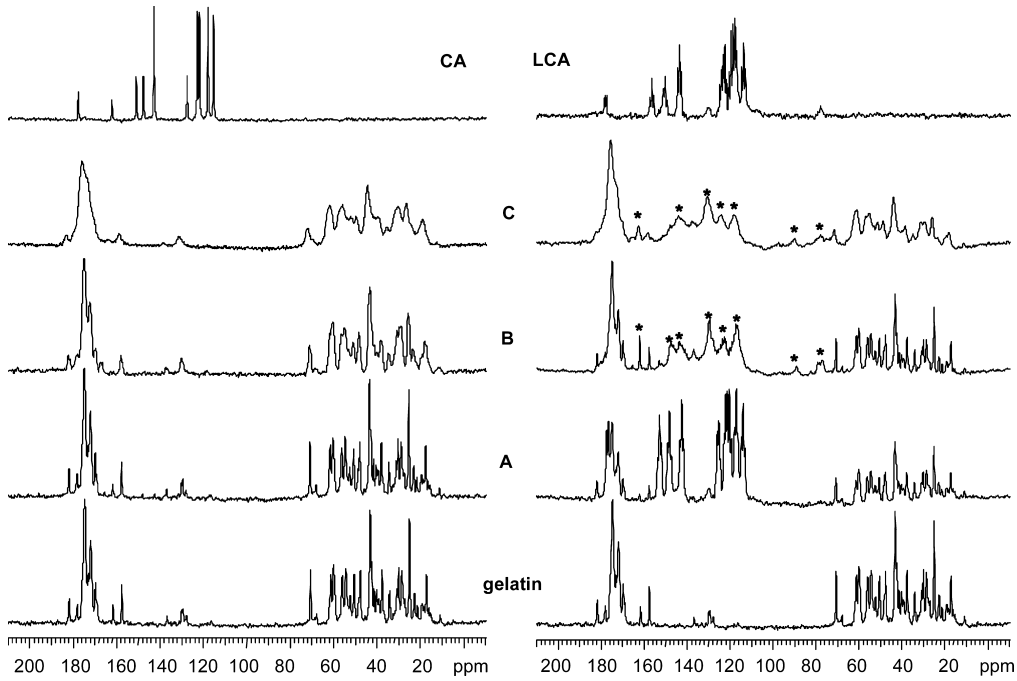
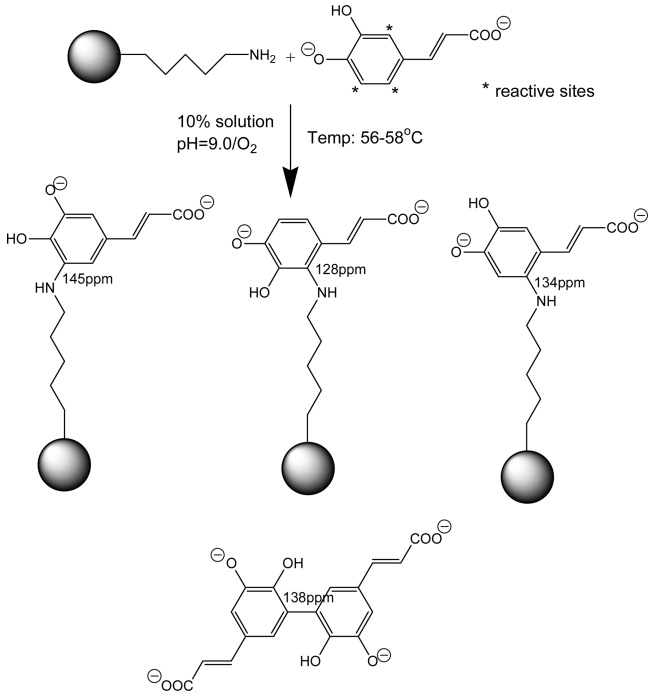


Figure 4. ^{13}C NMR solution spectra in aqueous solution for gelatin cross-linked with 1.5 wt % of unlabeled CA (left) or 97% ^{13}C-labeled CA (LCA, right) after reaction for 10 min (A), 20 min (B), and 30 min forming gel (C); *resonances were derived from LCA.

Scheme 3. Representative Products Formed in Reactions between Gelatin and Caffeic Acid



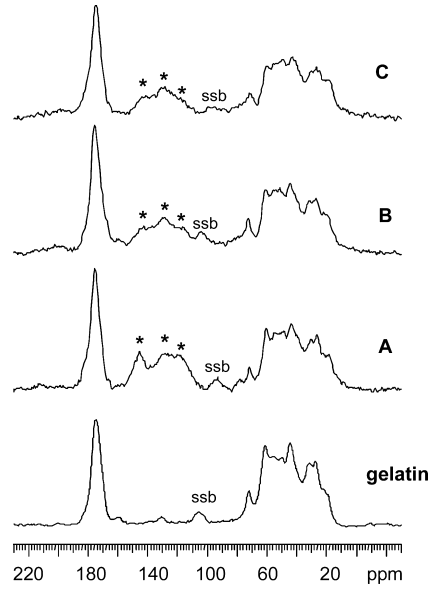


Figure 5. ^{13}C CP/MAS NMR spectra of solution cast gelatin samples after cross-linking reactions with 97% ^{13}C-labeled CA (LCA) for 10 min (A), 20 min (B), and 30 min forming gel (C); "ssb" represents spinning sideband and "*" peaks were derived from LCA.

thus provide further evidence of the formation C−N covalent linkages between LCA and gelatin.

The effect of pH and CA or TA concentration on gel times of gelatin solution (recorded when the conversion from clear solution to gel occurred during the reaction) are presented in Figures 7 and 8. Under reaction conditions of pH 9, 9.5, and 10 using 1.5 wt % of CA or TA (mass to dry gelatin) in a 10% gelatin solution, the gel time of the gelatin/CA system decreased abruptly as pH increased while that of gelatin/TA gradually reduced as pH increased (see Figure 7). Varying the proportional or reactant concentration also changed the gel time at pH 9 (see Figure 8). The effect appeared not to be influenced by the choice of reactant, that is, CA and TA and this may possibly be explained by formation of a high degree of CA oligomers during oxidation and these CA oligomers would behave similarly to TA in reactions with gelatin. The only data point where gel times are significantly shorter for CA than TA is at the low reactant (1.5 wt %) and solution concentration (10%). These results demonstrated that the reactivity could be controlled by varying the pH, the solution concentration and the amount or the species of cross-link agents used in the cross-linking reactions.

Properties of the Gelatin/CA and Gelatin/TA Gel and Film Materials. The hydrogel materials obtained in the reaction did not melt upon heating to 100 °C, being consistent with the formation of stable covalent bonds within the gelatin matrix.

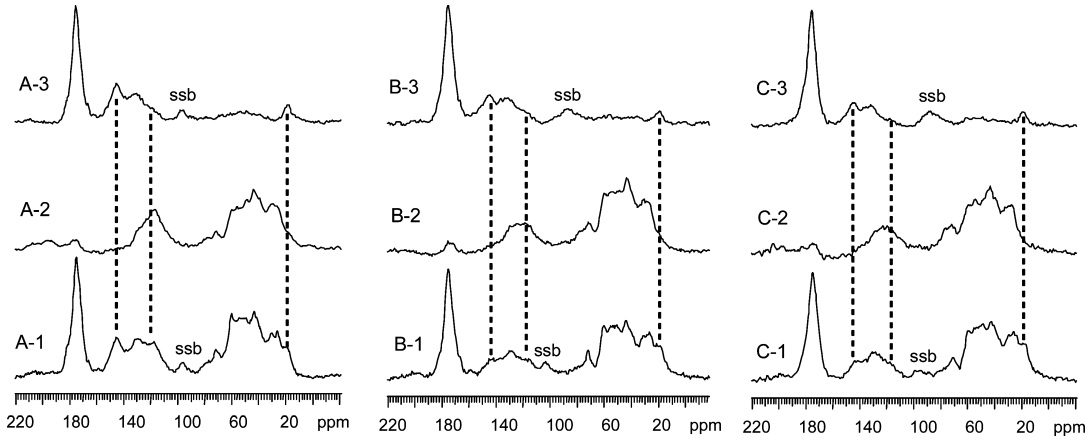


Figure 6. ^{13}C NMR spectra of solution cast gelatin samples after cross-linking reactions with 97% ^{13}C-labeled CA (LCA) for (A) 10 min, (B) 20 min, and (C) 30 min forming gel. (1) CP/MAS spectra with contact time of 1 ms. (2) CP/MAS spectra with contact time of 20 μs. (3) CP/MAS spectra with 10 ms spin locking time in ^{13}C channel before CP. "ssb" represents spinning sideband.

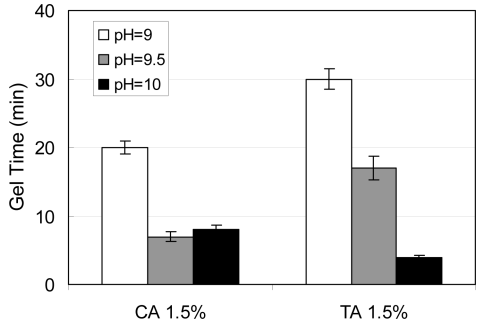


Figure 7. Gel times at pH 9−10 when reacting gelatin with 1.5 wt % of CA or TA in 10 wt % solutions.

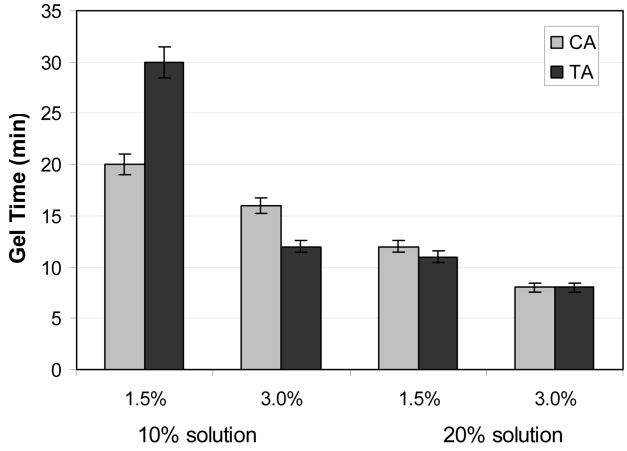


Figure 8. Gel times at pH 9 when reacting gelatin with 1.5 or 3.0 wt % of CA or TA in 10 or 20 wt % solutions.

This is in contrast to the gelatin gel formed during cooling which involving no chemical bonding. Strong intermolecular interactions between macromolecular chains and chemical cross-linking within the protein matrix will significantly restrict molecular motions and enhance dipolar interactions within the gel system. The ^1H spin−spin relaxation time (T_2) is sensitive to the molecular motions of protein hydrogels. If strong intermolecular interactions or chemical cross-linking has occurred, it will result in decreased ^1H T_2 values of the gel samples. The ^1H T_2 data of gelatin/CA and gelatin/TA gels obtained after reactions in 10% solution at pH 9 are listed in Table 1. All of these samples were aged at 5 °C for 24 h and then allowed to warm naturally to room temperature before ^1H T_2 measurement. After the reaction the gelatin-only gel had a ^1H T_2 value of 218 ms, while that of

Table 1. ^1H T_2 Data (ms) at Room Temperature of Gelatin Gel (10 wt %) Samples after Reactions at pH = 9

samples	reaction conditions	^1H T_2 of the gel at RT
gelatin	30 min, no O_2	218 ± 16
CA-1.5%	forming gel with O_2	139 ± 5
CA-3.0%	forming gel with O_2	133 ± 4
TA-1.5%	30 min, no O_2	179 ± 23
TA-1.5%	forming gel with O_2	90 ± 2
TA-3.0%	30 min, no O_2	217 ± 14
TA-3.0%	forming gel with O_2	92 ± 5

gelatin/CA gels was lower at 130−140 ms, suggesting a significant restriction of the molecular motions due to the cross-linking effect. For gelatin/TA gel, the ^1H T_2 value was even lower ~ 90 ms. The ^1H T_2 value of systems where no oxygen was bubbled into the reaction solutions was universally higher and confirmed that oxygen was necessary for the reaction.

Dynamic mechanical analysis (DMA) was conducted in tension mode for these gelatin films to observe any changes in viscoelastic response subsequent to reaction and these results are shown in Figure 9. In general, all gelatin films had adequate strength (storage modulus E' of 6−9 GPa) at room temperature and showed behavior typical of plasticized protein materials in that their storage modulus slowly decreased as temperature increased from −100 to 60 °C. Between −100 °C and ambient temperature, CA or TA modified gelatin films displayed slightly

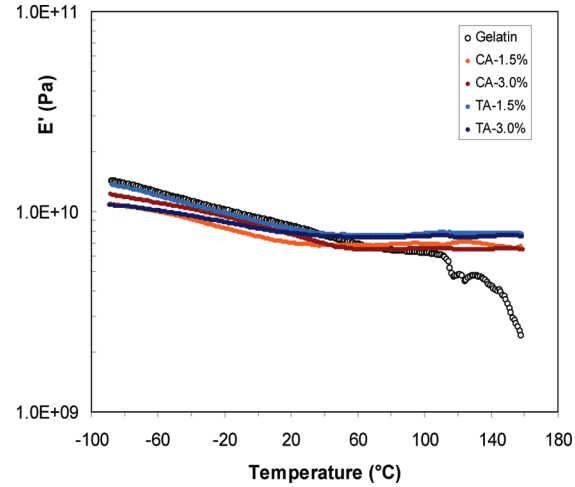


Figure 9. E' storage-modulus data measured by DMA: tension mode for gelatin and modified gelatin films.

Table 2. ^{1}H $T_{1\rho}$ Data (ms) of Gelatin/LCA Films

	LCA component			gelatin component		
samples	145 ppm	128 ppm	116 ppm	60 ppm	44 ppm	27 ppm
gelatin				4.7 ± 0.3	4.9 ± 0.2	4.9 ± 0.2
A	3.9 ± 0.1	4.1 ± 0.1		3.9 ± 0.1	4.0 ± 0.1	3.9 ± 0.1
B	4.2 ± 0.6	4.2 ± 0.3		4.2 ± 0.3	4.2 ± 0.3	4.2 ± 0.2
C	4.6 ± 0.6	4.4 ± 0.2		4.4 ± 0.2	4.3 ± 0.2	4.2 ± 0.2
LCA	32.9 ± 1.4		32.7 ± 1.1			

lower E' values than their unmodified counterpart suggesting the protein aggregative structure of gelatin was modified. On the other hand, the difference in moisture content of these samples might also play a role in determining modulus behavior. Although the moisture content was around 17−19 wt % for all samples, the value for modified samples (18−19%) was 1−2% higher than gelatin film (∼17%). As moisture is an efficient gelatin plasticizer, a small difference in moisture content could cause a change in mechanical properties. The E' of CA or TA modified gelatin films was constant from ∼60 to 160 °C, while in unmodified gelatin film there was a significant drop in E' between 100−160 °C due to glass transition (T_g) and melting of the sample. For the CA or TA cross-linked gelatin materials, their behavior is consistent with the formation of stable cross-linked protein networks. Small differences between gelatin/CA and gelatin/TA film performance are likely attributed to the character of the different cross-link structures (cross-link density, cross-link segment length, etc.) in the respective systems.

High resolution solid-state NMR also provides a powerful technique to explore the intermolecular interactions, molecular motions, and phase structures of protein materials, as demonstrated in our previous publications.[40–45] To assess the molecular motions and intermolecular interactions between gelatin and LCA, ^{1}H $T_{1\rho}$ data were measured through high-resolution ^{13}C resonances, as listed in Table 2. This parameter is sensitive to the molecular motions of polymer chains in the regions of tens of kHz. Only a single exponential $T_{1\rho}$ decay was obtained from all ^{13}C resonances in all samples. As seen in Figure 7, the resonances derived from LCA appear at a range of 110−150 ppm and have no overlap with the signals of gelatin at 20−60 ppm. Thus, the ^{1}H $T_{1\rho}$ data obtained at 110−150 ppm reflected the behavior of LCA component, while those observed at 20−60 ppm reflect the gelatin behavior in gelatin/LCA samples. The ^{1}H $T_{1\rho}$ data for gelatin obtained at different resonances were essentially identical (4.7−4.9 ms), indicating strong spin-diffusion interactions within the gelatin matrix and the material is homogeneous at the scale of the spin-diffusion path length within the $T_{1\rho}$ time, about 2−3 nm.[34,35,46] In the gelatin/LCA samples (A−C), the ^{1}H $T_{1\rho}$ of pure LCA (∼33 ms) significantly decreased and became similar to that of the gelatin component (∼3.9−4.2 ms). This suggests that LCA is homogeneously distributed in the gelatin matrix at the scale of 2−3 nm and that the whole gelatin/LCA materials are homogeneous with the domain sizes of phases (such as cross-linked regions, plasticized phases, high molecular LCA species, etc.) smaller than the scale of 2−3 nm. As only 1.5 wt % of LCA was used in the system, its ^{1}H $T_{1\rho}$ value was averaged out and became similar to those of gelatin in the gelatin/LCA samples, with gelatin as the majority component.

For gelatin/LCA samples at room temperature, significant cross-linking would result in a rigid polymer network and cause ^{1}H $T_{1\rho}$ data of the samples to increase. In contrast, plasticization effect derived from either water or LCA would mobilize the gelatin matrix and reduce the $T_{1\rho}$ value. It was noted that the ^{1}H $T_{1\rho}$ value of the gelatin component in the gelatin/LCA samples was shorter than that of unmodified gelatin possibly due to a weak cross-linking effect (low cross-link density) and slightly higher moisture content (plasticization effect) in the gelatin/LCA systems. Modification of aggregated gelatin structures may also influence the ^{1}H $T_{1\rho}$ values. However, the ^{1}H $T_{1\rho}$ values for samples B and C are higher than that of sample A, which is consistent with an enhanced cross-linking effect in B and C samples.

Conclusion

Cross-linking gelatin at a pH above 9 with natural phenolic compounds CA and TA resulted in formation of insoluble hydrogels. The mobility of these modified gelatin gels was lower than the original gelatin gel. The cross-linking reactivity could be controlled by varying pH, the concentration of the gelatin solution or the amount of CA or TA used in the cross-linking reaction. Oxidation mechanism played a key role in the cross-linking reaction and oxygen acted as an efficient and green oxidizer in the reaction. However, the oxygen bubbling might not be controlled constantly, and some bubbles might be trapped in the gel. The cross-linking chemistry and structures of the modified gelatin gel and cast film samples were studied by both solution and solid state high-resolution NMR technique to investigate small molecular model systems or using ^{13}C labeled caffeic acid (LCA) in the reaction with gelatin. Direct evidence was obtained confirming that chemical reactions occurred between phenolic reactive sites of the phenolic compounds and the amino groups in gelatin to form C−N covalent bonds as the cross-linking linkages in gelatin matrix. The phenolic cross-linker and the C−N cross-linking linkages were found to be homogeneously distributed within the gelatin matrix and the entire cross-linked network was homogeneous on a scale of 2−3 nm. This cross-linking resulted in the modified gelatin films showing no T_g/melting behavior, thus, the films maintain high modulus data at high temperatures.

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BM1001284