Development and Characterization of Genipin Cross-linked Gelatin Based Composites Incorporated with Vegetable-tanned Collagen Fiber (VCF)*

by

Jie Liu,[b]* Cheng-Kung Liu,[a]† And Eleanor M. Brown[a]

[a]*Eastern Regional Research Center, United States Department of Agriculture***

Wyndmoor, PA 19038, USA

[b]*School of Materials Science and Engineering, Zhengzhou University*

Zhengzhou, Henan 450001, China

Abstract

Collagen fibers obtained from solid fibrous wastes generated in tannery have a high potential of being used in developing green composites. Earlier studies in our laboratory demonstrate that nonwoven composites can be derived from collagen fiber network using paper-making technology. The purpose of this work was to fabricate green composites based on gelatin and vegetable-tanned collagen fibers (VCF), using a non-toxic and naturally occurring compound, genipin, as cross-linking agent. VCF were obtained from split hide that has been tanned with vegetable tannins by mechanical milling technique. The VCF content in the networks of gelatin was varied from 10 to 40% (w/w) in dry weight relative to that of the gelatin. The structure, mechanical properties, thermal properties and water resistance of the composite films have been investigated with Fourier transform infrared spectroscopy (FTIR), mechanical testing, thermogravimetric analysis and swelling measurement. The results from those studies revealed that VCF and gelatin could mix with each other homogeneously under alkaline condition and the mechanical properties of the composite films at wet state were improved significantly over that of pure gelatin film. In addition, the composite films showed higher water resistance and thermal resistance than the control. FTIR analysis confirmed the molecular interactions between gelatin and VCF and the formation of cross-links between primary amino groups on gelatin and VCF. Our results indicated that VCF can be used as environmentally friendly and cost-effective potential reinforcing agent for green composites, providing better properties than the original biopolymer matrix. The genipin cross-linked gelatin/VCF composite film can be a promising candidate for the biomedical and packaging applications.

Introduction

Collagen is the most abundant extracellular protein in various connective tissues in animal bodies. Due to its unique triple helical arrangements and fibrous structure, collagen has been found to have diverse applications and is also an important constituent of many food and cosmetic products in the form of gelatin.[1] Using hide and skin as raw materials, leather can be produced by stabilizing through tanning of the proteins, which is accompanied by introduction of additional cross-links into collagen. So far, leather has become one of the most essential commodities of our daily life through its extensive uses in shoes, upholstery, and wearing apparel. Tanning agents with specific active groups are generally used to bind the functional groups (COO^- and NH_3^+) of the collagen.[2] There are several types of tannage could be found in tanning industry, such as chrome, vegetable, and aluminum tannages, etc.[3] Vegetable tanning is an organic method using natural vegetable tannins. Tannins are natural polyphenolic compounds obtained from different kinds of plants, such as the chestnut wood (Castanea sativa), red quebracho (Schinopsis lorentzii), Chinese gallnut etc. Among the various tanning methods, vegetable tanning is considered the most ancient, the most classical and the most environmental-friendly process of making leather. Unlike its chrome tanned counterpart, vegetable-tanned leathers are more eco-friendly and can be recycled.[4] However, large amounts of solid wastes are generated during the transformation of raw hides and skins into leathers in tanneries, including vegetable tannage. According to the studies of other researchers, solid wastes such as trimmings and splits containing proteins and fats that constitute more than 60% of raw hide weight are disposed, which have potentially negative impact on the environment and human health.[5]

†Corresponding author e-mail address: ChengKung.Liu@ars.usda.gov.; Tel (215) 836-6924

Manuscript received August 18, 2017, accepted for publication October 3, 2017.

*Presented in part at the 113th Annual Convention of the American Leather Chemists Association, June 13-16, 2017.

**Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Recent studies have revealed that the solid wastes could be useful to the production of composite materials. After removal of the noncollagenous materials through liming, hair removal and bating steps, collagen fiber networks can be obtained from untanned hides as well as from tanned leathers. Earlier studies indicated that the stability and physical properties of untanned collagen fibers can be improved by cross-linking.[6] Further studies showed the possibility of fabricating nonwoven composites directly from vegetable-tanned collagen fiber network using paper-making technology. The results demonstrated that the collagen fiber sizes and compositions have significant effects on the physical properties of resultant mats and composites.[7]

In addition to these attempts to fabricate green composites, research efforts were also made to develop novel composites by using synthetic thermoplastic polymer as matrix and leather powder as reinforcing fillers. The acrylonitrile-butadiene-styrene (ABS) and leather powder composites were prepared by the extrusion of ABS with leather powder in corotating twin screw extruder.[8] Researchers reported that the tensile modulus, tensile elongation, and Charpy impact strength values are reduced significantly with the incorporation of leather powder into ABS. The unfavorable mechanical properties could be attributed to the poor interfacial bonding and incompatibility. In another study, chrome-tanned leather waste was disintegrated into dust and used as filler in acrylonitrile butadiene rubber (NBR). The resultant NBR/leather composites exhibited considerable improvements in some of their properties such as rheological and mechanical properties.[9]

Though many methods have been developed to fabricate composite materials using collagen fiber derived from solid fibrous leather wastes, there are still various challenges to overcome, particularly in the area of the type of solid waste used and its intrinsic properties, the dispersion state of collagen fibers in the polymer matrix and their interfacial interactions, and the regulation of network structure which is responsible for the final properties and applications of composites. The purpose of this work was to fabricate green composites based on gelatin and vegetable-tanned collagen fiber (VCF), using a nontoxic and naturally occurring compound, genipin, as cross-linking agent. Genipin is an ideal cross-linking agent in biomedical applications. The vegetable-tanned collagen fiber was obtained by grinding the vegetable-tanned splits and trimmings in an experimental mill. The effect of VCF loading on the microstructure, mechanical property, water solubility, swelling ability, and thermal property of the genipin-fixed gelatin matrix was experimentally evaluated.

Experimental

Materials

Porcine Gelatin (~300 Bloom, type A) was purchased from Sigma-Aldrich Chemical Co., USA. Genipin was provided by Challenge Bioproducts Co. Ltd., Taiwan. The vegetable-tanned split in the dry state was obtained from a vegetable tannery, cut into approximately 2.5×5.5 cm^2 pieces, and then ground into fibers using a Wiley Mill (Wiley Laboratory Mill, Model 4, Thomas Scientific, USA). The obtained vegetable-tanned collagen fibers (VCF) were stored at room temperature in sealed plastic bags until use.

Methods for the Preparation of Composites

The composite films used in this study were prepared by casting the gelatin/VCF mixed solution containing determined amount of genipin as illustrated in Figure 1. The VCF was first dispersed in de-ionized water by magnetic stirring at 24°C for 60 min. The pH of the suspension was adjusted drop-wise with 1.0 M NaOH to pH 9.5. Certain amount of gelatin was dissolved in de-ionized water at 50°C. Subsequently, gelatin solution was poured into the VCF dispersion and the resulting slurry was gently stirred for 30 min at 50°C. After achieving a homogeneous dispersion of the VCF suspended in gelatin solution, an aqueous genipin solution in a concentration of 4 g/L was added and stirred for 10 min at 40°C. Composite films were cast by pouring 75 g of the film forming solution into plastic Petri dishes and allowed to dry at room temperature for 72 h. Then the cross-linked dark blue composite films were repeatedly washed with distilled water to remove excess chemicals present in the films. The films were again dried at room temperature and the air-dried films were conditioned in environmental chamber at 20°C and 75% relative humidity (RH) prior to characterization. The obtained genipin cross-linked films were named G-Gel, G-Gel/VCF10%, G-Gel/VCF20%, G-Gel/VCF30%, and G-Gel/VCF40% when the weight percentage of added VCF was 0%, 10%, 20%, 30%, and 40% (w/w) in dry weight relative to that of the dry gelatin, respectively.

Figure 1. Schematic representation of the preparation of genipin cross-linked gelatin/VCF composite films.

Characterization

FTIR analysis

Fourier transform infrared (FTIR) spectra of the samples were collected using a Nicolet 6700 spectrometer, accumulating 60 scans and using a resolution of 2 cm^{-1}, in the wavenumber range of 4000-600 cm^{-1} by KBr pellet technique.

Degree of Cross-linking

The degree of cross-linking of genipin cross-linked film was determined with the ninhydrin assay.[10-11] The assay determines the amount of free amino groups remaining in the film before and after cross-linking. Weighed samples (~2.0 mg) to be assayed were allowed to rehydrate in 1.0 mL de-ionized water for 1 h, and subsequently heated to 100°C in a dry bath incubator (Isotemp 2052FS, Fisher Scientific, UK) with 1.0 mL ninhydrin reagent (2% solution, Sigma-Aldrich Chemical Co., USA) for 20 min. After the solution was cooled down to room temperature and diluted with 50% (v/v) isopropanol, the optical absorbance of the solution was recorded with a Cary 50 UV-vis spectrophotometer (Agilent, Santa Clara, CA, USA) at 570 nm. The amount of free amino groups in the sample is proportional to the absorbance of the solution after heating with ninhydrin. The concentration of free amino groups in each sample is determined from a standard curve of glycine concentration vs. absorbance.[12] The degree of cross-linking (DC) was calculated according to:

$$DC(\%) = \frac{M_{un} - M_{cr}}{M_{un}} \times 100$$

where, M_{un} and M_{cr} are the amounts of free amino groups in noncross-linked and cross-linked samples, respectively. Results reported here were averaged on three independent runs.

Swelling Property and Solubility in Water

The film samples (2×2 cm^2) were first dried at 37°C for 24 h in an incubator and accurately weighed. The samples were then directly immersed into 20 mL distilled water for 1-8 h at 24°C. Wet films were carefully wiped with filter paper to remove surface absorbed water followed by immediately re-weighing the films. The water uptake or swelling property of the films was calculated as:

$$SR(\%) = \frac{W_s - W_d}{W_d} \times 100$$

where SR is the swelling ratio of the samples; W_s and W_d are the weights of the films in their swollen and dry states, respectively. The swelling measurements were repeated three times for each type of film, and an average was taken as the result.

Water solubility was determined as the total soluble matter (TSM) of the film after immersion in distilled water. The film samples (2 × 2 cm^2) were dried at 100±2°C for 24 h in an oven, and weighed to determine the initial dry weight of the films (W_0). After 24 h agitation in 50 mL distilled water at 25°C , the specimens were taken out and dried at 100±2°C until final constant dry weight (W_f) reached. The TSM was calculated by the following equation:

$$TSM(\%) = \frac{W_0 - W_f}{W_0} \times 100$$

TSM tests were performed in triplicate for each film obtained.

Mechanical Tests

Tensile strength (TS), Young's modulus (YM), and elongation at break (EAB) of the films were measured with an Insight 5 mechanical property tester (MTS Systems Corp., Minneapolis, MN) equipped with a 250 N load cell at a rate of 50 mm/min. The specimens were in rectangular shape with dimension of 50×5×0.5 mm^3 and used a 25 mm gauge length. All specimens were preconditioned in environmental chamber at 20°C and 75% RH for at least 1 week and then stretched to failure. TS, YM and EAB in the wet state were measured immediately after taking out the specimens that had been soaked in water for 1 h. The tests were done at approximately 24°C and 50% RH. TS, YM, and EAB values were automatically collected using Testworks-4 data acquisition software. In each case five specimens were tested to obtain a representative value.

Thermogravimetric Analysis (TGA)

Thermogravimetric analysis of the films was carried out using a thermogravimetric analyzer (Q500-1708, TA, USA). Experiments were carried out under nitrogen atmosphere. The weight of the film samples varied from 6 to 8 mg, scanning range was maintained to 22-600°C and the heating rate was 10°C/min.

Scanning Electron Microscopy (SEM) Analysis

The film samples used for SEM were freeze fractured and glued to specimen holders using Duco cement. They were sputter-coated with an ultrathin layer of gold using a Scancoat Six Sputter coater before subjected to SEM. Surface and cross-section of samples were examined and photographed by scanning electron microscope (Model JSM 840A, JEOL USA, Peabody, MA) at an accelerated voltage of 10 kV.

Results and Discussion

FTIR Analysis

Figure 2 shows the FTIR spectra of gelatin, genipin cross-linked gelatin, and genipin cross-linked gelatin/VCF composites. The characteristic absorption bands of gelatin were observed in composites containing both VCF and genipin. The specific absorption peaks include 1638 cm^{-1} (C=O stretching vibrations) for amide I; 1539 cm^{-1} (N-H bending vibrations) for amide II, 1400 cm^{-1} (C-N stretching vibrations); and 1237 cm^{-1} (N-H bending vibrations) for amide III. The appearance of an amide I

mode indicated that all composites adopt a predominantly helical configuration and also confirmed by the appearance of amide II at 1539 cm^{-1}.[13] Mi et al. reported that genipin undergoes ring-opening polymerization prior to cross-linking with polymers under basic conditions.[14] The cross-linking reaction here is believed to occur between amino groups and terminal aldehyde groups on genipin or polymerized genipin via a Schiff-base reaction, which leads to the formation of cross-links between primary amino groups on polymers, including both gelatin and VCF. Therefore, the intermolecular interactions between gelatin and VCF may occur by the formation of covalent cross-linking bonds and hydrogen bonds at the interface. The specific bands corresponding to C-N vibration at 1400 cm^{-1} became broader and stronger for genipin cross-linked films, which might indicate that the genipin cross-linked films contained higher amount of amide bonds due to the formation of C-N bonds during the formation of heterocyclic genipin-cross-linked compound.[15] The formation of the heterocyclic compound was further confirmed by the increase in the intensity of C-N band at 1082 cm^{-1} at the expense of the C-O band at 1031 cm^{-1}.[16] The cross-linking reaction also affects the vibrational frequencies of the amide bond and therefore affects the amide I and amide II bands of the infrared spectrum of gelatin. The broadening and shifting of carbonyl band (1638 cm^{-1}) to higher wavenumbers indicated increased intermolecular associations for genipin cross-linked gelatin/VCF composites. Similar results have been reported by other researchers.[17-18]

From the spectra, the peaks situated around 3435 cm^{-1} of gelatin film might be related to the combination of N-H and O-H stretching vibration. The addition of genipin resulted in the shifting of the band to 3418 cm^{-1}, which could be an indicative of a hydrogen bonding interaction between polymer molecules in the film.[19] It is also possible that the presence of tannins creates new hydrogen bonding interactions between hydroxyl groups of tannin molecule and polar groups of gelatin. [20] Further, at fixed genipin concentration, the band slightly shifted from 3418 cm^{-1} to 3412 cm^{-1} and the peaks appeared broader with the increase of VCF content from 0% to 40%, suggesting that the intermolecular interactions between gelatin and VCF may occur by the formation of additional hydrogen bonds and hydrophobic interactions at the interface.[19] This is in agreement with the observed broadening and shifting of carbonyl bands (1638 cm^{-1}) for increasing amounts of VCF in the composites.

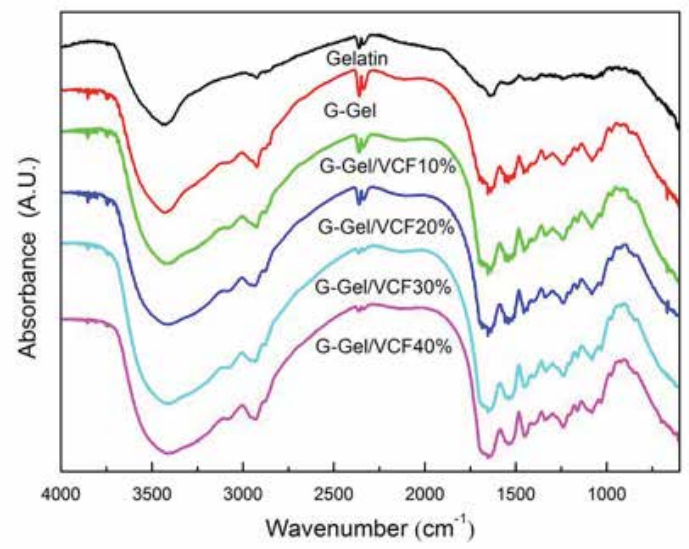

Figure 2. FTIR spectra of gelatin, genipin cross-linked gelatin, genipin cross-linked gelatin/VCF composites.

Degree of Cross-linking

The degree of cross-linking is a measure of the effectiveness of genipin as cross-linker for gelatin and VCF in case there are still free amino groups remaining in the fiber. The degree of cross-linking of the cross-linked samples was determined using a ninhydrin assay. The determinations in all five cross-linked films indicated a degree of cross-linking of 51.4 to 61.8% after genipin cross-linking (Table I). The degree of cross-linking was found to increase with the increasing VCF content. The maximum degree of cross-linking attainable for gelatin/VCF composites was observed to be 54.2 to 61.8% for all content of VCF (10 to 40%) examined. In addition to the cross-linking effect of genipin, tannins also tend to have a strong affinity for gelatin, and the uses of tannin as cross-linker have been reported to improve the physical and chemical properties of gelatin by other researchers.[20-21] The increase of degree of cross-linking with respect to the pure genipin-cross-linked gelatin matrix may be attributed to the cross-linking effect of small amounts of tannin released from the VCF under alkaline condition. Also, the side chain amino groups of gelatin may interact with those tannins which were still immobilized on collagen fibers by an aldehydic cross-linking reaction. It can be envisaged that interactions between gelatin and two cross-linking agents, genipin and tannins, give rise to the formation of different kinds of cross-links. Thus, with higher VCF contents, more free amino groups in composites were likely to react with the cross-linking agents, leading to an increase of the degree of cross-linking.

Swelling Property and Water Solubility

The swelling ability of gelatin-based film is an important aspect to evaluate its property for package, controlled drug release system, medical dressing, et al. The effect of VCF contents on

Table I
Degree of cross-linking of genipin cross-linked gelatin/VCF composites.

VCF content (w/w %)	0	10	20	30	40
Degree of cross-linking (%)	51.4	54.2	54.6	57.4	61.8

swelling ratio (SR) was analyzed and the results are presented in Figure 3. It can be seen that the cross-linked gelatin/VCF composites had lower water uptake compared to the G-Gel matrix. After 8 h of immersion, the SR of the G-Gel film was found to be 450%, whereas due to the incorporation of 10%, 20%, and 30% VCF, the SR values of the composite films were found to be 437%, 337%, and 309%, respectively. Composites which contain more VCF tend to decrease the SR. The significant decrease in the SR of the composite films can be explained by two factors. First, the increase of weight percentage of less swellable VCF within the composites from 10% to 40% reduces the water uptake capability of the composite films. Second, with the increase of the degree of cross-linking from 51.4% to 57.4%, the composites with more cross-linking points possess a greater elastic restorative force which decreases the volume at equilibrium. This behavior is consistent with the most popular theory (Flory-Rehner theory) for predicting the equilibrium swelling of cross-linked polymer networks, where hydrogel volume at equilibrium is affected by the balance between restorative forces and osmotic pressure.[22] However, with further increase of the VCF content from 30% to 40%, the swelling ratio of the films increases from 309% to 395%. This may be attributed to the formation of a much looser polymer matrix with further increase in VCF content. The formation of cross-links between primary amino groups of gelatin and genipin is responsible for a more compact and sustainable hydrogel compared to its tannin cross-linked counterpart. More VCF in the composites will inevitably influence the cross-linking reaction between gelatin and genipin, which is unfavorable for fabricating well cross-linked composites. This hypothesis is in agreement with that of Bigi and coworkers, who suggested that the reaction between genipin and gelatin may be prevented by a shielding effect of the gelation tertiary structure, which prevents genipin to attain a 100% degree of cross-linking.[23] Therefore, although fewer free amino groups in the gelatin/VCF film with 40% VCF were found by ninhydrin assay, this sample still exhibits an increase of swelling ratio at equilibrium. Similarly, Huq et al. observed a significant increase after a constant decrease in SR with the increase of the nanocrystalline cellulose content in a cross-linked alginate gel matrix.[24]

Total soluble matter (TSM, %) value is also a measure of the resistance of composite films against surrounding medium. Figure 4 shows the water solubility as TSM values of control cross-linked gelatin film as well as composite films. Unlike noncross-linked gelatin film which is completely soluble in water, genipin cross-linked gelatin film was slightly soluble in water, consistent with the observation of other authors. [25] The obtained TSM values of films gradually decreases with the increase of VCF content before reaching a lowest value at 20% VCF content, indicating the potential of VCF in reducing the water solubility of gelatin films. Previous researches also have reported that fillers improve water resistance of biopolymer matrix in the film or sheet form.[26-27] However, these values did not comply with a simple rule of mixture of soluble and insoluble components. It was observed that there is a slight increase with the further increase of VCF content from 20% to 40%. This can also be explained by the mechanism of the influence of tannins on the cross-linking structure of the matrix as discussed in swelling tests. As a result, gelatin could not fully react with genipin to form effective cross-linking bridges with the further increase of VCF content. This would in turn form looser polymer matrices between the embedded collagen fibers, resulting in the slightly increase of the TSM.

Mechanical Properties of the Composites

The effects of VCF content on the tensile strength (TS), Young's modulus (YM), and elongation at break (EAB) of the films in dry

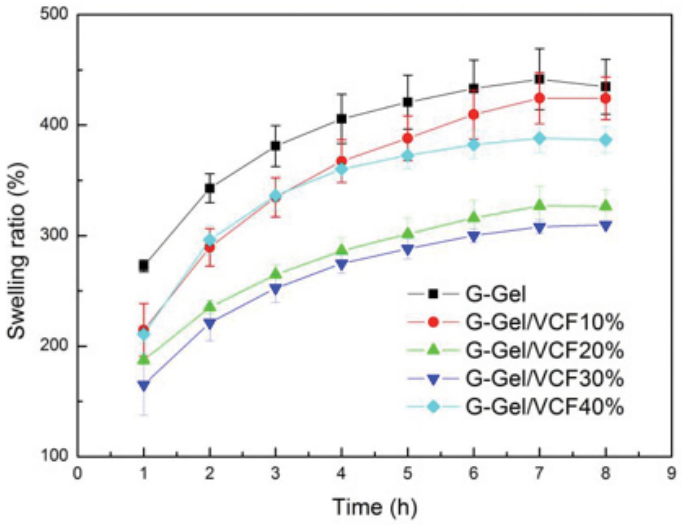

Figure 3. Effect of VCF content on the swelling ratio (SR) for the genipin cross-linked gelatin and gelatin/VCF composites.

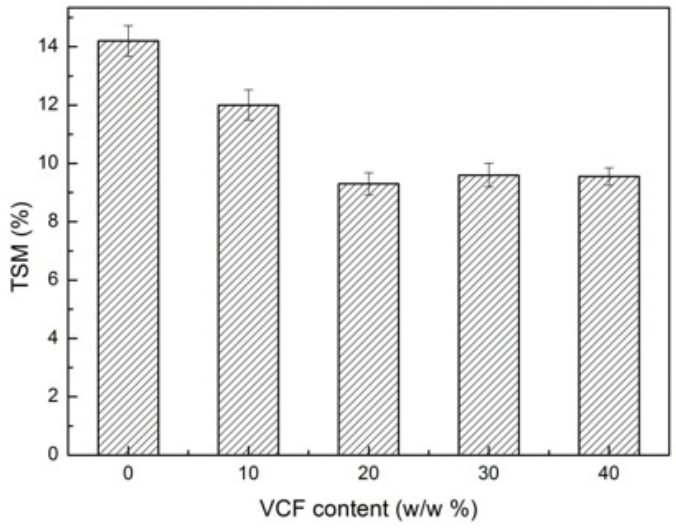

Figure 4. Effect of VCF content on the total soluble mass (TSM) for the genipin cross-linked gelatin and gelatin/VCF composites.

and wet state are shown in Figure 5. In the dry state, the TS of pure genipin cross-linked gelatin films were found to be 100.2±5.3 MPa. The TS of pure gelatin decreased with the incorporation of VCF. With 20% VCF, the TS of the composites decreased to 72.1±0.4 MPa, a decrease of 28% compared to the cross-linked gelatin film (Figure 5a). This is attributable to the presence of VCF bundles in the gelatin matrix, as shown in Figure 7 (SEM). Similar observations were also made in other fiber filled composites that showed lower mechanical properties due to the possible agglomeration at high content of fiber in the matrix.[28] Furthermore, the TS values of the composites were constant with VCF content beyond 20 wt%. This behavior is expected to result from the overlapping and tangling effects resulting from the increase in fiber volume fraction as well as the interaction between the fibers. Additionally, the incorporation of VCF may weaken the gelatin matrix by preventing the self-assembly of gelatin triple helices and the structuration of matrix. Figure 5c shows the effect of VCF content on the EAB of dry gelatin-based composites. The EAB value of the cross-linked gelatin film was found to be 11.5±1.7%. A significant decrease down to 8.2±1.7% in the EAB value was observed for 30% VCF loading, which means a relative decrease of 29% compared to that of the control sample. This behavior has also been reported for the natural silk fiber reinforced gelatin composites prepared

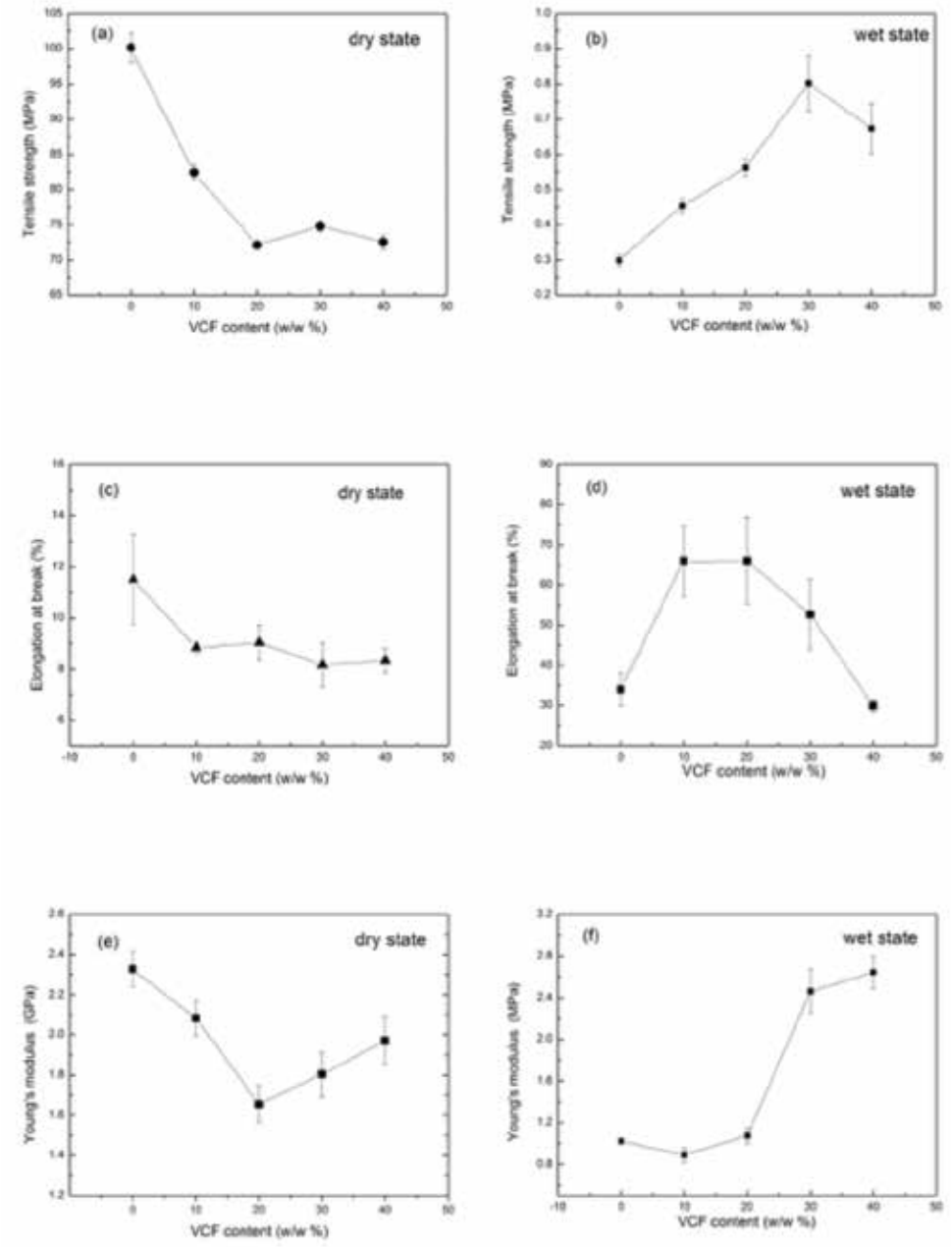

Figure 5. Effect of VCF content (w/w %) on (a, b) tensile strength, (c, d) elongation at break, (e, f) Young's modulus of genipin cross-linked gelatin/VCF film, as a function of VCF content in dry and wet state.

by compression molding process.[29] All of the VCF-containing specimens showed lower values of YM than those of the control film. It was observed that the lowest YM value of the composite films was found to be 1.66 GPa, due to the incorporation of 20% VCF. VCF filled composite films became less brittle as the VCF content increases. The decrease in YM with increasing VCF content can probably be attributed to the decreased stiffness of the film by the addition of VCF. With the further increase of VCF content, composite films may have more cross-linking points on the surface of the fibers compared with films loaded with lower content of VCF, which is in accordance with our results in the degree of cross-linking. Therefore, the YM increases at higher content of VCF (Figure 5e).

Figure 5b, d, and f show the mechanical properties of genipin cross-linked gelatin/VCF composite films upon submersion of these films in distilled water for 1 hour. It was found that the TS of composite films decreased significantly to range between 0.30±0.02 MPa and 0.80±0.08 MPa on average after water-immersion. This can be explained by the plasticizing effect exerted by the water absorbed into the polymer matrix. Water molecules interact strongly with the hydrophilic groups (such as hydroxyl groups and amino groups) on gelatin, resulting in weakening of intermolecular hydrogen bondings. Interestingly, compared with the pure genipin cross-linked gelatin film, the TS of the composite films increased gradually with the increasing loading of VCF from 10 to 30 wt%. This suggests that the addition of VCF could effectively improve the mechanical strength of the swollen films. The strong dependence of the TS on the VCF content is probably related to the enhanced interactions between gelatin and VCF via non-covalent bondings with the help of water. However, higher content of VCF (40%) may be responsible for looser gelatin matrix and higher swelling ratio as discussed above, which could decrease the effective properties of gelatin matrix and facilitate lowering the mechanical properties. The EAB values of the native cross-linked gelatin films were found to be increased from 11.5% to 34.0% after water-immersion (Figure 5c, 5d). Further, a significant increase up to 66±9% in the EAB value of the composite films was observed for films having a 20% VCF loading. Gelatin/VCF composite films in the wet state lose their YM to about one thousandth those of the dry films. Nevertheless, with the increase of VCF content, the YM values of gelatin-based composite films increased from 1.00 MPa to 2.64 MPa. The increased stiffness of the films by the incorporation of VCF is responsible for the increased YM values of VCF reinforced films. The significantly improved mechanical performances of the composite films in the wet state are very important for their potential application as food/medical packaging and biomaterials. Our results demonstrated that genipin cross-linked gelatin/VCF composites are versatile materials which can have tuned mechanical properties under different environments through careful control over the VCF content.

Thermal Property

Thermogravimetric analysis (TGA) was performed in order to analyze the thermal properties of the cross-linked gelatin and gelatin/VCF films. The TGA and DTG curves consist of three major regions as the water loss, pyrolytic, and char decomposition regions, as shown by Figure 6. The first region until 260°C is related to the loss of low molecular mass compounds, mainly adsorbed and structurally bound water, and other volatile impurities.[30] In this region, there are more weight losses in the curves for those samples incorporated with VCF, suggesting more water absorption and coordination capacity of the composites due to the ability of VCF to hold water by coordinate link or hydrogen bond. The main thermal stage occurs in the second region between 260°C and 400°C, where a weight loss of around 50% was observed. This stage refers to thermal degradation of the polymeric chains of gelatin and VCF.[31] It is noticed that the weight losses of gelatin/VCF composites are much less than that of the G-Gel sample in this pyrolytic region. The best thermal stability is shown by G-Gel/VCF40% composite, it loses only 58% while the G-Gel sample

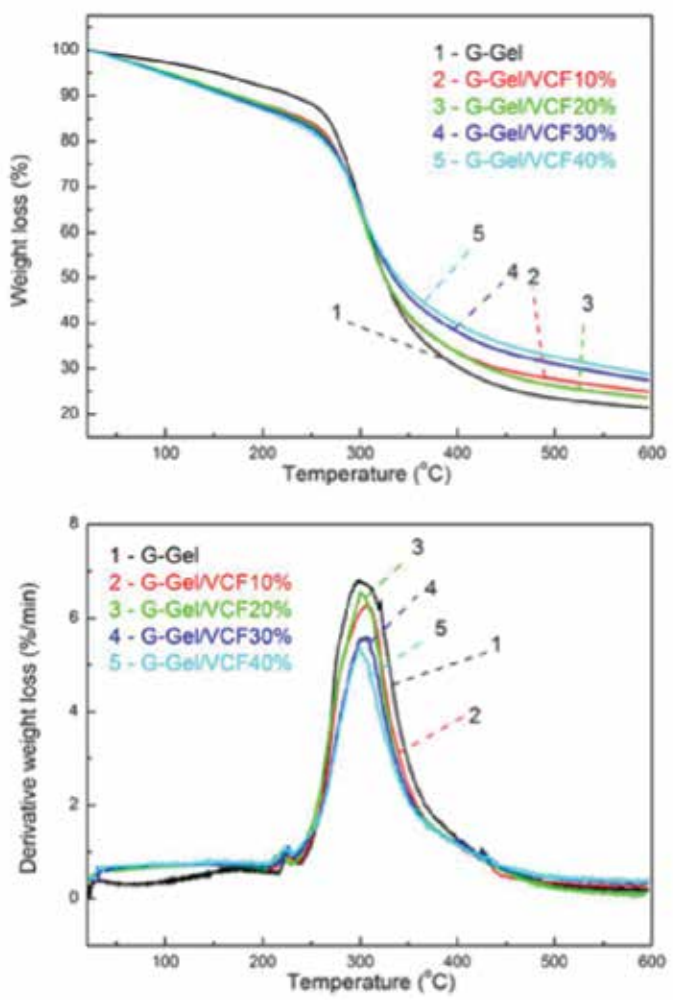

Figure 6. (a) TG and (b) DTG curves of genipin cross-linked gelatin and gelatin/VCF composites.

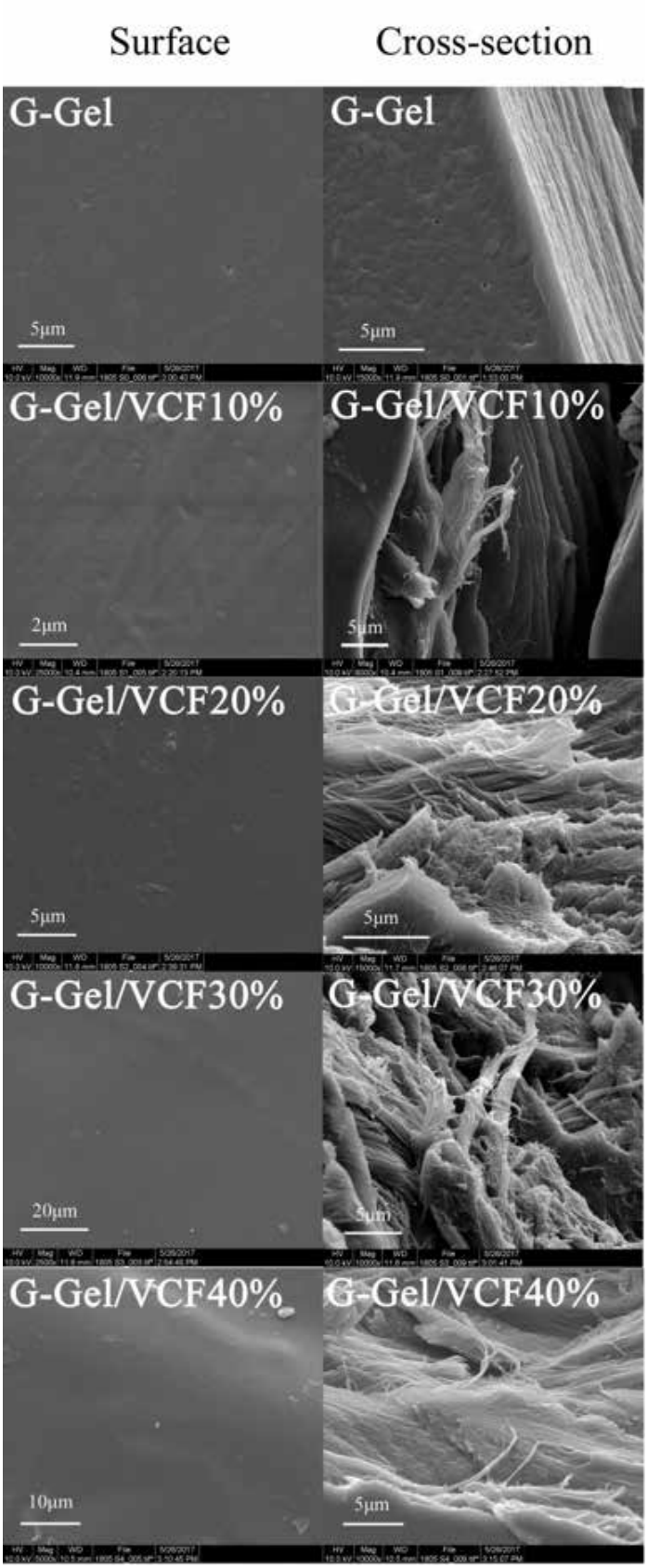

Figure 7. SEM images of the surfaces and cross-sections of the genipin cross-linked gelatin and gelatin/VCF composites.

loses 70% up to 400°C, proving the strong interaction between gelatin and VCF. The third stage started at 400°C show a steady mass loss, which refers to the carbonization of polymeric materials. The ash (residue at 600°C) increases from 22% without VCF to 32% at maximum VCF content used. The investigation indicates that the inclusion of VCF in gelatin matrix has a favorable effect on the thermal stability in nitrogen atmosphere.

Morphology

Scanning electron microscopy (SEM) was carried out for extensive morphological inspection of surface and cross-section in gelatin/VCF composite films. Figure 7 represents the SEM images of gelatin/VCF composites with different VCF content. From the SEM images of G-Gel film, it was found that the cross-linked gelatin film had a relatively smooth and continuous surface, suggesting no filling materials as compared to samples containing VCF. The surface morphologies of composite films incorporated with 10-40% VCF were overall similar to control, regardless of the VCF contents. Figure 7 also shows the composites containing VCF content from 10% to 40% which has remarkably different cross-section surfaces. It can be observed that the composites incorporated with VCF had a very rough surface, and fiber bundles pull out is clearly found. In spite of these fiber bundles, SEM images also reveal the presence of long and thin collagen fibers dispersed in the gelatin matrix. The decrease in TS of the gelatin/VCF composites in the dry state could be due to the presence of randomly distributed VCF bundles or fiber aggregates embedded into the gelatin networks. Indeed, fiber bundles act as stress concentration points within the polymer matrix and can in some cases, reduce the mechanical properties of original polymer as demonstrated by other researchers.[32]

Conclusions

We successfully prepared genipin cross-linked gelatin/VCF composite materials which are characterized in terms of chemical structure, degree of cross-linking, swelling behavior, solubility, thermal property and mechanical characteristics as well as surface and cross-section morphology. It was shown that the addition of VCF provides better mechanical properties at wet state for the gelatin matrix. The swelling behavior and solubility can be controlled through adjustment of the VCF contents. The composites have shown much less mass losses and greater char residues than that of genipin cross-linked gelatin matrix during the pyrolysis process. Our results indicate that VCF can be useful to develop green composites providing better properties, than the original biopolymer matrix. This work contributes to the development of genipin cross-linked gelatin/VCF composites as a promising candidate for use in future biomedical and packaging applications.

Acknowledgments

The authors wish to thank ARS, ERRC scientists: Joseph Uknalis for SEM photos, Jianwei Zhang for TGA tests, Ryan Marsico for FTIR analysis, and Nickolas P. Latona for sample preparations.

References

1. Gómez-Guillén, M. C., Pérez-Mateos, M., Gómez-Estaca, J., López-Caballero, E., Giménez B. and Montero P.; Fish gelatin: a renewable material for developing active biodegradable films. *Trends in Food Science and Technology* **20**, 3-16, 2009.
2. Brown, E. M. and Dudley R.L.; Approach to a tanning mechanism: Study of the interaction of aluminum sulfate with collagen. *JALCA* **100**, 401-409, 2005.
3. Brown, E. M. and Shelly, D. C.; Molecular modeling approach to vegetable tanning: preliminary results for gallotannin interactions with the collagen microfibril. *JALCA* **106**, 145-152, 2011.
4. Krishnamoorthy, G., Sadulla, S., Sehgal, P. K. and Mandal, A. B.; Green chemistry approaches to leather tanning process for making chrome-free leather by unnatural amino acids. *J. Hazard Mater.* **215-216**, 173-182, 2012.
5. Ozgunay, H., Colak, S., Mutlu, M. M. and Akyuz, F.; Characterization of leather Industry wastes. *Polish J. of Environ. Stud.* **16**, 867-873, 2007.
6. Liu, C.-K., Latona, N. P., Taylor, M. M. and Latona, R. J.; Effects of bating, pickling and cross-linking treatments on the characteristics of fibrous networks from un-tanned hides. *JALCA* **108**, 79-85, 2013.
7. Liu, C.-K., Latona, N. P. and Taylor, M. M.; Preparations of nonwoven and green composites from collagen fibrous networks. *JALCA* **109**, 35-40, 2014.
8. Ramaraj, B. Mechanical and thermal properties of ABS and leather waste composites. *Journal of Applied Polymer Science* **101**, 3062-3066, 2006.
9. El-Sabbagh, S. H. and Mohamed, O. A.; Recycling of chrome-tanned leather waste in acrylonitrile butadiene rubber. *Journal of Applied Polymer Science* **121**, 979-988, 2011.
10. Cui, L., Jia, J. F., Guo, Y., Liu, Y. and Zhu, P.; Preparation and characterization of IPN hydrogels composed of chitosan and gelatin cross-linked by genipin. *Carbohydrate Polymers* **99**, 31-38, 2014.
11. Yao, C. H., Liu, B. S., Chang, C. J., Hsu S. H. and Chen Y. S.; Preparation of networks of gelatin and genipin as degradable biomaterials. *Mater. Chem. Phys.* **83**, 204-208, 2004.
12. Lai, J. Y. and Ma, D. H.; Glutaraldehyde cross-linking of amniotic membranes affects their nanofibrous structures and limbal epithelial cell culture characteristics. *International Journal of Nanomedicine* **8**, 4157-4168, 2013.
13. Boskey, A. and Camacho, N. P.; FT-IR imaging of native and tissue engineered bone and cartilage. *Biomaterials* **28**, 2465-2478, 2007.
14. Mi, F. L., Shyu S. S. and Peng, C. K.; Characterization of ring-opening polymerization of genipin and pH-dependent crosslinking reactions between chitosan and genipin. *Journal of Polymer Science Part A: Polymer Chemistry* **43**, 1985-2000, 2005.
15. Butler, M. F., Ng Y. F. and Pudney, P. D. A.; Mechanism and kinetics of the cross-linking reaction between biopolymers containing primary amine groups and genipin. *Journal of Polymer Science: Part A: Polymer Chemistry* **41**, 3941-3953, 2003.
16. Chen, S. C., Wu, Y. C., Mi, F. L., Lin, Y. H., Yu, L. C. and Sung, H. W.; A novel pH-sensitive hydrogel composed of N, O-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery. *Journal of Controlled Release* **96**, 285-300, 2004.
17. Hameed, N., Glattauer, V. and Ramshaw, J. A.; Evaluation of polyvinyl alcohol composite membranes containing collagen and bone particles. *Journal of the Mechanical Behavior of Biomedical Materials* **48**, 38-45, 2015.
18. Muyonga, J. H., Cole, C. G. B. and Duodu, K. G.; Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (Lates niloticus). *Food Chemistry* **86**, 325-332, 2004.
19. Xie, Y. L., Zhou, H. M. and Qian, H. F.; Effect of addition of peach gum on physicochemical properties of gelatin-based microcapsule. *Journal of Food Biochemistry* **30**, 302-312, 2006.
20. Peña, C., de la Caba, K., Eceiza, A., Ruseckaite, R. and Mondragon, I.; Enhancing water repellence and mechanical properties of gelatin films by tannin addition. *Bioresource Technology* **101**, 6836-6842, 2010.
21. Cao, N., Fu, Y. and He, J.; Mechanical properties of gelatin films cross-linked, respectively, by ferulic acid and tannin acid. *Food Hydrocolloids* **21**, 575-584, 2007.
22. Kamata, H., Li, X., Chung, U. and Sakai, T.; Design of hydrogels for biomedical applications. *Advanced healthcare materials* **4**, 2360-2374, 2015.
23. Bigi, A., Cojazzi, G., Panzavolta, S., Roveri N. and Rubini, K.; Stabilization of gelatin films by crosslinking with genipin. *Biomaterials* **23**, 4827-4832, 2002.
24. Huq, T., Salmieri, S., Khan, A., Khan, R. A., Tien, C. L., Riedl, B., Fraschini, C., Bouchard, J., Uribe-Calderon, J., Kamal, M. R. and Lacroix, M.; Nanocrystalline cellulose (NCC) reinforced alginate based biodegradable nanocomposite film. *Carbohydrate Polymers* **90**, 1757-1763, 2012.
25. Chiono, V., Pulieri, E., Vozzi, G., Ciardelli, G., Ahluwalia, A. and Giusti, P.; Genipin-cross-linked chitosan/gelatin blends for biomedical applications, *J Mater Sci*: *Mater Med* **19**, 889-898, 2008.

26. Cao, X., Chen, Y., Chang, P. R., Stumborg, M. and Huneault, M. A.; Green composites reinforced with hemp nanocrystals in plasticized starch. *Journal of Applied Polymer Science* **109**, 3804-3810, 2008.
27. Abdollahi, M., Alboofetileh, M., Behrooz, R., Rezaei, M. and Miraki, R.; Reducing water sensitivity of alginate bio-nanocomposite film using cellulose nanoparticles. *International Journal of Biological Macromolecules* **54**, 166-173, 2013.
28. Khan, R. A., Salmieri, S., Dussault, D., Calderon, J. U., Kamal, M. R., Safrany, A. and Lacroix M.; Production and properties of nanocellulose-reinforced methylcellulose-based biodegradable films. *Journal of Agricultural and Food Chemistry* **58**, 7878-7885, 2010.
29. Shubhra, Q. T. H., Alam, A. K. M. M., Beg, M. D. H.; Mechanical and degradation characteristics of natural silk fiber reinforced gelatin composites, *Materials Letters* **65** , 333-336, 2011.
30. 30. Guo, J., Ge, L., Li, X., Mu, C. and Li, D.; Periodate oxidation of xanthan gum and its cross-linking effects on gelatin-based edible films. *Food Hydrocolloids* **39**, 243-250, 2014.
31. Cucos, A., Budrugeac, P., Simultaneous TG/DTG–DSC–FTIR characterization of collagen in inert and oxidative atmospheres, *Journal of Thermal Analysis and Calorimetry* **115**, 2079-2087, 2014.
32. Bhattacharya, M.; Polymer nanocomposites-a comparison between carbon nanotubes, graphene, and clay as nanofillers. *Materials* **9**, 262-297, 2016.