Cross-linking of Gelatin with Epichlorohydrin in a Sodium-Acetate-Trihydrate/Urea Deep Eutectic Solvent System

by

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Abstract

To improve the cross-linking efficiency of gelatin molecules, sodium acetate trihydrate/urea deep eutectic solvent with 30% water (SAT/U-DES$_{30W}$, based on DES weight) was proposed as the solvent of gelatin in this paper. The system could overcome the gelation effect of gelatin at low temperature and expose more active groups of gelatin molecules, resulting in better cross-linking effect. Firstly, the optimal reaction concentration of epichlorohydrin (ECH) was determined by boiling water solubility of cross-linking products, and the cross-linking efficiency of gelatin under two systems was judged together with the Glass transition temperature method. Secondly, the water resistance of the cross-linked product was investigated by swelling capacity and water contact angle. The water absorption rate of GE$_W$ and GE$_{DES}$ decreased from 4614% to 1500% and 929% at 12 h, respectively, and the water contact angle also obviously decreased, indicating that the cross-linking efficiency of gelatin under DES system was higher. Finally, the cross-linking mechanism of gelatin and ECH were discussed and it was found the thermal stability of GE$_{DES}$ was significantly improved, which would provide a theoretical basis for gelatin-based materials.

Introduction

In the leather industry, a large amount of leather wastes is generated during tanning. Initially, leather wastes were disposed of by discarding in a landfill. This way not only caused a waste of resources, but also seriously polluted the environment. In this context, the researchers have transformed the waste into resources by hydrolyzing the leather shavings to obtain gelatin.[1] The molecular weight of gelatin acquired by the method was low but exhibited a wide distribution and its aqueous solution shows high viscosity. Gelatin is widely used in food, pharmaceuticals and cosmetics industry due to its good film-forming properties, emulsifying properties and biocompatibility. There are a large number of hydrophilic groups in gelatin molecules, the molecules will absorb water and swell rapidly when dissolved in water. And the elevated temperature will break the helix structure of molecular chains to promote its dissolution. However, the strong hydrophilicity and instability of gelatin limit its further application. Cross-linking reaction is generally regarded as an effective method to overcome these problems, because cross-linking reactions will form a dense structure among gelatin chains to enhance the water resistance and stability. But gelatin molecules exhibit an aggregated state in aqueous solution,[2] which forms a large number of hydrogen bonds and van der Waals forces between chains, resulting in the difficulty exposing active groups and hydrophobic bonds inside the molecules and low modification efficiency in water phase. In addition, there is always a certain amount of water in the bulk gelatin, named structural water, which contribute to the helix structure of gelatin. However, the presence of such water complicates the relationship between the macromolecular structure and its solution properties.[3] Cooling of a gelatin solution induces partial conformational change of gelatin chains into helix and then the sol-gel transition occurs, leading to the difficulty of the cross-linking reaction in gelatin molecules at room temperature.[4] Although gelatin can be dissolved in some organic solvents, the application of these solvent systems is still limited due to toxicity and instability. At present, scholars have begun to turn their attention to ionic liquids and deep eutectic solvents (DES) to solve these problems of gelatin molecules in aqueous solution. For instance, Horinaka et al have investigated the rheology behavior of gelatin/ 1-ethyl-3-methylimidazolium dimethyl phosphate.[5] Grønlien et al have explored the behavior of collagen in a natural DES solution.[6] However, most of these studies only focus on the interaction mechanism between gelatin and solvents, and few studies concentrate on the modification effect of gelatin in the systems.

In previous work of this laboratory, sodium acetate trihydrate (SAT) and urea were heated in a molar ratio of 1:2 to obtain SAT/U-DES. The changes of physicochemical properties and microstructure of "water-in-DES" system were investigated. Then SAT/U-DES was used to dissolve gelatin and it was found SAT/U-DES with 30% water (SAT/U-DES$_{30W}$), was the optimal dissolution system for gelatin. SAT/U-DES$_{30W}$ system could effectively overcome the gel effect of gelatin solution and did not destroy the molecular structure of gelatin.[7] Therefore, this work intends to carry out subsequent cross-linking reaction under the system.

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At present, modification methods for gelatin are mainly either physical or chemical with the later proving to be more effective. The common cross-linking agents include aldehydes, epoxides, carbodiimides, and natural molecules. Therein, the polarization of the epoxy charge and the epoxy ring tension of epichlorohydrin (ECH) make it highly reactive. Thus, ECH can be used to react with the reactive groups in gelatin molecule via its epoxy group to form new chemical bonds and a denser network, which can make the peptide structure of gelatin more stable and also improve the properties of gelatin-based products.

The objectives of this work were to cross-link gelatin with ECH in SAT/U-DES_{30W}, and to compare the cross-linking effect with that in water phase. It was expected that the SAT/U-DES_{30W} system can significantly improve the cross-linking efficiency of gelatin, the cross-linking products obtained showed higher water resistance and stability than those cross-linked under water phase, which could provide a certain theoretical basis for the diversified application of gelatin.

Material and Methods

Materials

Sodium acetate trihydrate (SAT), urea and epichlorohydrin (ECH) were purchased from Chengdu Jinshan Chemical Reagent Co. Ltd and they are all analytical purity. Gelatin (Industrial Grade, M_w = 20~60 kDa) was acquired from Key Laboratory of Leather Chemistry and Engineering, Sichuan University.

Cross-linking reactions under SAT/U-DES_{30W} and water system

On the basis of the dissolution system for gelatin previously explored in the laboratory, the SAT/U-DES with 30% water content was used to dissolve gelatin. Exactly 40.8 g of SAT, 36.0 g of urea and 30% w/w water content (23.04 g, based on SAT/U-DES weight) were mixed in a round-bottomed flask and heated at 60°C, with constant mechanical stirring for 1 h until forming a transparent homogeneous liquid. Then 20% w/w of gelatin was introduced into the system under continuous stirring until dissolved completely. Finally, ECH with different weight fractions (based on gelatin weight) was added to the system and cross-linked with gelatin molecules for 2 h under SAT/U-DES_{30W} system, and the sample was named GE_{DES}. What's more, the sample under water system was prepared under the same reaction conditions, and it was labeled as GE_W.

GE_{DES} was transferred to the 300 Da dialysis tubing for 24 h dialysis and dried in a polytetrafluoroethylene mold at 45°C to obtain solid GE_{DES} sample. The solid gelatin and GE_W samples were also prepared in the same way for subsequent performance tests.

Boiling water solubility

The solid GE_{DES} and GE_W samples were completely dried and placed in a boiling water bath at 100°C for 1 h, then taken out and dried to constant weight. The weight of the samples before and after boiling were determined, and the solubility was calculated to determine the cross-linking degree of gelatin.

Glass transition temperature

The solid gelatin, GE_W and GE_{DES} were dried completely and 5-10 mg of samples were taken under nitrogen atmosphere to test the glass transition temperature at a heating rate of 10°C/min and a scanning range of 30~150°C.

Swelling capacity and water contact angle

The swelling capacity of solid gelatin, GE_W and GE_{DES} were determined using the gravimetric method. The samples after drying were immersed in distilled water at room temperature for 12 h, they were weighed after blotting excess surface water with filter papers every two hours. The water absorption rate (%) of the samples was calculated as follows:

$$\text{Water absorption rate (\%)} = \frac{W_2 - W_1}{W_1} \times 100\% \quad (1)$$

where W_2 is the weight of the swollen sample and W_1 is the weight of the dried sample.

The water contact angle (WCA) of gelatin film, GE_W film and GE_{DES} film obtained were carried out by the sessile drop method using an optical tensiometer, equipped with the image analysis OneAttension software. A droplet of Mili-Q water (5 μL) was deposited on the film surface (2 cm^2, 25°C) with a precision syringe. The droplet image was recorded by a video camera (after 0s, 30s, 60s), and the droplet profile was numerically solved and fitted to Young-Laplace equation by the OneAttension software. The test was repeated three times for each sample and the results were averaged.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR was conducted using a Nicolet iS10 FTIR spectrometer (PerkinElmer, USA). The gelatin and solid GE_{DES} were oven dried and milled into powder. Then the dried samples were blended with potassium bromide powder and pressed into tablets before spectrum acquisition. The pellet was examined using a Spectrum One FTIR spectrometer. The scan was conducted between 4000 and 500 cm^{-1} with a resolution of 2 cm^{-1} and the background spectrum was collected before each scan.

X-ray diffraction (XRD)

The gelatin and GE_{DES} film were dried and made into 30 mm × 30 mm film, placed in an X-ray diffractometer to test the crystal structure. The voltage was 40 kV, the current was 40 mA, the diffraction Angle was 5°-50°, and the scanning rate was 2°/min.

Scanning electronical microgram (SEM)

The surfaces and cross-sections of gelatin film and GE_{DES} film were observed under field emission scanning electron microscope. The samples were treated with gold spraying in vacuum before observation and the test voltage was 5 kV.

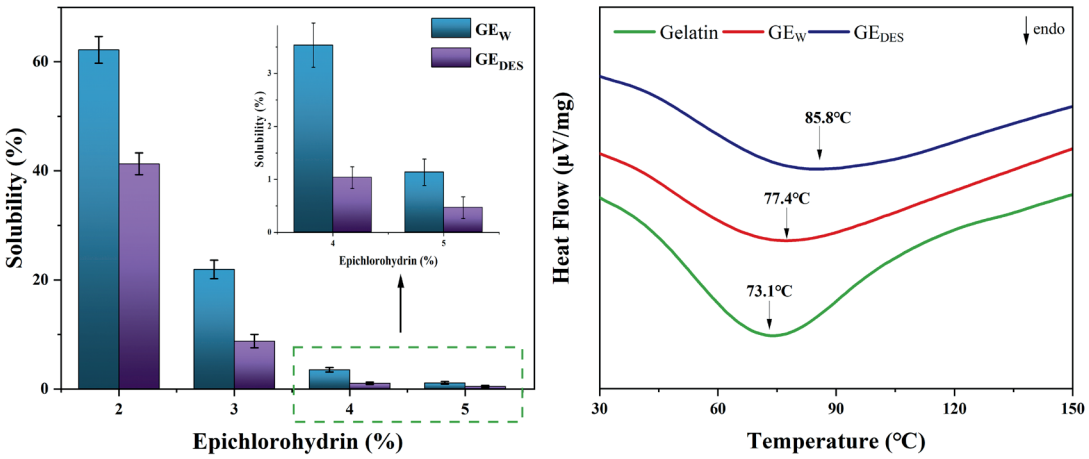

Figure 1. **(a)** The solubility of GE_W and GE_{DES} with different ECH concentrations in boiling water. **(b)** DSC curves of gelatin, GE_W and GE_{DES}

Thermo-gravimetric analysis (TGA)

Thermogravimetric analysis was carried out using a TG209F1 thermal analyzer (Netzsch, Germany). The dried samples were heated from 50°C to 600°C at a heating rate of 10°C/min under a continuous stream of nitrogen. During this pyrolysis, the apparatus automatically recorded TG curves and saved data for analysis.

Results and Discussions

Cross-linking degree analysis

Boiling water solubility

The cross-linking degree of ECH to gelatin can be estimated from the solubility of samples in boiling water. Figure 1(a) shows the solubility of solid GE_W and GE_{DES} samples in boiling water at 100°C. When the weight fraction of ECH was 2.0%, the solubility of GE_W reached 65.2%, indicating that the cross-linking degree of gelatin and ECH was too low and most of gelatin molecules still existed in the dissolved state. With the increase of ECH content, the degree of cross-linking increased and the solubility of cross-linking products gradually decreased. When the ECH content reached 4.0%, the solubility of GE_W reduced to 3.1%. It could be explained that ECH and gelatin molecules formed a dense network structure by cross-linking reaction and blocked the infiltration of water molecules. The solubility of GE_{DES} was only 0.8% under the same ECH content conditions (4.0%). With a further increase of ECH content, the solubility of GE_{DES} remained constant, suggesting that the cross-linking reaction between gelatin and ECH reached saturation as the content of ECH was 4.0% in SAT/U-DES_{30W} system. According to the overall solubility trend, gelatin and ECH can display higher cross-linking degree under SAT/U-DES_{30W} system than that in water phase, resulting in lower solubility of the products. For subsequent experiments and performance characterization, the products cross-linked with 4% ECH was adopted.

Glass transition temperature

The thermal stability of gelatin molecules will increase with the increase of cross-linking degree, so DSC can be used to characterize the Glass transition temperature of gelatin, GE_W and GE_{DES} to determine their cross-linking degree.[8] As was shown in Figure 1(b), the Glass transition temperature of gelatin was 73.1°C, representing the transition of gelatin molecules from glassy state to high elastic state.[9] The Glass transition temperature of GE_W increased to 77.4°C due to the chemical cross-linking between gelatin and ECH. When the external chemical cross-linking existed, the cross-linking agent will covalently bond among the molecules in gelatin, which makes it tend to be stable and difficult to be destroyed by high temperature. In addition, it was observed that the enthalpy changes in the gelatin denature process was significantly greater than that in the GE_W denature process. Therefore, compared with GE_W, the phase transition temperature of gelatin molecules in GE_{DES} was higher and reached 85.8°C, GE_{DES} showed better thermal stability than that of GE_W, indicating that gelatin and ECH have better cross-linking effect under SAT/U-DES_{30W} system.

Swelling capacity

Figure 2 showed the swelling capacity of solid gelatin, GE_W and GE_{DES} in distilled water. It could be observed that the swelling weight of the samples increased with the increasing length of

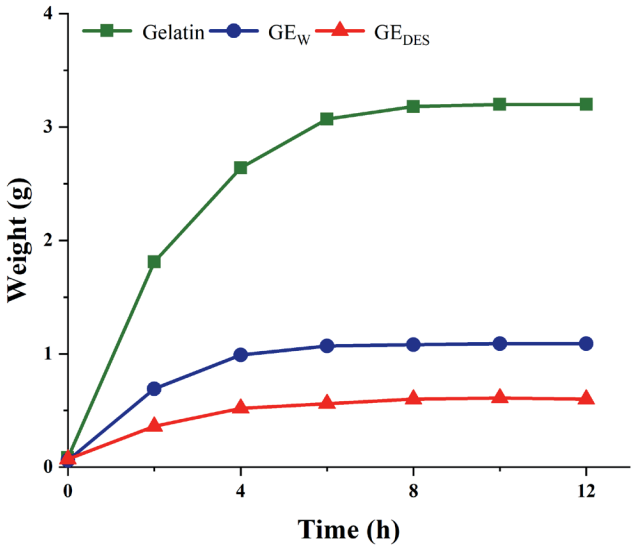

Figure 2. The swelling capacity of gelatin, GE_W and GE_{DES}.

time, and then leveled off until an equilibrium was reached. The initial weight of all three samples was 0.07 g and they reached different weights after 12 h water absorption expansion. The weight of gelatin reached 3.30 g after water absorption and the swelling rate reached 4614%. The surface of gelatin molecules contained a large number of hydrophilic groups, leading to its high swelling capacity. The water absorbed by gelatin could be divided into two states. First, water molecules were combined with the polar groups in gelatin by hydrogen bonds, called *hydration water*. Second, the free water existed among the gelatin chains, called *swelling water*.[10] After cross-linked with ECH, the weight of GE_W and GE_{DES} reached 1.12 g and 0.73 g, respectively, it could be explained that ECH can cross-link more groups inside gelatin molecules and form denser network than that in the water system. Compared with original gelatin, the cross-linked samples exhibited much lower swelling capacity, owing mainly to the network structure formed effectively via intermolecular chemical coupling. The structure prevented gelatin free-chains movement ability, leading to the water resistance increase. Moreover, the solid gelatin after cross-linked did not dissolve in water phase even at high temperature, revealing the improvement in water resistance.

Water contact angle

The surface hydrophobicity of gelatin film and GE_W film and GE_{DES} film under SAT/U-DES_{30W} and water system was determined by measuring the water contact angle (Table I). A high WCA ($\theta > 65°$) represents a hydrophobic surface, whereas a small WCA ($\theta < 65°$) indicates a hydrophilic one.[11] The WCA of the gelatin film was slightly below 65°, exhibiting low hydrophilicity. The functional groups of hydrophilic amino acids (serine, threonine, and glutamic acid) of gelatin remain positioned in the film-forming aqueous matrix while the hydrophobic ones (leucine, valine, and phenylalanine), driven by their hydrophobic nature, are preferably realigned towards the air-side interface, contributing to the formation of a hydrophobic surface.[12,13] ECH reacted with hydrophilic groups in gelatin molecules, such as amino and carboxy groups to form secondary amines and esters, greatly enhancing its hydrophobicity. And the decrease of the WCA of GE_W film and GE_{DES} film within 60 s was much smaller than that of gelatin film, because the cross-linking of ECH to gelatin matrix would delay the diffusion of water on the film and the absorption of water by the film. In addition, the WCA of GE_{DES} displayed smallest change trend within 60 s, which further indicated that SAT/U-DES_{30W} system can improve the reaction accessibility of gelatin.

FTIR

To explore the cross-linking mechanism between gelatin and ECH, the infrared spectra of gelatin and GE_{DES} were determined, as shown in Figure 3(a). The amide region (amide A, B, I, II, III) was the main characteristic peaks of protein, which can significantly exhibit the functional groups within the protein molecule. Firstly, amide A band was aroused from the characteristic of the stretching vibration of N-H group coupled with hydrogen bonds. Compared with gelatin, the absorption peak of N-H group in GE_{DES} shifted from 3382 cm^{-1} to 3425 cm^{-1} due to the reaction of ECH and amino group in gelatin molecule. Next, amide B band at 3081 cm^{-1} was attributed to N-H bending vibration.[14] The absorption band at 1652 cm^{-1} corresponded to the C=O stretch vibration in amide I, which represented a random coil structure of gelatin. The amide II at 1535 cm^{-1} and amide III at 1240 cm^{-1} were mainly attributed to the N-H in plane bend coupled with C-N stretching variation and N-H bending combined with the C-N stretching vibration.[15] The peak at 1403 cm^{-1} of GE_{DES} was slightly stronger than that of gelatin, this could arise from esters formed in the reaction of the epoxy groups of ECH with carboxyl groups in gelatin molecule, resulting in the formation of new cross-linked network structures, which in turn led to the observed enhanced water resistance.[16] The decrease in all of the absorption peak intensities of GE_{DES} also confirmed the reaction of ECH and gelatin. The absorption band of GE_{DES} at 1030 cm^{-1} was obviously broadened because new C-O-C absorption peak was generated by the addition of ECH. The cross-linking mechanism between gelatin and ECH is shown in Figure 3(c). Moreover, the absorption bands at 2933 cm^{-1} and 2873 cm^{-1}(-CH_2 asymmetric and symmetric stretching variation), 1449 cm^{-1} (-CH_2 bending variation), 1332 cm^{-1} (-CH_2 wag of proline & glycine) of gelatin and GE_{DES} were observed. It demonstrated that the main amide structure of gelatin modified by ECH under SAT/urea system has not changed or degraded. Instead, the presence of urea in DES would expose the polar groups inside the gelatin molecule and promote the degree of cross-linking with ECH.

Table I

The WCA of gelatin film, GE_W film, and GE_{DES} film

Time (s)	Water contact angle (θ, °)		
	Gelatin film	GE_W film	GE_{DES} film
0	92.8	100.2	99.3
30	66.1	89.4	97.3
60	59.2	88.9	96.5

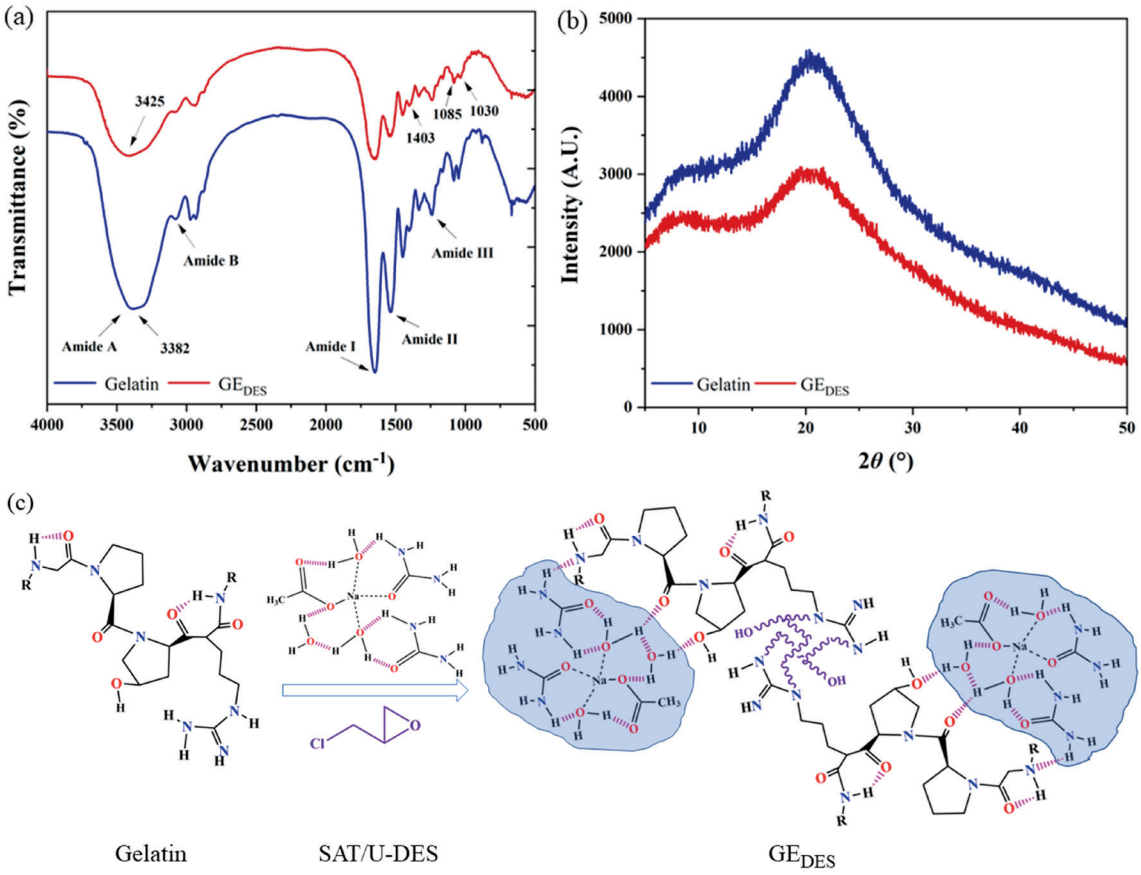

Figure 3. FTIR spectra **(a)** and X-ray diffraction patterns **(b)** of gelatin and GE_{DES}, **(c)** schematic diagram of cross-linking mechanism between gelatin and ECH.

XRD

The X-ray diffraction spectra of gelatin and GE_{DES} were shown in the Figure 3(b). Two major diffraction peaks were observed in both curves. The 2θ values of the first diffraction peak represented the distance among collagen molecules, the intensity of the diffraction peak represented relative triple helix content in gelatin.[17] The 2θ values of the first diffraction peaks of gelatin and GE_{DES} were 8.75 and 8.14. According to the Bragg equation (d(Å) = λ/2sin θ), the distances between collagen molecules in gelatin and GE can be calculated as 1.014 nm and 1.089 nm, respectively. The increase of distance among gelatin molecules might be due to the formation of covalent cross-linking between ECH and gelatin. Besides, the intensity of the first diffraction peak in GE_{DES} curve showed a smaller increase than that in gelatin curve after cross-linking by ECH. The literature has reported that covalent cross-links among collagen molecules might affect the measurement of triple helix content under neutral conditions.[8] The second diffraction peaks showed at 2θ = 20°, were one of the main characteristic peaks of gelatin. The gelatin molecule was long-range disorder and short-range order and the better the short-range order degree was, the narrower the diffraction peak will be.[18] It can be observed from the two curves that GE_{DES} showed a wider and weaker peak compared to gelatin. On the one hand, covalent cross-links formed new hydrogen bonds and hydrophobic bonds inside the gelatin molecules, which destroyed the amorphous structure of the gelatin molecule, leading to a decrease in the intensity of the diffraction peak. On the other hand, the cross-links made the short-range structure of gelatin molecules disordered, resulting in the broadening of diffraction peak.

SEM

Figure 4 shows the micromorphology of surfaces and cross-sections microstructure of gelatin film and GE_{DES} film. As can be seen from the Figure 4(a,b), the surfaces of gelatin film without cross-linking were relatively rough. After cross-linking, the GE_{DES} film displayed a smoother surface, which was attributed to the fact that cross-linking between molecules stabilized the longitudinal and transverse aggregation among collagen fibers, resulting in a more disorderly structure and a denser network structure. The cross-sections of gelatin film and GE_{DES} film were shown in Figure 4(c,d). It was observed that there existed many obvious cracks and pores on the cross-sections of gelatin film without cross-linking. The cracks and pores reduced significantly and the cross-sections became smoother after cross-linking. This was because ECH and gelatin molecules rearranged the structure of collagen fiber by covalently cross-linking. However, it was found that the GE_{DES} film exhibited uneven smoothness, which might be related to the difference of cross-linking effect caused by the aggregation of the cross-linking agent.[19]

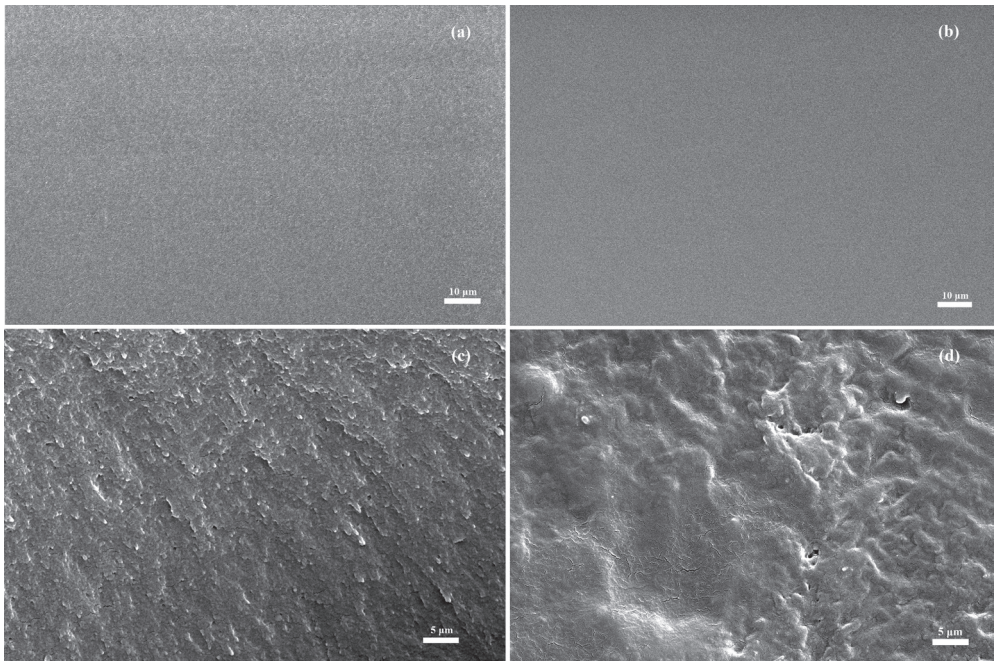

Figure 4. SEM images of surfaces **(a, b)** and cross-sections **(c, d)** of gelatin film and GE_{DES} film

TGA

Figure 5 revealed the thermal degradation behaviors of gelatin and GE_{DES}. The curves can be divided into two main stages ((I) 50~260°C, (II) 260~500°C). The first stage weight loss at the range of 50~260°C was observed on both curves, which associated with the evaporation of water inside gelatin molecules. The weight loss of GE_{DES} (12%) was lower than that of gelatin (20%) and the downward trend was relatively gentle, indicating the GE_{DES} displayed higher thermal stability at the range of 50~260°C. Its thermal stability in the temperature range can basically satisfy the demand for material application. In the second stage, major weight loss stage occurred from 260 to 500°C owing to the degradation of highly interacted peptides in gelatin molecules, the decomposition of unstable chemical bonds, and the breaking of intramolecular hydrogen bonds, electrostatic interactions and covalent bonds between amino acid residues.[20] Moreover, the temperature of gelatin with a maximum weight loss rate (311°C) was slightly lower than that of GE_{DES} (318°C). And its total weight loss (83.5%) was higher than that observed for original gelatin (67.5%) by 16%. The phenomenon could be explained that ECH cross-linked with the active groups in gelatin molecule to form dense network structure, making it difficult to decompose, and thus resulting in higher thermal stability.

Conclusion

In this paper, the cross-linking efficiency and mechanism of ECH and gelatin in SAT/U-DES_{30W} were investigated. First, the cross-linking efficiency was explored by denature-temperature and boiling water solubility of GE_W and GE_{DES}. Next, the water resistance of cross-linked products was analyzed by water contact angle and swelling capacity of GE_W and GE_{DES}. The results showed that the cross-linking efficiency of ECH to gelatin in DES system was better than that in

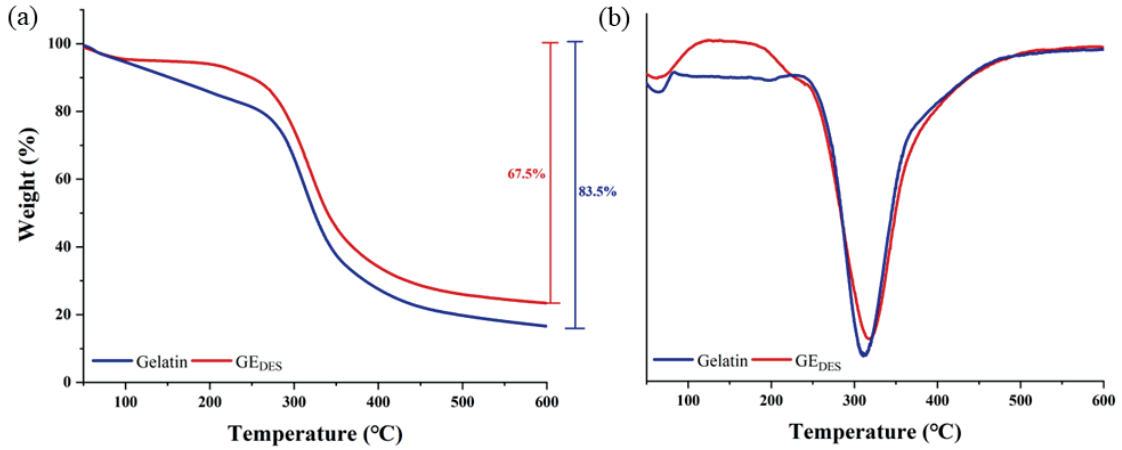

Figure 5. TG curves **(a)** and DTG curves **(b)** of gelatin and GE_{DES}.

water system. Finally, the cross-linking mechanism between gelatin and ECH was investigated by means of FT-IR, XRD and SEM. The epoxy group of ECH displayed high reactivity and could react with active groups such as amino groups in gelatin molecules, forming a dense network between gelatin molecular chains by covalently cross-linking. In addition, due to the intermolecular cross-linking, the thermal stability of cross-linked products has been significantly improved, which can meet the application in industrial production.

Credit authorship contribution statement

Yuming Cui: Investigation, Methodology, Data curation, Writing-original draft. Min He: Data curation, Formal analysis. Yanqing Wang: Methodology, Supervision. Hui Chen: Conceptualization, Methodology, Investigation, Writing-review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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