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Anti-degradable gelatin films crosslinked by active ester based on cellulose

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Abstract

Functionalization of microcrystalline cellulose (MCC) with EDTA dianhydride (EDTAD) was achieved firstly by esterification reaction. N-hydroxysuccinimide activated MCC-EDTAD ester (MEN), a novel macromolecule crosslinker based on MCC, was synthesized to modify gelatin films. The reaction between gelatin and MEN was verified by the residual free amino test, FTIR and XRD spectra. The introduction of MEN into gelatin decreased the film degradation ratio and increased its thermal stability, flexibility, hydrophobicity, light barrier performance and water uptake ability. Additionally, SEM images further proved the successful surface grafting reaction and degradation phenomenon. The unique gelatin film material with advanced properties broke up the limitation of blending modification method for gelatin with macromolecule and broadened its application as novel sustained-release material.

Keywords: Gelatin, Microcrystalline cellulose, EDTAD, Crosslinking, Sustained-release material

1. Introduction

Gelatin is a peptide molecule polymeric material, obtained from a hydrolytic treatment of collagen under acidic or alkaline conditions. The triple helix structure of collagen partially separates, ruptures and non-uniform mixture of polypeptides with different amino acid are formed [1]. Because of its good biological properties and low toxicity, gelatin is widely used as different kinds of materials, such as sponges, films, microballoon, scaffolds, nanoparticles and bandages etc [2-6]. However, the relatively weak thermal stability, poor mechanical properties and easily-degradable quality limit the potential application of gelatin as a practical material [7]. Microcrystalline cellulose (MCC), a linear polysaccharide combining of β-glucoside keys, is usually blended with gelatin to overcome obstacles of the biopolymer matrix [8-10]. Its excellent properties, such as renewable origin, biodegradable-ability of their components, environmental-friendly and non-toxic character further broaden its usages [10-13]. Ethylenediamine tetraacetic dianhydride (EDTAD) is used as chelating reagent in common studies [14]. Its biodegradable behavior and special molecular structure, which consists of two anhydride groups that can react with hydroxyl or amine groups, ensure its function in modification of biomaterials [15-16].

Recent years, a great number of researchers worldwide have been devoting to modification of gelatin with various crude macromolecules, such as cellulose, chitosan, starch, montmorillonite, polyvinyl alcohol, zeolite etc [17-22]. Jridi et al. [23] investigated the physical, structural, antioxidant and antimicrobial properties of gelatin/chitosan composite films and chose the best proportion of the two components to be applied as food package material; Li et al. [24] prepared an active gelatin-based films incorporated with five kinds of natural antioxidants and compared the function of these extracts on the antioxidant, physical and mechanical properties of the films; Alves et al. [25] studied the effect of three components (gelatin, cellulose, starch) on the biodegradation, water vapor permeability and mechanical properties of the starch/ cellulose/gelatin nanocrystals films by orthogonal experiments; Andrade et al. [26] reported a new edible coating materials containing gelatin and cellulose nanofibers, and evaluated the wetability of the coating film on banana and eggplant epicarps.

Unfortunately, the existing modification way of gelatin-based composite films with natural polymers, especially cellulose, are mostly prepared by blending method, in which the hydrogen bond or electrostatic interactions is used to explain the mechanism of the polymer matrix. Not exact chemical reaction happened between gelatin and original cellulose. Therefore, proper chemical modification on cellulose is needed to make the crosslinking reaction with gelatin possible. Cheng et al. [27] oxidized cellulose by periodate oxidation to obtain 2, 3-dialdehyde cellulose (DARC), which then reacted with collagen via the Schiff base reaction between -NH_2 in collagen and -CHO in DARC backbone to obtain DARC/Col composite films; Li et al. [28] employed the same oxidation process to oxidize carboxymethyl cellulose and the product with two aldehyde groups reacted with gelatin to prepare edible film material.

Lately, a novel crosslinker N-hydroxysuccinimide (NHS) active ester, which is synthesized by reaction between carboxylic acid and NHS in the presence of carbodiimide [29], attracted extensive attention mainly due to their cytocompatibility, biocompatibility and availability [30-31]. Furthermore, Gil's group [32] concentrated on modifying sugarcane bagasse, which was the raw material of cellulose, with EDTAD to gain the ester group that used as an absorbent material. In light of these researches, the hydroxyl and/or carboxyl function groups in these three biological polymers (gelatin, cellulose and EDTAD) further guaranteed the chemical reaction to produce materials with new properties [33].

In this paper, microcrystalline cellulose was modified with EDTAD to get a new type of cellulose ester MCC-EDTAD (ME). And then, a novel macromolecule crosslinker N-hydroxysuccinimide activated MCC-EDTAD ester (MCC-EDTAD-NHS, MEN) was firstly synthesized in the presence of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC) to react with gelatin (Scheme 1), and the biological polymer film with new qualities was recorded. Testing instruments, such as FTIR, XRD, TGA-DSC, mechanical property, contact angles and residual amino group test were applied in our present study. Additionally, in vitro degradation studies, light barrier properties and water uptake measurement of crosslinked gelatin films were investigated. On the basis of these results, the comparison of thermal stability

and light barrier properties between MEN modified gelatin films (Gel-MEN) and cellulose blending films (Gel/MCC) were explored.

Scheme 1 here

2. Experimental

2.1 Materials

Gelatin (type A, obtained from pigskin, with an approximate molecular weight of 50,000 and isoelectric point at pH=8 determined by fluorescence measurements) was obtained from Sinopharm Chemical Reagent Co., Ltd. MCC (extra pure, average particle size 90 μm), NHS (AR, 98%) and EDC (AR, 99%) were purchased from Energy Chemical Technology Co., Ltd (Shanghai). Glycerol (AR, 99%), DMF (AR, 99.5%), EDTA disodium salt (AR, 99%), acetic anhydride (AR, 98.5%) and other agents were obtained from Tianjin Fu Yu Fine Chemical Co., Ltd. All chemicals and reagents were used as received without further purification.

2.2 Preparation of MEN

2.2.1 Synthesis of EDTA dianhydride (EDTAD)

The EDTA dianhydride was prepared using the method described by Gil [34] with EDTA disodium salt and acetic anhydride as ingredients. 25 g EDTA disodium was dissolved in 250 ml distilled water to get the clear solution, and then HCl was added dropwise until precipitation of EDTA occurred. The precipitate was vacuum filtered and rinsed with 99% EtOH, 99% diethyl ether, subsequently dried in an oven at 70 ^{o}C and cooled in a desiccator prior to use.

For the preparation of EDTA dianhydride, 18 g EDTA was suspended in 50 ml pyridine and 25 ml acetic anhydride was added. Then the mixture was heated under reflux and kept stirring at 65 ^{o}C for 24 h. After reaction, the solid obtained was vacuum filtered, rinsed in diethyl ether and dried under vacuum at 50 ^{o}C. The prepared EDTAD was characterized by ^{1}H-NMR spectrum (Bruker Advance 400 spectrometer) and FTIR spectrum (Nicolet NEXUS 470 FT-IR spectrometer).

2.2.2 Synthesis of MCC-EDTAD (ME)

The functionalization of MCC with EDTAD was carried out according to Gil's group [35] with slight modification. 9 g MCC and 3 g EDTAD were suspended in 100 ml DMF, and then the mixture was shaken and heated under reflux at 75 ^{o}C for 24 h. The modified materials were elaborated by filtration under reduced pressure, washed in a row with DMF, distilled water, saturated $NaHCO_3$ solution (in order to release

carboxylate and amine functions), distilled water, and then with ethanol. After dried under vacuum at 50 °C, the mass percent gains were calculated by equation (1).

$$Weight\ gain(\%) = \frac{m_{modified} - m_{unmodified}}{m_{unmodified}} \times 100 \quad (1)$$

2.2.3 Synthesis of NHS active MCC-EDTAD ester (MEN)

MEN was synthesized by the method of Li [36] with a bit improvement. Mixed solution was prepared by dissolving 12.5 mmol ME, 50.0 mmol NHS, 50.0 mmol EDC together in 200 ml distilled water and gently stirred at 40 °C for 1 h. After reaction, the solid was vacuum filtered, and then washed with distilled water several times, dried under vacuum at 50 °C to get purified MEN. The mass percent gains were also calculated by equation (1). The ME and MEN obtained were characterized using FTIR spectrum (Nicolet NEXUS 470 FT-IR spectrometer), Elemental Analyzer (Vario EL Ⅲ, Elementar Analysensysteme, Germany) and TGA-DSC (Q600SDT, TA, USA) .

2.3 Modification process and film formation

Gelatin solution (3%, w/v) was prepared by dissolving gelatin powder in distilled water and then heated at 45 °C for 2 h under continuous stirring. Glycerol was added as plasticizer at a certain concentration (15% of dry gelatin weight). The dosage of MEN was determined by mass ratio with gelatin, which meaned $m_{MEN}/m_{gelatin}$=0%, 5%, 10%, 15%, 20%, 25%, 30%. So the corresponding modified gelatin samples were named as Gel, Gel-5%MEN, Gel-10%MEN, Gel-15%MEN, Gel-20%MEN, Gel-25%MEN and Gel-30%MEN, respectively. Various weight of MEN powder was dissolved in distilled water under stirring for 12 h at room temperature to produce a suspension liquid. Then the solution was added dropwise to gelatin liquids, and acetic acid (3% of water volume) was dripped into the whole system to promote the start of the interfacial reaction. These mixtures were gently stirred for 12 h at 45 °C.

To cast the films, 30 g gelatin reaction solution was transferred into a teflon dish and placed at room temperature for 2 h, then put in oven at 40 °C until films dried. The dried films were peeled off and stored in a desiccator with relative humidity ≤20%. Besides, one part of gelatin reaction solution was freeze dried at -55 °C, 70 Pa with vacuum freeze drier (FD-1A-50, Beijing, China) and the lyophilized powder was characterized by FTIR spectrum (Nicolet NEXUS 470 FT-IR spectrometer).

2.4 XRD analysis

XRD analysis of samples were performed on an X-ray diffractometer (D8-ADVANCER, Bruker AXE, Germany) with a thin film attachment using Cu-Kα radiation (λ=0.1541 nm) at a current of 40 mA and an accelerating voltage of 40 kV. The patterns were recorded from 10° to 60°.

2.5 Determination of residual amino group in gelatin

The residual $-NH_2$ groups in modified gelatin solution was determined by the improved Van Slyke method at 45 ^{o}C [37-38]. Sample solutions were mixed with acetic acid, sodium nitrite and stirred for 45 min. The residual primary amine (mol/g) was calculated according to the volume of N_2. All samples were tested in triplicate.

2.6 In vitro degradation studies

The degradation study of gelatin films was carried out in vitro by incubating in phosphate buffer (pH 7.40) at 37 ^{o}C for different intervals (1, 3, 5, 7, 9, 12 and 24 h), which was developed from method of Haroun [39]. The gelatin films were dried at 60 ^{o}C to constant weight prior to use and marked as m_0. After different degradation time, the samples were washed with distilled water after filtrated under vacuum and dried at 60 ^{o}C to constant weight (m_t). The degradable performance was examined by the weight remaining from Equation (2).

$$\text{Weight remaining}(\%) = \frac{m_t}{m_0} \times 100 \qquad (2)$$

2.7 Scanning electron microscopy (SEM) of gelatin films

The microstructures of the prepared films were investigated by Quanta 200 environmental scanning electron microscope (SEM, FEI Company, Holland). Before observation, the film surfaces were coated with Au using SEM coating device. More than ten micrographs were taken from different zones of each surface film.

2.8 Thermo gravimetric analysis

The thermal stability of gelatin film was determined by thermogravimetric and differential thermal scanning calorimetry synchronous apparatus (TGA-DSC, Q600SDT, TA, USA). The gelatin film samples (approximately 2.5 mg) were weighed accurately into aluminium pans and sealed. The endothermal curve of the crushed film was recorded from 20 ^{o}C to 500 ^{o}C at a scanning rate of 10 ^{o}C/min under

nitrogen atmosphere. Additionally, the thermal stability of Gel/MCC blending films was also studied as compared with Gel-MEN films.

2.9 Mechanical testing

Prior to investigating the mechanical properties, films were conditioned for 48 h at 20 ^{o}C and 50±5% RH condition. Tensile strength (T_s), elasticity modulus (E_m) and elongation at break (E_{ab}) were determined as described by Benjakul [40] with a slight modification, using the Microcomputer Controlled Electronic Tensile Testing Machine (WDL-005, Jinan, China) equipped with tensile load cell of 300 N. Samples with initial grip length of 25 mm were used for testing and the cross-head speed was set at 10 mm/min. The thickness of each film was measured by Vernier Caliper (0.02 mm/150 mm, Shanghai, China).

2.10 Contact angles measurement

The water contact angles (CAs) of all films were measured by the Sessile drop method using a DSA100 contact angle measuring system from Krüss. The gelatin reaction solution was coated on the surface of glass sheet to obtain films with thickness about 0.1 mm, and then stored in a desiccator with relative humidity ≤20%.

2.11 Light barrier properties and transparency

The ultraviolet and visible light barrier properties of the films (1 cm×2 cm) were measured by an ultraviolet-visible spectrophotometer (UV-7504C, Shanghai, China) at selected wavelengths from 200 to 800 nm following Fang's method [41]. The transparency value of films was calculated by the Equation (3), where T was transmission (%) at each wavelength and x was film thickness (mm). According to the equation, high transparency values indicate good light barrier performance.

$$\text{Transparency value} = -\log T/x \quad (3)$$

2.12 Water uptake measurement

The water uptake measurement of the films was determined in the light of Kavoosi [42] and Tang [43] with a little development. Rectangular specimens sized 15 mm×10 mm with a thickness of 0.1 mm were prepared. The samples were conditioned at 20 ^{o}C in a desiccator containing silica gel (RH 20%±5%) three days to constant weight (W_i). Then, the film samples were transferred into desiccators at 100% relative humidity (supersaturated salt solution of $CuSO_4 \cdot 5H_2O$) at 20 ^{o}C for eleven days to

absorb water until the weight reached to equilibrium. The weight of samples at the adsorption time of t was noted as W_t. The amount of water adsorbed at different intervals and equilibrium were calculated as Equation (4). All tests are the means of at least three measurements.

$$Water\ Absorption\ (\%) = \frac{W_t}{W_i} \times 100 \quad (4)$$

3. Results and Discussion

3.1 Characterization of MEN

3.1.1 Spectra of EDTAD

^{1}H NMR (400 MHz, DMSO, Fig. S1): δ 3.691 (s，8H), 2.657 (s，4H), 3.080 (s, DMSO), 2.496-2.488 (m, DMSO), which were in accordance with the characteristic peaks of H in the ideal product. The FTIR spectrum (Fig. S2) further proved the dianhydride structure with two groups of splitting peaks. The peaks in high frequency region splitted at 1813, 1759 and 1689 cm^{-1}, gap of 60 cm^{-1} between adjacent peaks. The low frequency groups splitted at 1139, 1074, and 991 cm^{-1} with the same interval. Additionally, the bands at 1245 and 1400 cm^{-1} related to C-O and C-N stretch respectively were also evidence of EDTAD structure.

3.1.2 Spectra of MCC, ME and MEN

The FTIR spectra (Fig. S3) fully depicted the functional groups of MCC, ME and MEN. Compared with MCC, the appearance of strong bands at 1741 cm^{-1} in ME can be attributed to axial deformation of the ester bond, and bands at 1633, 1406 cm^{-1} are attributed to asymmetric and symmetric axial deformations of carboxylate. These bands confirmed the successful functionalization of MCC with EDTAD via formation of ester linkages. For MEN, absorption peaks at 1706, 1210 and 811 cm^{-1} represented γ-dicarbonyl stretching vibration, C-N stretching and C-C vibration respectively. Specially, the reinforce of ester carbonyl band at 1742 cm^{-1} and the weakening of carboxy carbonyl band at 1600 cm^{-1} further proved the structure of the active ester.

3.1.3 Elemental analysis and thermal properties of MCC, ME and MEN

As can be seen in Table 1, there was a considerable increase in nitrogen content with 1.92% after functionalization of MCC with EDTAD. Accompanied by the

significant weight gain of 72.50%, the modified material (ME) with EDTAD incorporated was obtained. Similarly, the element N increased to 2.48% in MEN, 0.50% higher than that of ME, which meaned the esterification reaction happened between ME and NHS with a five-membered nitrogenous ring linked. Also, the weight gain of 30.80% further proved the truth.

The initial decomposition temperature at 5% weight loss (T_i), the maximum weight loss temperature (T_m), the glass transition temperature (T_g) and the char residue at 500 ^oC of MCC, ME and MEN are recorded in table 1 (Fig. S4). T_i of MCC and ME was 309.10 ^oC and 271.45 ^oC, respectively while MEN was 222.54 ^oC, which suggested a reduction in thermal stability. It can be related to the reaction activity of the three materials with $-NH_2$ in gelatin, which was in accordance with the results of residual amino group below. To summarize, the difference of each item further certified the introduction of EDTA and NHS into MCC, which agreed with FTIR and elemental analysis.

Table 1 here

3.2 Confirmation of MEN crosslinking with gelatin

3.2.1 FTIR spectra analysis of MEN, gelatin and Gel-MEN film

FTIR spectra of the pristine gelatin (curve a), pristine MEN (curve b), and Gel-25%MEN film (curve c) are compared in Fig. 1. In the case of pristine gelatin, the C=O stretching vibration appearing at 1664 cm^{-1} demonstrated amide I band, while the amide band Ⅱ was indicated by N-H bending vibration observed at 1535 cm^{-1}. Besides, aliphatic C-H bending vibrations were observed at 1450 cm^{-1} and bands at 1331, 1230 cm^{-1} declared the C-N bond stretching vibrations. Gel-MEN showed all the characteristic peaks of gelatin and MEN, such as 1643 and 1546 cm^{-1}. This indicited the successful reaction between galatin and crosslinker MEN along with a representative peak at 1741 cm^{-1}, which clearly indicated the amidation reaction between $-NH_2$ in gelatin and active ester base in MEN.

Fig. 1 here

3.2.2 X-ray diffraction studies of MEN, gelatin and Gel-MEN film

In order to examine the effect of MEN on crystal structure and crystallinity of

gelatin, XRD patterns of freeze-dried gelatin films are investigated. Data on 25% MEN formulation are presented as a representative example. As shown in Fig. 2, curve (a) was the XRD pattern of MEN, which displayed the typical XRD pattern of the native cellulose with the main diffraction signals at around 14.9^o, 16.2^o, 22.5^o and 34.3^o [44]. The curve (b) only showed an extensive broadening peak in the 2θ range of 15-25°, which was a typical XRD pattern of pure gelatin originated from α-helix and triplehelical structure [45-46]. The XRD pattern of Gel-25%MEN film is given in Fig. 4(c), in which the characteristic peaks of MEN (22.6^o) and the characteristic broad diffraction peak of gelatin were observed respectively. It suggested that the gelatin was modified with MEN after crosslinking reaction, which was consistent with the FTIR results.

Fig. 2 here

3.2.3 Free -NH_2 in Gel-MEN film formation solution

Fig. 3 indicates the changing curve (a) of residual primary amino in Gel-MEN film formation solution and gelatin liquid blending with ME (Gel/ME, curve b) against the ratio δ ($\delta = m_{(MEN/ME)}/m_{(dry\ gelatin)}$). For Gel-MEN, the dosage of crosslinker played an important role in the content of free -NH_2 while the free -NH_2 changed slightly no matter how much ME was added. It suggested the stability of ester group in ME which was not active enough to react with -NH_2 in gelatin. This meaned that amine groups in gelatin did not act as nucleophiles to break ester bonds in ME but could break ester bonds in MEN. All these results confirmed the reaction process (scheme 1) we proposed were correct. Interestingly, after activation by NHS, the active ester MEN could consume -NH_2 in gelatin and dose dependent. The amount of free -NH_2 decreased sharply with the ratio δ increased from 0 to 25%, and then decreased slightly when the ratio further increased. Specially, the amount of free -NH_2 reduced down to a minimum value about 1.74×10^{-4} mol/g when δ=30%. All these proved that the whole system conquered the forbiddance of interfacial reaction. Compared with former interface reaction study by Xu [47], in which gelatin was modified by glycidol and the maximum -NH_2 conversion rate was 42%, the -NH_2 conversion rate in this work was 28% higher than that reported.

Fig. 3 here

3.3 Performance of Gel-MEN films

3.3.1 Degradation properties in vitro

As sustained-release material, the composite films are expected to degrade with a proper rate to match special needs and keep activity within service life. The degradation behavior of films in a physiological environment plays an important role in application as sustained-release material. The in vitro degradation performance of Gel and Gel-MEN films in phosphate buffered saline (PBS, pH 7.40) at different intervals was investigated. As shown in Fig. 4, the blank gelatin degraded rapidly because of the large number of hydrophilic amino and carboxyl groups in gelatin backbone. Besides, the physical structure of gelatin which possessed higher porosity and leaner pore-wall contributed to the minimum weight remaining of 15% at 24 h. The composite polymer Gel-MEN degraded proportionally slow because of the incorporation of cellulosic crosslinker MEN. The weight remainings of Gel-25%MEN and Gel-30%MEN at 24 h were 57% and 58% respectively, 40% greater than that of original gelatin films. The amido bond formed between MEN and gelatin was stable enough to resist adverse factor outside. It was reasonable to consider that strong hydrogen bond and electrostatic interaction between gelatin polypeptide and hydrophilic hydroxyl or carboxylic groups in MEN also depressed PBS medium diffusion and protected gelatin polypeptide chains from degradation. Meanwhile, the presence of MEN, a macromolecule crosslinker based on cellulose, also served as physical crosslinking sites, which enhanced the stability of the network. To conclude, MEN improved the anti-degradation performance of gelatin films and this guaranteed its potential usage as sustained-released material in many fields, such as food packages inside, medical engineering, controlled-release fertilizer in agriculture and so on.

Fig. 4 here

3.3.2 Morphology evaluation

SEM photographs of blank films revealed a dense, smooth and compact structure without any embossment or hole in Fig. 5 (a). The magnification times of first row (a_1,

b_1, c_1) was lower than that of second row (a_2, b_2, c_2). The introduction of cellulosic crosslinker MEN destroyed the homogeneous film surface with slice-like or rod-like macromolecule grafted on the covering of gelatin (Fig. 5 (b)). The inset of Fig. 5 (b_2) clearly displayed the feature of MEN.

Besides, the SEM images provided very good evidence in favor of the in vitro degradation of test sample (Gel-25%MEN). It can be seen from Fig. 5 (b) and (c) that the film surface was almost plane and even, though combined with some sags and crests before the degradation started. One hour after degradation, the porous structure with irregularities and apertures can be observed on the surface of the composite film, which confirmed that the internal structure of Gel-MEN polymeric film was started to degrade in the liquid medium. It can be assumed that the degradation of films was gradually penetrating deeper from the surface [48].

Fig. 5 here

3.3.3 Thermal stability

The thermal gravimetric analysis (TGA) and differential thermogravimetrie curves (DTG) of composite films are performed in Fig. 6 to investigate the thermal stability. Curve a (Gel) and curve b (Gel/glycerol) were almost similar with two representative peaks at 190.00-220.00 ^{o}C and 321.44 ^{o}C, which corresponded to the initial decomposition temperature at 5% weight loss (T_i) and maximum weight loss temperature (T_m) of gelatin. The special peak at 250.07 ^{o}C in curve b was due to the blending of glycerol as plasticizer in blank gelatin film. The DTG patterns of Gel-MEN presented three steps for weight loss at the temperatures around 100 ^{o}C, 250.07 ^{o}C and 320~350 ^{o}C, involving one strong and two weak endothermic peaks. The first weight loss at the temperature around 100 ^{o}C and the second weight loss at about 250.07 ^{o}C were similar to that of curve b. The third weight loss with a strong endothermic peak at 320~350 ^{o}C was due to the incorporation of active ester MEN into gelatin, and exhibited positive correlation with the dosage of crosslinker. This demonstrated that the crosslinking effect of the cellulose-based crosslinker improved thermal stability of the material to some degree as found in the literature. On the one hand, the crosslinking reaction between gelatin and MEN with amido bonds formed

made the macromolecule structure more stable and impregnable. On the other hand, the hydrogen bond and electrostatic interaction of special groups in gelatin and MEN further strengthened the structure. All these provided effective reinforcement layer to endure thermal degradation. As Fig. 6-1 and 6-2 shows, T_m reached maximum of 349 .26°C when δ (δ= $m_{(MEN)}$ /$m_{(dry\ gelatin)}$) was 25%.

The thermal properties of Gel-25%MEN and Gel/25%MCC in the presence of glycerol were compared in Fig. 6-1 and 6-2, in which curve b consisted of four decomposition stages. The four peaks at 104.42, 192.23, 250.72 and 359.12 °C were resolved into four different components of water, gelatin, glycerol and MCC, respectively. The obvious peak at 193.23 °C that almost disappeared in curve a indicated the severe phase separation in Gel/MCC system. However, compared with the typical T_m (309.10 °C) of MCC [49], the rising decomposition temperature of 359.12 °C in curve b may caused by the hydrogen bond formed between gelatin and MCC, which increased the thermal stability of Gel/MCC films.

Fig. 6 here

3.3.4 Mechanical properties

Mechanical properties, especially elasticity modulus (E_m) and elongation at break (E_{ab}) are particularly crucial for sustained-materials used in many fields. Table 2 shows the thickness, tensile strength (T_s), E_m and E_{ab} of the Gel-MEN composite films. The decreased T_s indicated that the modified films yielded lower stress than pure gelatin film. The tensile strength increased with the crosslinker adding from Gel-5%MEN to Gel-20%MEN, but decreased in Gel-25%MEN and Gel-30%MEN. And thse results were in accordance with the form work reported by Azeredo [50]. This may caused by the fact that when the amount of the macromolecule crosslinker was high, adding them to film may induce the development of a heterogeneous structure with the presence of discontinuous areas, which produced lower tensile strength. Similarly, Martucci [51] reported that the addition of dialdehyde starch in gelatin resulted in lower T_s values than control film and explained this apparently anomalous behavior. The fact that polymeric nature of dialdehyde starch did not introduce severe restrictions within gelatin matrix as usually occured with short chain

dialdehyde such as formaldehyde or glutaraldehyde, and some degree of phase separation in gelatin- dialdehyde starch films could reduce the T_s, too. Fortunately, E_m and E_{ab} tended to predict better elasticity and flexibility that indicating the new material was not fragile any more. The E_{ab} of Gel-25%MEN was 31.96%, thirty times of blank gelatin film while the E_m of Gel-25%MEN was 448.72 MPa, a quarter of blank one, which was 1736.11 MPa. The reason was that the active ester group in MEN could form covalent bonds with amino in gelatin polypeptide, and the hydrophilic groups could form hydrogen bonds. And all the newly formed bonds weakened the protein-protein interactions which was effective to stabilize the gelatin network. Besides, MCC, the base of the crosslinker MEN, demonstrated to be an effective nano-reinforcement for biopolymer films that can drastically influence the mechanical properties of biomaterials [50]. All these contributed to better flexibility of the new biologic polymer matrix.

Table 2 here

3.3.5 Hydrophobicity analysis by contact angles

Gelatin was a kind of hydrophilia material because of the functional groups: amino, carboxyl, hydroxyl and so on. The water-sensitve property limited its application in many fields and the hydrophobization was needed. Ninan [21] reported a new material of gelatin/zeolite porous scaffold and the contact angle was found to increase from 88.6 ^{o}C to 108.0 ^{o}C with the increasing concentration of zeolites in gelatin. The hydrophobic effect of the crosslinker MEN on gelatin was confirmed by the results of water contact angle measurements (Fig. S5). The pure gelatin film (Fig. S5, a) with a typical contact angle of 77.8^{o} was because of the hydrophilic groups exposed in gelatin chains. After crosslinked by MEN, the Gel-MEN films (Fig. S5, b and c) presented a sharp increase to 125.1^{o} and 135.5^{o}, respectively. This was due to the replacement of some surface amino groups in gelatin polypeptide with active ester groups in MEN. Besides, the hydrogen bonds formed between hydrophilic groups of gelatin backbone and MCC-based crosslinker also contributed to good hydrophobicity of modified gelatin films. The modified films with perfect hydrophobicity overcame the permanent weakness of water-sensitive in the application of gelatin. And the

advanced properties broadened its usage as more kinds of material.

3.3.6 Water uptake studies

The hydrophilic property of gelatin can be controlled in two ways. One was hydrophobization referred above, and the other was expected to absorb water molecules. This happened because of two beneficial structure features of gelatin. One advantage of gelatin materials was its highly hygroscopic nature due to which it swelled and transformed to any shape easily in humid environment. The other was the porosity in the network that allowed more water to enter inside, because which the porous gelatin films showed higher swelling capacity. Water uptake (%) of pure gelatin films and Gel-MEN composites tested during 11 days are shown in Fig. 7. The water uptake (%) can be controlled by incorporating different dosages of MEN in the polymer matrix. In the case of Gel-15%MEN, the water uptake (%) reached the maximum among all MEN crosslinked gelatin films. This was attributed to the increase in pore size of gelatin film with the presence of MEN. Additionally, the Gel-MEN composites showed an increase in the swelling capacity till the 11th day, which indicated a better ability of water absorbing. By comparison, the original gelatin film acquired maximum swelling capacity on the 10th day and thereafter percentage of water uptake was found to be invariant even decline. This event suggested that introduction of MEN into gelatin provided effective channel for water molecules to diffuse into the polymer matrix, thereby swelling ability increased. Uncontrolled swelling properties can badly affect the mechanical property, so it was advantageous to tune the swelling capacity [52].

Fig. 7 here

3.3.7 Light resistance performance

Many researches indicated that ultraviolet radiation was one of the main reason causing skin hurting, light aging and skin cancer. Hence the low light transmission also made the active gelatin film possess some health function [24]. Light transmission at the selected wavelengths from 200 to 800 nm in UV and visible ranges and transparency values of gelatin films are shown in Table 3 (& Fig. S6). Comparison of the results with control films revealed that lower light transmittance (T)

was found in Gel-MEN composite films. And the films with 30%MEN displayed the lowest values among them. This revealed that the addition of MEN improved the UV barrier properties of gelatin films, resulting from the amido bonds by Schiff base formation between the active ester groups in MEN and the amino groups of lysine or hydroxylysine side groups in gelatin. Based on transparency values (T_v, Table 3), the more crosslinker led to the greater T_v, which represented the opacity of resulted films. The opacity was highly influenced by the crystalline content of a sample: more compact polymer chains made it more difficult for light passing through and then the opacity of films was increased [53]. All these indicated that protein-based films were considered to exhibit high UV barrier properties, owing to their high content of aromatic amino acids which absorbed UV light.

Table 3 also displays the light barrier properties of Gel/15%MCC and Gel/25%MCC blending films, as compared with the corresponding mass ratio of Gel-MEN films. The Gel/MCC blending films exhibited better transparency while worse light resistance performance. This fact may be an indication that MCC nanoparticles were homogeneously distributed in the matrix because they are white, and light incident on the film surface was reflected in larger quantity due to the white particles [25]. The light barrier properties of films were relatively important while used as sustained-materials for food packaging or food coating. The polymer matrix in this work just matched these needs.

Table 3 here

4. Conclusion

In summary, the structure and conformation of gelatin were modified by the macromolecule crosslinker MEN. The FTIR spectra, elemental analysis and TGA values verified the structure of MEN. Reaction between $-NH_2$ in gelatin and active ester in MEN was confirmed by residual primary amino test, FTIR and XRD spectra, which broke the limitation of blending modification method for gelatin with macromolecule. Dose-dependent effect of crosslinker was investigated through degradation in vitro, in which the weight remaining decreased with the increase of MEN dosage. The SEM images further proved the successful surface grafting reaction and the degradation phenomenon in PBS medium. The decomposition temperature

obtained from TGA curves increased to 350 ^{o}C compared with the native film of 320 ^{o}C. Besides, TGA patterns of Gel-MCC composites exhibited serious phase separation. The mechanical properties changed to some degree with higher E_{ab} and lower E_m, which suggested better flexible and shatter-proof. The contact angles with high value of 135.5^{o} indicated good hydrophobic properties. The swelling ability after absorbing water could be regulated by adding different weight of crosslinker. The light barrier performance was improved since the introduction of MEN compared with both pure gelatin film and Gel/MCC composites. Giving the application status of gelatin, our study is the extension of existing NHS crosslinking technique and will broaden the application of gelatin films as sustained-released material in food industry, medicine, agriculture and so on .

Acknowledgment

We gratefully acknowledge the promotive research fund for young and middle-aged scientists of Shandong Province (BS2014NJ012), the National Natural Science Foundation of China (NO.21276149) and the Program for Scientific Research Innovation Team in Colleges and Universities of Shandong Province.

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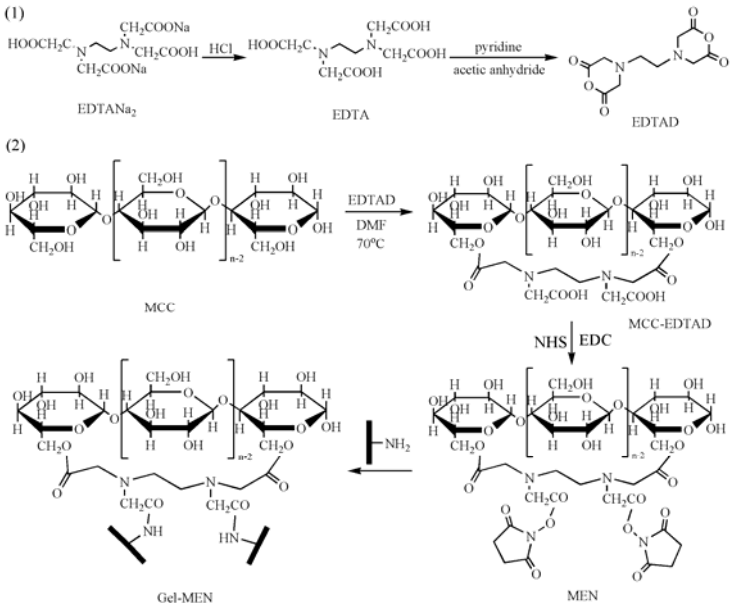

Scheme 1 The formation process of crosslinked gelatin with MEN.
(1). The synthetic route of EDTA anhydride (EDTAD);
(2). The preparation path of gelatin modified with MEN.

Figure and Table captions:

Fig. 1 The FTIR spectra of MEN, gelatin and Gel-MEN

Fig. 2 The XRD pattens of MEN, gelatin and Gel-MEN

Fig. 3 Residual amino group content of gelatin solution modified by MEN (a) and ME (b) with different dosages

Fig. 4 Effect of macromolecule crosslinker on in vitro degradation of the Gel-MEN composite films

Fig. 5 Film Surface morphology of Gel (a_1, a_2), Gel-25%MEN (b_1, b_2) and Gel-25%MEN after 1 h degradation (c_1, c_2)

Fig. 6 TGA and DTG curves of modified gelatin films with different dosage of crosslinker (6-1, 6-2) and comparison curves between Gel-25%MEN and Gel/25%MCC blending films (6-3, 6-4)

Fig. 7 Water uptake properties of gelatin films incorporated with different dosage of MEN

Table 1 Elemental analysis and thermal property values of MCC, ME and MEN

Table 2 Mechanical performance of different Gel-MEN films

Table 3 Light transmission and transparency values of different Gel-MEN films

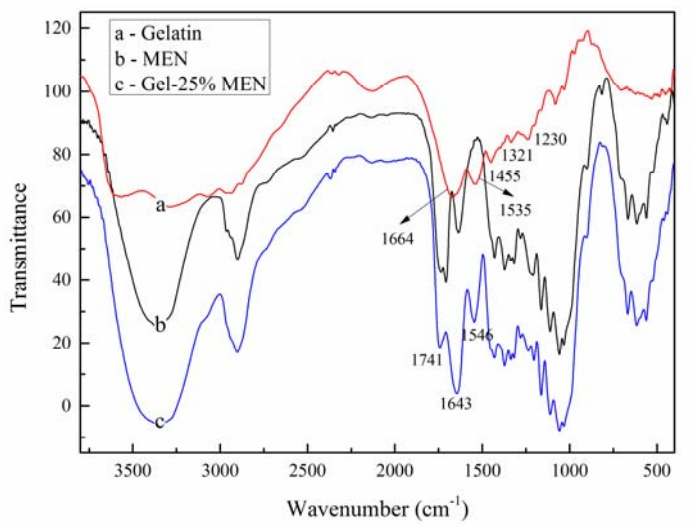

Figure 1

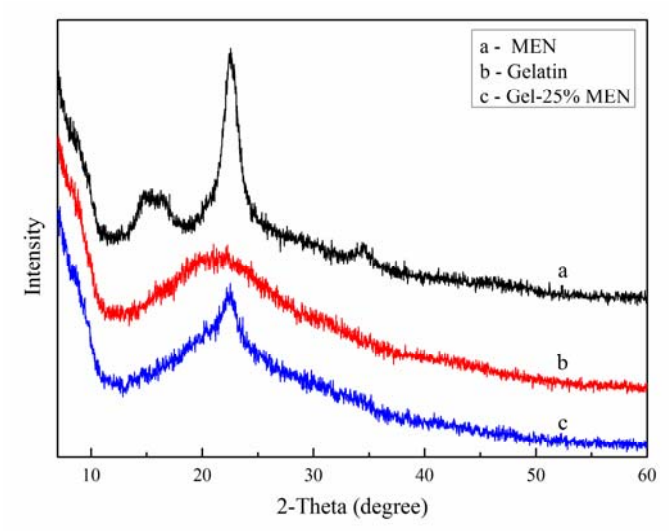

Figure 2

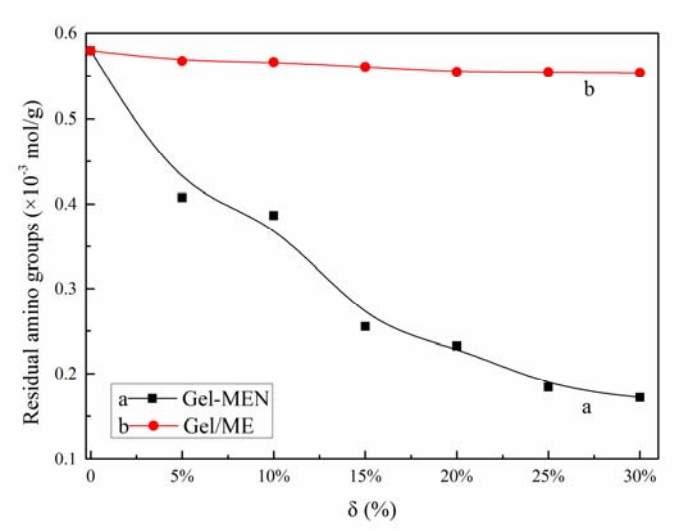

Figure 3

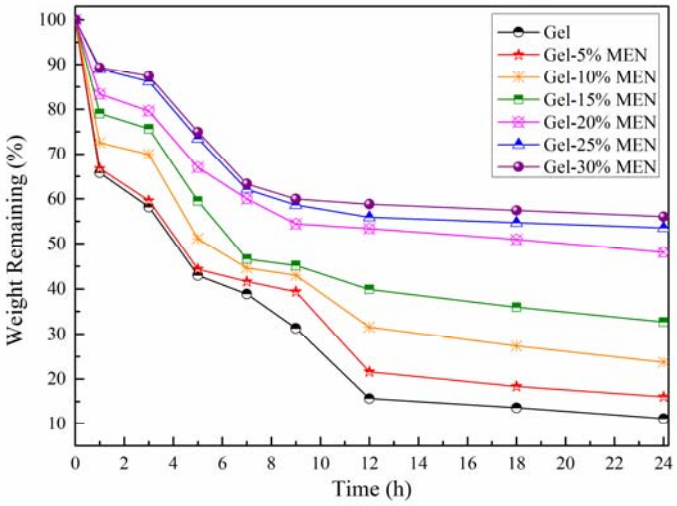

Figure 4

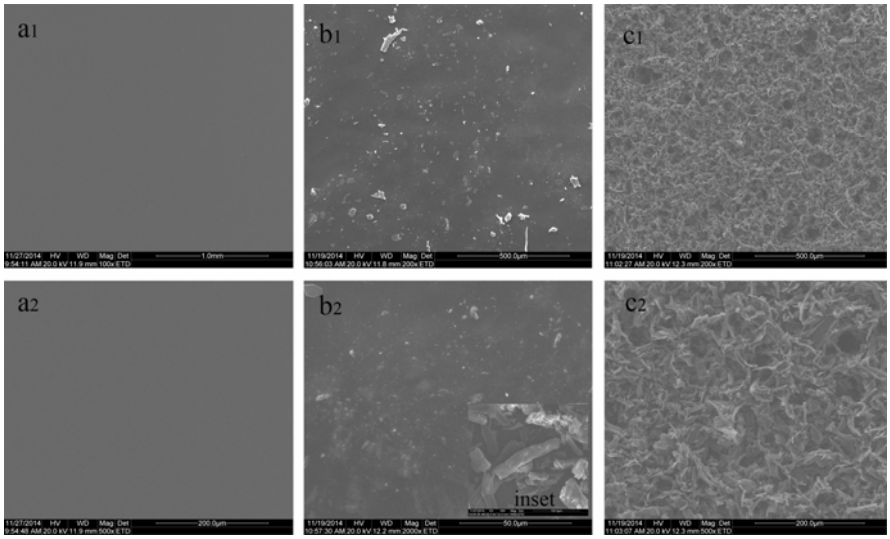

Figure 5

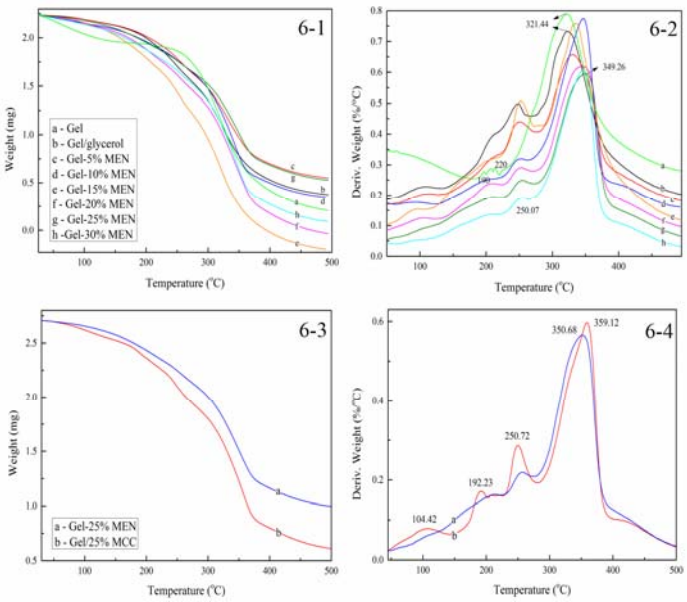

Figure 6

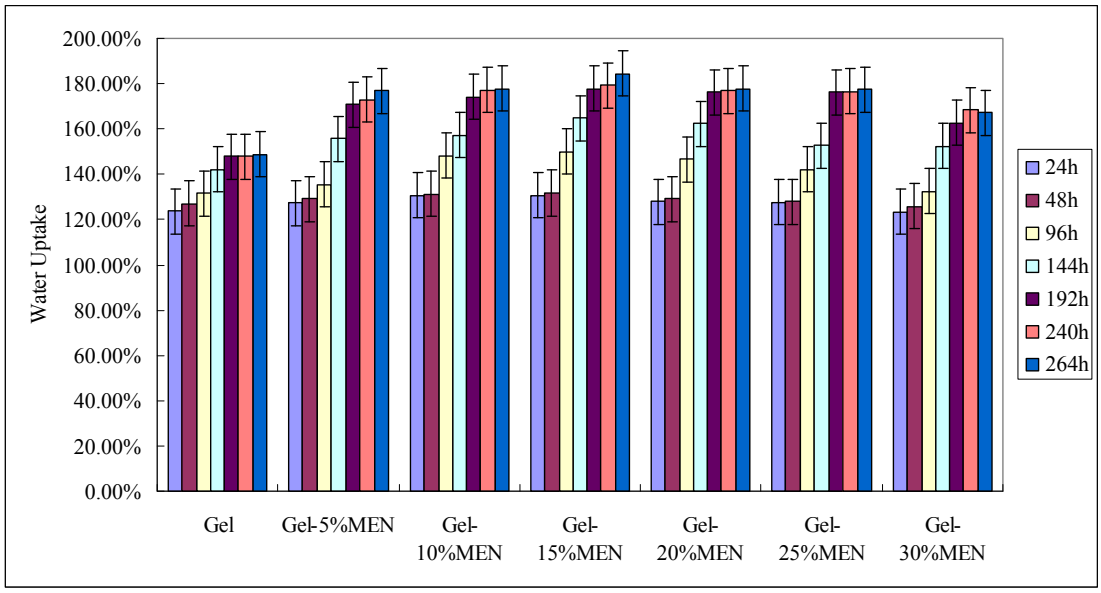

Figure 7

Table 1

Materials	C (%)	H (%)	N (%)	Weight gain (%)	T_i (^oC)	T_m (^oC)	T_g (^oC)	Residue (%)
MCC	42.21	6.40	0.11	-	309.10	364.08	340.68	15.28
ME	42.29	6.45	1.92	72.50	271.45	331.50	342.70	33.01
MEN	42.97	6.65	2.48	30.80	222.54	377.31	364.09	10.45

Table 2

Films	Thickness (mm)	Tense Strength (MPa)	Elongation at Break (%)	Elasticity Modulus (MPa)
Gel	0.10	24.17	1.84	1736.11
Gel-5%MEN	0.18	15.28	7.64	435.73
Gel-10%MEN	0.20	16.17	11.52	468.75
Gel-15%MEN	0.20	18.25	12.44	595.24
Gel-20%MEN	0.28	18.10	28.64	525.21
Gel-25%MEN	0.26	13.97	31.96	448.72
Gel-30%MEN	0.20	14.08	83.08	476.19

Table 3

Films	Wavelength (nm)										Transparency Value	
	200	280	350	400	450	500	550	600	700	800	280	600
Gel	2.2	1.5	43.2	55.0	59.7	62.6	64.4	66.5	67.6	68.5	10.13	0.98
Gel-MEN5%	1.5	5.8	29.8	34.4	35.4	36.4	37.0	37.8	38.4	39.0	7.73	2.64
Gel-MEN10%	0.6	2.6	12.4	14.4	15.1	15.5	15.8	16.2	16.4	16.4	11.32	5.65
Gel-MEN15%	0.2	0.2	4.4	5.8	6.3	6.6	6.7	7.1	7.1	7.2	13.49	5.74
Gel-MEN20%	0.1	0.2	2.2	3.0	3.5	3.6	3.7	3.7	3.7	3.7	13.49	7.16
Gel-MEN25%	0	0	0.6	1.0	1.3	1.4	1.5	1.6	1.6	1.6	---	8.98
Gel-MEN30%	0	0	0.4	0.6	0.9	1.0	1.1	1.1	1.1	1.1	---	9.79
Gel/MCC15%	1.9	9.3	34.6	39.6	40.9	42.3	43.3	45.1	46.5	47.7	10.32	3.46
Gel/MCC25%	1.4	5.3	26.6	31.7	33.0	34.4	35.5	36.9	38.0	39.3	12.78	4.32