DE GRUYTER

Chemical Papers 70 (4) 505–514 (2016)
DOI: 10.1515/chempap-2015-0226

ORIGINAL PAPER

Preparation and properties of gelatin films incorporated with *N*-hydroxysuccinimide-activated end-bit binary acid

Chen Zhuang, Fu-Rong Tao*, Yue-Zhi Cui

Shandong Provincial Key Laboratory of Fine Chemicals, Qilu University of Technology, Jinan 250353, China

Received 26 June 2015; Revised 24 September 2015; Accepted 3 October 2015

A series of novel cross-linkers, *N*-hydroxysuccinimide (NHS)-activated end-bit binary acid (NHS-C_4, C_5, C_6, C_8, C_{10}, C_{14}), were synthesised to modify gelatin films and the crosslinking effects were compared. Homogeneous films with the exception of the film crosslinked by NHS-C_{14} were observed and the thickness was measured using a scanning electron microscope. The section feature influenced by different film-treatment conditions was also recorded. The differential scanning calorimetry results indicated higher thermal stability. The water contact angles confirmed enhanced hydrophobicity. NHS-C_6, which was used as a probe crosslinker, exhibited the best crosslinking effect that the content of the free -NH_2 achieved was the lowest out of all the crosslinkers. The biodegradation results of gelatin films modified by NHS-C_6 exhibited better degradation-resistance and excellent stability. In addition, the optimal experimental conditions were 45 °C for 12 h when [NHS-C_6]/[-NH_2] = 2.5.
© 2015 Institute of Chemistry, Slovak Academy of Sciences

Keywords: gelatin, crosslinking, NHS-C_n, hydrophobicity, homogeneity film

Introduction

Gelatin is generated from collagen in skin, ligaments, tendons, etc. by partial acid or alkaline hydrolysis. The triple helix of collagen is cracked into gelatin polypeptide chains with a different molar mass without interruption of the repeating typical tripeptide units Gly–X–Y. Also, as a natural nutritious high protein food thickener, gelatin is widely recognised as a biomaterial because of its good physico-chemical and biological properties and its high mechanical strength. However, the rapid biodegradation rate and low thermal stability of the non-crosslinked gelatin cannot match the demand of applications in many cases; this is one of the crucial factors limiting further use of this material (Ma et al., 2004; Kozlov & Burdygina, 1983).

Chemical crosslinking is an effective method for reducing the biodegradation rate and optimising the thermal properties of gelatin-based materials (Zeeman et al., 1999; Chang et al., 2012), which involves the formation of either intra- or intermolecular ionic or covalent bonds between the gelatin's amino acid residues. Traditionally, glutaraldehyde, pluronics and diisocyanate (Fathima et al., 2004; Homenick et al., 2001; Vijayakumar & Subramanian, 2014) have been most extensively used for gelatin crosslinking. However, these crosslinkers entail toxicity and side reactions, in particular cytotoxicity and long-term calcification when gelatin is crosslinked with glutaraldehyde. Hence, the attention of researchers has been drawn to readily-prepared crosslinkers with lower toxicity, such as successive epoxy, biological extracts (heparin and transglutaminase) (Niu et al., 2014; Sztuka & Kołodziejska, 2009), carbodiinide (Velmurugan et al., 2013; Sloviková et al., 2008) and *N*-hydroxysuccinimide (NHS) active esters (Saito et al., 2004). Kajiyama (2004) described a new method for the synthesis of activated poly(maleic acid) (Su-PMA) by the reaction between synthesised α,β-PMA and NHS in the presence of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC), and presented an evaluation of the gelation properties of Su-

*Corresponding author, e-mail: taofurong17329@126.com

Brought to you by | New York University Bobst Library Technical Services
Authenticated
Download Date | 2/28/16 7:56 AM

PMA with collagen. Wissink et al. (2001) investigated heparin immobilisation using a non-cytotoxic crosslinked collagen substrate for endothelial cell seeding and resulted in a material containing 14 free primary amino groups per 1000 amino acid residues (E/N14C). The influence of molar ratios of EDC to heparin carboxylic acid groups (Hep-COOH) on heparin covalently immobilised to E/N14C was evaluated. Iwata et al. (1998) investigated the capacity of NHS-activated poly (L-glutamic acid) (NHSPLGA) to crosslink gelatin and enable tissues to adhere with good tissue compatibility and high bonding strength.

Almost all these studies focus on cytotoxicity in order to satisfy the requirements in drug carriers for tumour treatment, as scaffolds for tissue engineering or as injectable materials for tissue augmentation (Lai et al., 2007; Guan et al., 2006), but the microscopic surface texture, hydrophobicity and biodegradation condition were not studied. In addition, end-bit binary acids, as a wide range of different carbon chain compounds, have attracted extensive attention because of their reactivity. Succinic acid (C_4) is frequently used as an intermediate in dye-production and the medical industry, and the esterification reaction with alcohol has been widely researched (Delhomme et al., 2012; Dudáš et al., 2014). Glutaric acid (C_5), as an initiator for synthesising resin and rubber, is used to produce glutaric anhydride (Tsai et al., 2011). Adipic acid (C_6) is widely used to synthesise active esters while suberic acid (C_8), sebacic acid (C_{10}) and tetradecanedioic acid (C_{14}) are used as ingredients of high-molecular polymer, nylon and plastics (Krištofič et al., 2000; Xie et al., 2010; Park et al., 2012; Andrianov et al., 1967; Makedonopoulou & Mavridis, 2001). Although many studies have focused on these end-bit binary acids, there have been few investigations of the esterification reaction with NHS, especially C_8, C_{10} and C_{14}. In addition, studies of gelatin crosslinked by the active ester formed between C_8, C_{10}, C_{14} with NHS and a comparison of these film materials have been infrequent; this study serves to remediate this deficiency.

In the current work, one kind of crosslinking reagents (NHS-C_n) was prepared to crosslink gelatin and the effects were compared. The thermal properties, homogeneity in microstructure and hydrophobicity proved to be greater than ever and, in particular, the section properties influenced by different film-treatment conditions were also recorded. Test items, such as differential scanning calorimeter (DSC), water contact angles (CAs), biodegradation, residual amino groups test and viscosity were applied in the present study. In addition, the optimal reaction conditions of NHS-C_6 were explored.

Experimental

Gelatin (type A, obtained from pigskin, with an approximate molecular mass of 50000 and isoelectric point at pH = 8 determined by fluorescence measurements), oxalic acid (C_2, AR, 99 %), malonic acid (C_3, AR, 99 %) and succinic acid (C_4, AR, 99.5 %) were obtained from Sinopharm Chemical Reagent (China). Glutaric acid (C_5, AR, 99 %) and adipic acid (C_6, AR, 99 %) were sourced from Aladdin (China). Suberic acid (C_8, AR, 99 %), sebacic acid (C_{10}, AR, 99 %), tetradecanedioic acid (C_{14}, AR, 98 %), NHS (AR, 98 %) and EDC (AR, 99 %) were purchased from Energy Chemical Technology (China). Dimethylsulphoxide (DMSO; AR, 99 %), acetone (AR, 99 %), methanol (AR, 99 %) and other agents were obtained from Tianjin Fu Yu Fine Chemical (China). All chemicals and other reagents were used as received without further purification. The ^{1}H NMR spectra were recorded on a Bruker Advance 400 (Germany) spectrometer and the chemical shift is given in δ relative to internal standard (TMS). The FT-IR spectra were recorded on a Nicolet NEXUS 470 FT-IR (Thermo Scientific, USA) spectrometer.

NHS-C_n was prepared as described by Chen et al. (2011) with a small modification; end-bit binary acid (15 mmol each; C_2, C_3, C_4, C_5, C_6, C_8, C_{10}, C_{14}) and NHS (40 mmol) were dissolved in acetone (25 mL), and then EDC (30 mmol) was added to the solution. The clear mixture was gently stirred at 25 ℃ for 24 h. After the acetone was removed by rotary evaporation under reduced pressure, the residue was washed several times with deionised water and then dried under vacuum at 50 ℃. The crosslinker thus prepared was characterised by the ^{1}H NMR and FT-IR spectra.

To prepare the crosslinked gelatin films, gelatin was first dissolved in distilled water (3 mass %) then heated to 45 ℃ for 2 h to ensure complete dissolution. The mole (-NH_2) was set as 1 artificially; meanwhile the molar mass of the crosslinkers was determined by the free amino content in gelatin (5.25×10^{-4} mol g^{-1}) (Meng et al., 2013), i.e. the different mole of the crosslinker was formulated by the corresponding times (0, 0.5, 1, 1.5, 2 2.5, 3, 3.5) of mole (-NH_2). Hence, the final [crosslinker]-to-[-NH_2] ratios were 0 : 1, 0.5 : 1, 1 : 1, 1.5 : 1, 2 : 1, 2.5 : 1, 3 : 1 and 3.5 : 1. These corresponding gelatin samples were marked as Gel (0), Gel (0.5), Gel (1), Gel (1.5), Gel (1), Gel (2.5), Gel (3) and Gel (3.5), respectively. NHS-C_n solutions with different concentrations were prepared by dissolving in DMSO, then the mixture was added drop-wise to gelatin solutions. These solutions were stirred gently for 12 h at 45 ℃. NHS-C_n with two active ester groups was used to crosslink gelatin by the reaction between the amino and active ester groups (Fig. 1, NHS-C_6 as an example). After the reaction, 30 g of the gelatin reaction solutions (Gel (2.5) of all crosslinkers) was poured into a teflon® dish to cast the films. The conditions for formation of all the films, which were first placed in the mould at 30 ℃ for 2 h and then at 40 ℃ until dry, had to remain constant. The dry films were peeled off and dipped in methanol for 36 h to remove

Brought to you by | New York University Bobst Library Technical Services
Authenticated
Download Date | 2/28/16 7:56 AM

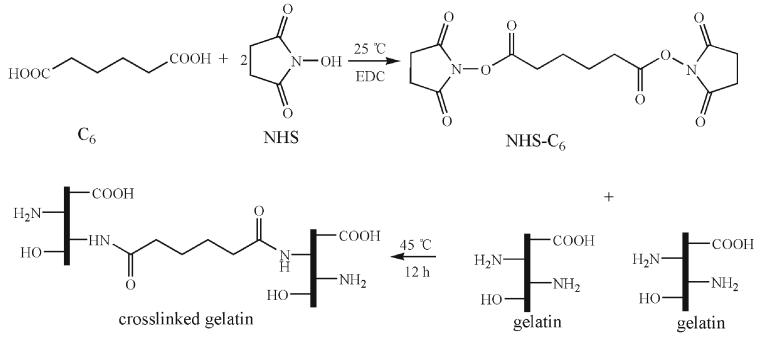

Fig. 1. Synthetic routes of crosslinked gelatin with NHS-C_6.

the DMSO. After drying, the films were stored in a desiccator. In addition, the purified films crosslinked by NHS-C_4 were dissolved in water at 45 ℃ for 2 h and poured into teflon® dishes to cast the films once again. However, the films modified by other crosslinkers did not undergo this two-step film-forming procedure due to their low solubility in water.

The content of the free -NH_2 groups was determined by the improved Van Slyke method at 45 ℃ (Van Slyke, 1911; Li, 2012). The testing solutions were mixed with acetic acid and sodium nitrite then stirred for 45 min. The residual primary amine (mol g^{-1}) was calculated according to the volume of N_2. The measurement was performed three times at each point.

DSC (Q600SDT, TA Instrument, USA) was used to detect the denaturation temperature (T_d) of the gelatin films. The gelatin film samples (approximately 2.5 mg) were accurately weighed into aluminium pans and sealed. The endothermal curve of the crushed film was recorded from 20 ℃ to 500 ℃ at a scanning rate of 10 ℃ min^{-1} under a nitrogen atmosphere.

The microscopic surface texture of the prepared films was investigated at different magnifications (× 100 and × 1000) using Quanta 200 environmental SEM (FEI, The Netherlands), and the section feature was recorded at × 500. The film surfaces were coated with Au using a metal-spraying device prior to observation. More than ten micrographs were taken at an acceleration voltage of 15 kV from different zones of each surface film under investigation. In addition, the section features of the films were recorded at the micron level.

The CAs of all the films were measured by the Sessile drop method using a DSA100 contact angle measuring system from Krüss (Germany). Films with a thickness of approximately 0.2 mm were obtained from the aqueous solution of gelatin (Gel (2.5)) applied onto the surface of a glass sheet then stored at ambient temperature until dry.

The biodegradation study of the gelatin film modified by NHS-C_6 was carried out according to the method described by Haroun et al. (2011) with some modifications. The gelatin films with constant mass (m_0) were incubated in vitro in a phosphate buffer (pH 7.40) at 37 ℃ for different periods (1 d, 4 d, 7 d, 10 d, 13 d and 15 d). After degradation, the films were washed with water and dried at 105 ℃ to constant mass (m_t). The biodegradable percentage (D) was examined by the mass loss according to Eq. (1). The measurement was performed three times at each point.

$$D = \frac{m_0 - m_t}{m_0} \times 100\ \% \tag{1}$$

The inherent viscosity of the gelatin solution crosslinked by NHS-C_6 was determined using an Ubbelohde viscosimeter at 30 ℃ with the gelatin (10 mL) solution added to the viscosimeter (ø 0.43 mm, China), and the efflux time (t) was recorded (Ogawa et al., 2004). The inherent viscosity at the given temperature was calculated according to Eq. (2);

$$\frac{\eta}{c} = \frac{t}{t_w - 1} c^{-1} \tag{2}$$

where t_W was the efflux time of distilled water and c (g mL^{-1}) defined as the mass concentration. All samples were tested in triplicate.

Time and temperature were explored as the optimal conditions on the basis of the above studies. The reaction gelatin solutions crosslinked by NHS-C_6 were allowed to react at 45 ℃ for different time intervals (4 h, 8 h, 12 h, 16 h, 20 h, 24 h) and, similarly, at different temperatures (30 ℃, 35 ℃, 40 ℃, 45 ℃, 50 ℃, 55 ℃) for 12 h. A double coordinate system was obtained by investigating the residual primary amine and inherent viscosity.

Results and discussion

Spectra of NHS-C_n

Fig. 2 shows the ^{1}H NMR spectrum of NHS-C_6. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.90 (s, 4H), 2.69 (s, 4H), 2.92 (s, 8H), 7.27 (s, $CDCl_3$), which are in accordance with the characteristic peaks of H in the ideal product. The FT-IR spectrum further confirmed the ester structure, which exhibits the bands at 1790 cm^{-1},

Brought to you by | New York University Bobst Library Technical Services
Authenticated
Download Date | 2/28/16 7:56 AM

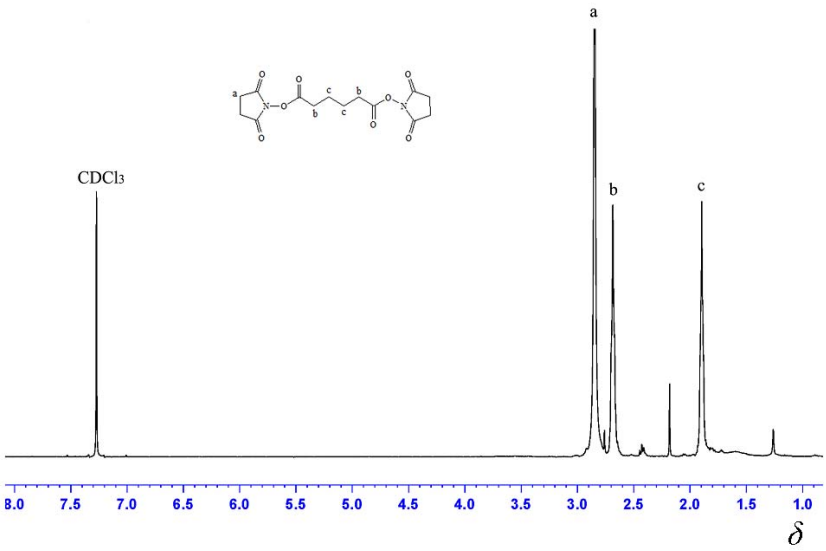

Fig. 2. ^{1}H NMR spectra of NHS-C_6.

1732 cm^{-1} and 1628 cm^{-1} due to the stretching vibration of C═O. The same protocol as was used for the preparation of NHS-C_6, was followed for the other end-bit binary acid crosslinkers. Their ^{1}H NMR and FT-IR spectral characteristics were as follows:

NHS-C_4: ^{1}H NMR (400 MHz, $CDCl_3$), δ: 2.85 (s, 4H), 3.09 (s, 8H), 7.27 (s, $CDCl_3$); FT-IR, $\tilde{\nu}$/cm^{-1}: 1642.65, 1783.95, 1749.6.

NHS-C_5: ^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.69 (s, 2H), 2.17–2.24 (m, 4H), 2.76–2.85 (m, 8H), 7.27 (s, $CDCl_3$); FT-IR, $\tilde{\nu}$/cm^{-1}: 1636, 1745, 1704, 1780.

NHS-C_8: ^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.41–1.50 (m, 4H), 1.64–1.70 (m, 4H), 2.63 (t, 4H), 2.84 (t, 8H), 7.27 (s, $CDCl_3$); FT-IR, $\tilde{\nu}$/cm^{-1}: 1645, 1748, 1710, 1785.

NHS-C_{10}: ^{1}H NMR (400 MHz, DMSO), δ: 1.24–1.34 (m, 8H), 1.59–1.65 (m, 4H), 2.64 (s, 4H), 2.80 (s, 8H), 2.58; FT-IR, $\tilde{\nu}$/cm^{-1}: 1746, 1784, 1831.

NHS-C_{14}: ^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.38 (s, 8H), 1.72–1.79 (m, 4H), 2.61 (s, 4H), 2.85 (s, 8H); FT-IR, $\tilde{\nu}$/cm^{-1}: 1644, 1742, 1779, 1703.

Unfortunately, when the oxalic acid (C_2) and malonic acid (C_3) reacted with NHS, respectively, although with EDC motivating carboxyl groups, its own decarboxylation which could not be prevented, precluded the esterification reaction, which resulted in a zero result after rotary evaporation under reduced pressure with or without water washing.

Free -NH_2 content of gelatin solution

Fig. 3 indicates the influence of different end-bit binary esters on the degree of crosslinking with gelatin, in which NHS-C_6 and NHS-C_8 exhibit the best crosslinking effect while NHS-C_4, -C_5, -C_{10}, and -C_{14} are the least effective. There may be two reasons for this. One is that succinic acid (C_4) and glutaric acid

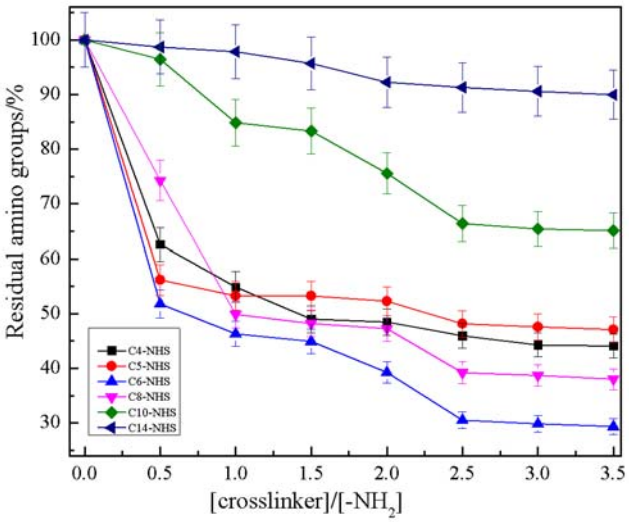

Fig. 3. Residual amino group content of gelatin solution crosslinked by NHS-C_n.

(C_5) tend to partly decarboxylate to form unsaturated monobasic acid because of the electron-withdrawing group -COOH. In addition, C_4 and C_5 are also inclined to dehydrate due to forming a stable five-membered and six-membered ring, respectively; all of these contributed to the poorer crosslink function. Secondly, the long distance between the two carboxyl compounds (C_{10}, C_{14}) favoured many adverse reactions, such as decarboxylation, dehydration, bending and folding of long carbon chains, which was decisive in the worst crosslinking effects of sebacic acid (C_{10}) and tetradecanedioic acid (C_{14}). The minimal side reactions were jointly attributed to the greatest crosslinking degree of adipic acid (C_6) and sacid (C_8), especially C_6.

Specifically, the effect of the crosslinker concentra-

Brought to you by | New York University Bobst Library Technical Services
Authenticated
Download Date | 2/28/16 7:56 AM

tion on the residual amino groups was analysed using the example of NHS-C_6. Fig. 3 shows that the amount of free -NH_2 in gelatin decreased sharply with a small quantity of NHS-C_6 added, and then decreased slightly when the ratio ranged from 0.5 : 1 to 1.5 : 1 then somehow rapidly decreased when the x variable ≥ 1.5. Interestingly, the amount of free -NH_2 reduced to a minimal value of approximately 30 % when [NHS-C_6]/[-NH_2] = 2.5 and an unusual slight decrease was observed after the ratio exceeded 2.5. The almost linear decreasing trend when the crosslinker was added was due to the high reactivity between -NH_2 and the active ester group. The reason why the free -NH_2 reduced slowly when $0.5 \leq$ [NHS-C_6]/[-NH_2] ≤ 1.5 and could not react completely may be the kinetically arrested state which resulted from self-aggregation by triple helix (Xu et al., 2012). The gelatin self-aggregation was previously suggested as the reason for the incompatibility with other biopolymers and the incomplete reaction with crosslinkers. The wrap behaviour in the gelatin solution, which produced a large-scale conformation with larger molecular mass, was associated with active ester hydrolysis in near-neutral or alkaline solutions, jointly hindered the reaction between the free -NH_2 and the dual-active ester.

Thermal stability

Fig. 4 shows the DSC curves of the gelatin films incorporating NHS-C_n, in which T_d of all the crosslinked films were higher than that of the blank film. However, there was no significant difference between the various carbon chain-lengths of the crosslinkers (NHS-C_n). In particular, the gelatin film modified by NHS-C_4 is shown in Fig. 4a, in which the endothermic peak was broad and obtuse with two representative peaks: 295.62 ℃ and 333.8 ℃. The first peak of 295.62 ℃ represented the partial denaturation of the modified gelatin films along with the decomposition of NHS-C_4, and then decomposed again at 333.8 ℃ after combination. In addition, the melting point of NHS-C_{14} was 148.6 ℃, as shown in the inset of Fig. 4b.

In order to study the influence of crosslinker concentration on the thermal stability of the original and crosslinked gelatin films, the thermogram of gelatin films modified by NHS-C_6 is shown in Fig. 4c, in which T_d ranged from 285 ℃ to 290 ℃, 60 ℃ higher than that of the native film. This was an endothermic process in which a phase transition took place involving changes in the lattice and long-range order. The increase in T_d could be related to the adding of crosslinkers, which was due to the decreased entropy of the transition and the rupture of hydrogen bonds to achieve a rearrangement of the triple helix into a random configuration (Grinberg & Tolstoguzov, 1972). It was evident that the curve changed slightly with variations in the crosslinker concentration, which indicated that the concentration of the

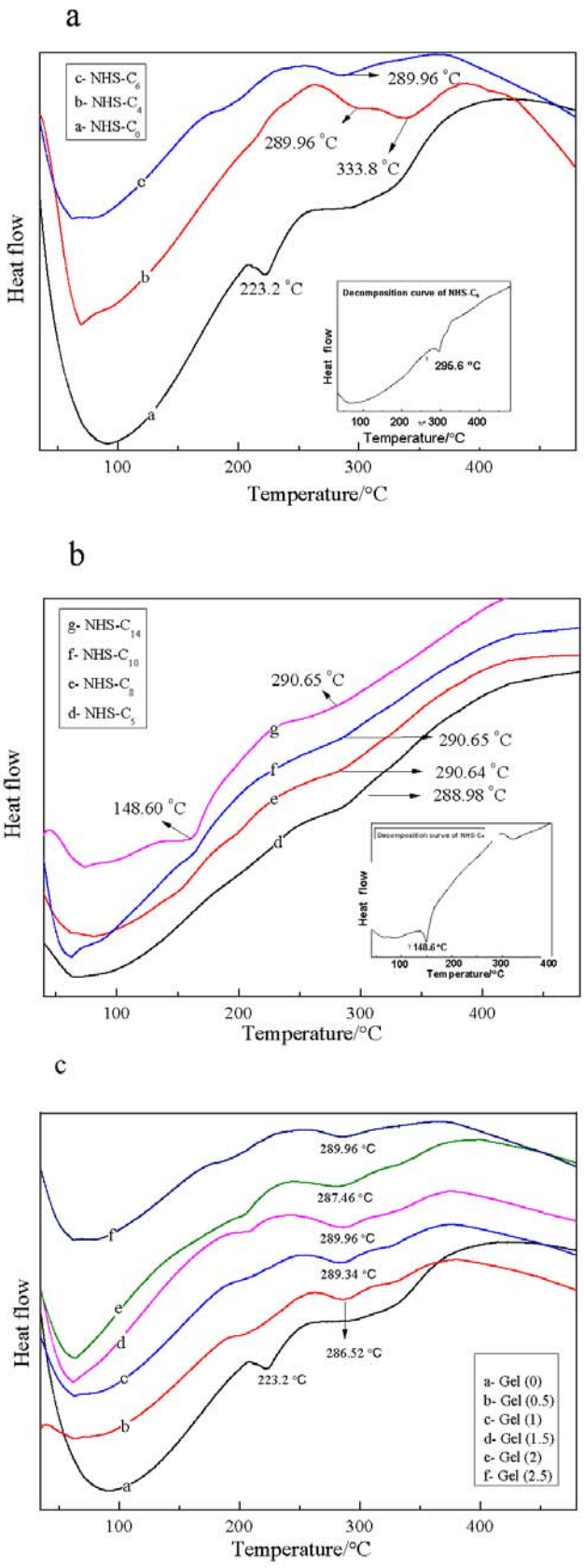

Fig. 4. DSC thermograms of gelatin films with different crosslinkers.

Brought to you by | New York University Bobst Library Technical Services
Authenticated
Download Date | 2/28/16 7:56 AM

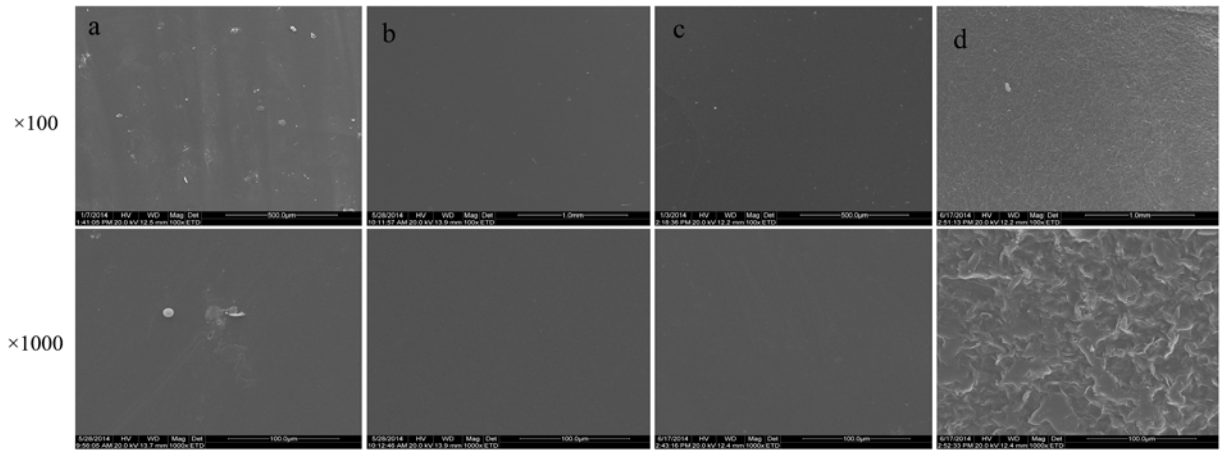

Fig. 5. Surface structure of prepared gelatin films with NHS-C_n; $n = 0$ (a), $n = 4$ (b), $n = 6$ (c) and $n = 14$ (d).

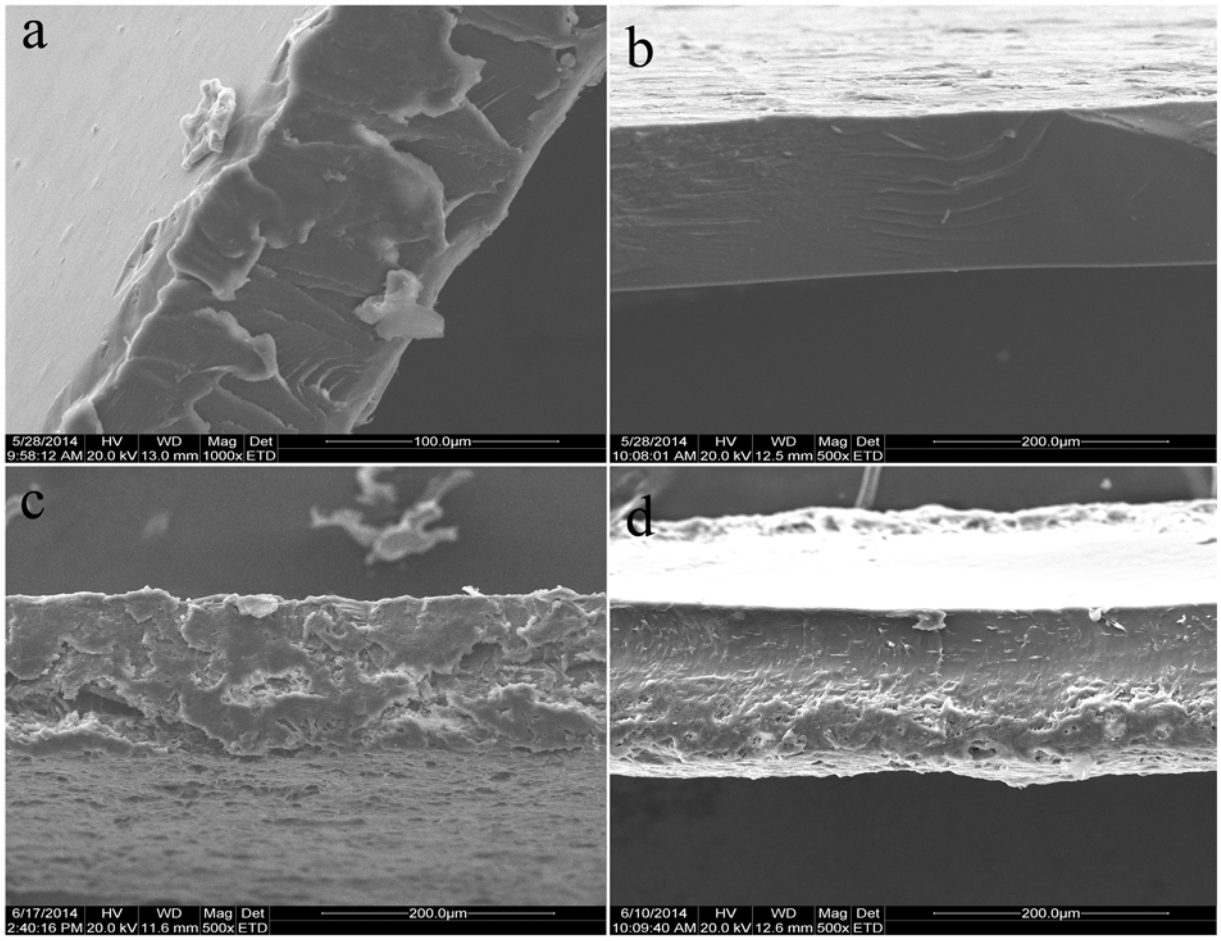

Fig. 6. Section properties of films crosslinked by NHS-C_n: $n = 0$ (a); $n = 4$ (b); with two-step film-forming $n = 6$ (c); soaked in methanol for purification $n = 6$ (d) (without any purification).

crosslinker did not significantly affect the thermal stability.

Surface and section morphology

Fig. 5 shows the surface morphology of crosslinked gelatin films prepared by adding NHS-C_n ($n = 0$, 4, 6, 14 as examples) with Gel (2.5). The original films (Fig. 5a) presented an uneven and chaotic image filled with balls and hollows both at × 100 and × 1000. With the addition of NHS-C_n ($n = 4, 6$), the randomly distributed fragments were cracked into small pieces

Brought to you by | New York University Bobst Library Technical Services
Authenticated
Download Date | 2/28/16 7:56 AM

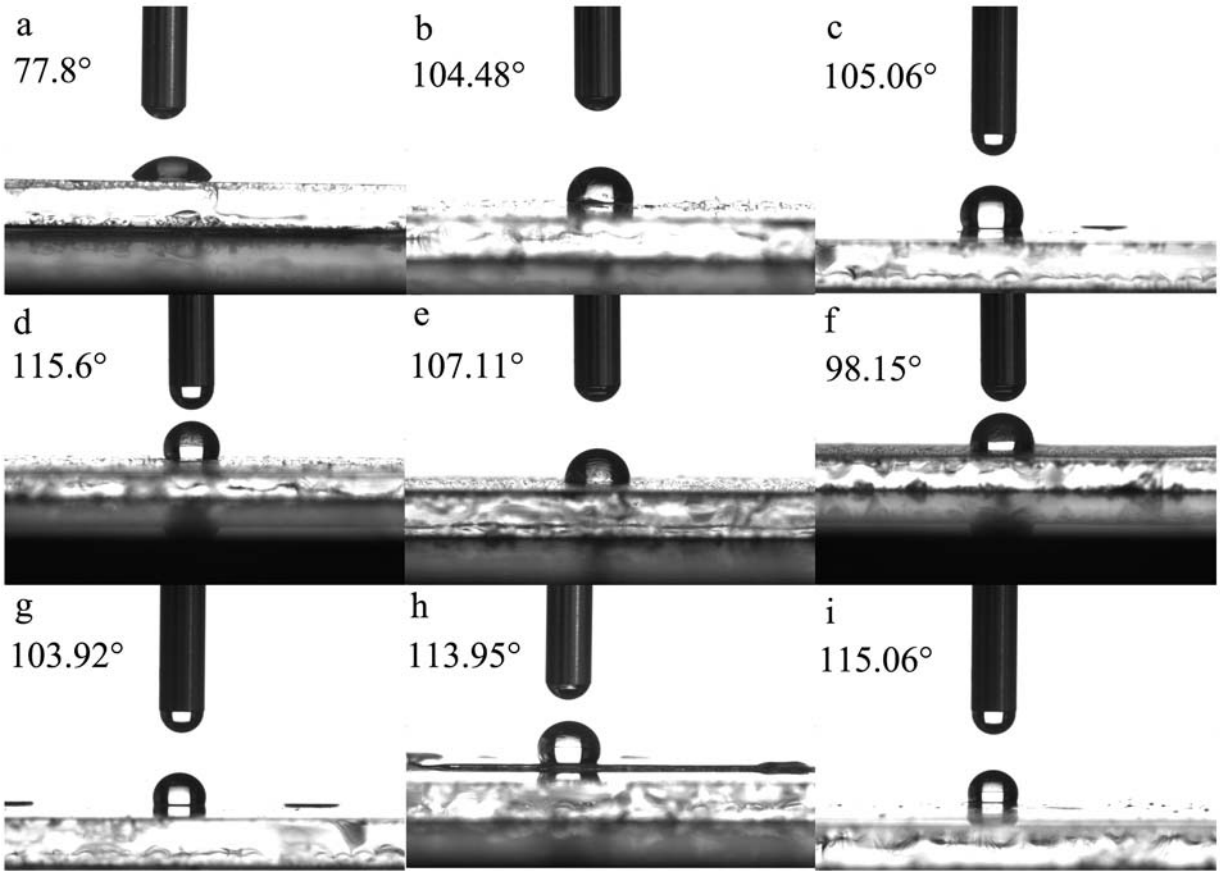

Fig. 7. Contact angles of films modified with NHS-C_n: $n = 0$ (a); $n = 4$ (b); $n = 5$ (c); $n = 8$ (d); $n = 10$ (e); $n = 14$ (f); $n = 6$ (g–i); films modified with NHS-C_6 at different ratio Gel (1.5) (g); Gel (2.5) (i).

and more homogeneous films were formed, which may be attributed to modification by crosslinkers (Figs. 5b and 5c). At × 1000, the same image was observed, in which the microcosmic ball structure of the original film and the flakes in the crosslinked films was clearly recorded. However, when NHS-C_{14} was added to the gelatin films, an unusual surface structure appeared and a flower-like pattern was exhibited at higher magnification (Fig. 5d). That was in accordance with the macroscopic phenomenon, where films crosslinked by NHS-C_n ($n = 4$, 6) were almost transparent and became absolutely white when NHS-C_{14} was incorporated.

In addition, Fig. 6 shows the thickness and section property of crosslinked films at the level of 200 μm, which are 30–100 μm thicker than the original film where the thickness was 108.33 μm (Fig. 6a). Interestingly, there are two phenomena revealed by Fig. 6. First, the influence of the two-step film-forming process on the section feature of crosslinked film. The film crosslinked by NHS-C_4 (Fig. 6b) was more orderly and symmetrical than the NHS-C_6 crosslinked film (Fig. 6c); this was due to the two-step film-forming process of NHS-C_4 crosslinked film. The reason why the film incorporating NHS-C_6 and other crosslinkers lacked this process was on account of the low solubility of the modified films in water.

Another interesting feature was the influence of DMSO on the film section shape in Figs. 6c and 6d, which were the purified film (soaked in methanol) and the original crosslinked film without any purification, respectively. As can be seen in Fig. 6d, the film section was divided into two layers. The upper was dense and close while the lower was full of floccules, which may be caused by the greater density of DMSO that led to its sedimentation during the film-forming process. Moreover, the loose and porous structure with many layers in Fig. 6c probably contributed to the removal of DMSO.

Contact angles

Fig. 7 shows images of the water contact angles by different crosslinkers NHS-C_n, where the angles increase with the length of NHS-C_n when $n \leq 8$ while they decrease with the carbon chain exceeding 10. It was certain that the longer carbon chain led to stronger hydrophobicity and all these explained the greater contact angles when compared with the blank film. However, when the chain length of the crosslinker was too long to obey the law, just as Figs. 7a–7i

Brought to you by | New York University Bobst Library Technical Services
Authenticated
Download Date | 2/28/16 7:56 AM

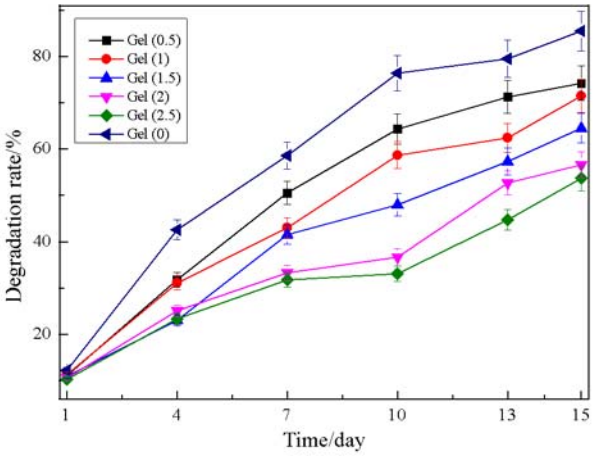

Fig. 8. Biodegradation of crosslinked gelatin films by NHS-C_6.

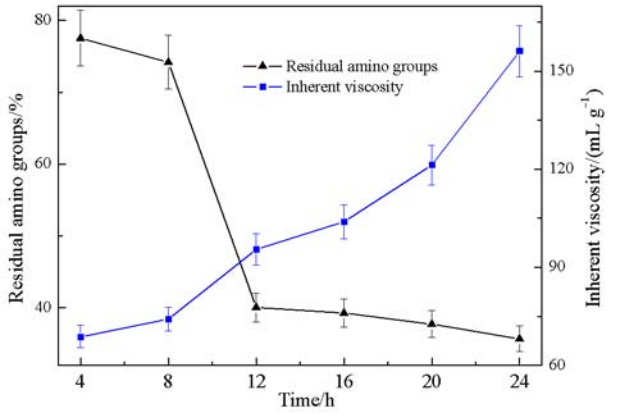

Fig. 9. Residual amino group content and inherent viscosity of gelatin solution crosslinked by NHS-C_6 at different intervals.

showed, the abnormal phenomena of NHS-C_{10} and NHS-C_{14} could be analysed as follows. First, the reduction in the crosslinking level, which was in accordance with the graph of residual amino group (Fig. 3), accounted for the smaller angles. Second, the uneven surface contributed to the decreased angles and this can be inferred from the SEM images (Fig. 5). Finally, the long crosslinker and gelatin chains may intertwine and cluster together, resulting in many vesicular structures and these vesicles provided chances for water to permeate the films.

The influences of the crosslinker concentration on the contact angles are shown in Figs. 7g–7i using NHS-C_6 as an example. Compared with the original film in which the angle was 77.8°, the contact angles increased to 103.92° with a small amount of the crosslinker (Fig. 7g, Gel(1.5)) added, and the more the crosslinker reacted, the larger were the contact angles (Fig. 7i, Gel(2.5)).

Biodegradation analysis

The biodegradation rates of the gelatin films crosslinked by different concentrations of NHS-C_6 are summarised in Fig. 8. The modified films degraded more slowly than the original films and achieved their minima when [NHS-C_6]/[-NH_2] = 2.5. Seven days after the start of the biodegradation, the crosslinked films degraded more slowly than the blank film; this resulted from the gelatin being a readily-degradable material which partially degraded when in contact with an aqueous medium and lost their fibrous structure. After seven days, all the modified films displayed much slower degradation rates due to the incorporation of NHS-C_6 into films. In addition, the hydrophobic structure of NHS-C_6 hindered the biodegradation of films, which could also be indicated by water contact angles above. In summary, incorporation of the crosslinker NHS-C_6 reduced the biodegradation rate of the modified films and the lowest rate was at the ratio of [NHS-C_6]/[-NH_2] = 2.5.

Optimal experiments

Time and temperature exhibited important influences on the gelatin modification. Figs. 9 and 10 show the effects of the two factors on the NHS-C_6-modified gelatin solution. When time was the sole variable, free -NH_2 decreased with the crosslinker added while viscosity increased when the time was adjusted from 4 h to 12 h. This may be explained from three aspects. First, the sharp reduction in free -NH_2 from 4 h to 12 h was attributed to the crosslinking procedure between the residual primary amino and NHS-C_6, and the somewhat slow rate after 12 h may due to the molecular cluster in the crosslinked gelatin solution. Second, the thixotropy, which meant the viscosity was dependent on time, was a reversible phenomenon. The external force that caused by deformation of liquid flowing decreased with time prolonged (Bakoš et al., 1993). Finally, the crosslinking, which introduced the macromolecule, contributed to the change of the entire test. That is to say, the longer chains led to a greater molecular mass and greater friction force, and all these resulted in greater viscosity.

Fig. 10 shows the content of free -NH_2 and changes in viscosity with temperature. The free -NH_2 decreased rapidly when the temperature was less than 45 ℃ and rose sharply above 50 ℃. The crosslinking by NHS-C_6 led to the reduction while the hydrolysis of the gelatin chains at higher temperature contributed to the rising trend. The fact that the higher temperature increased the distance between molecules reduced the molecular attraction as well as internal friction. All of these led to a declining trend in viscosity as the temperature increased, hence lower viscosity. In addition, as the temperature rose, the average velocity of the liquid molecular movement increased, which led

Brought to you by | New York University Bobst Library Technical Services
Authenticated
Download Date | 2/28/16 7:56 AM

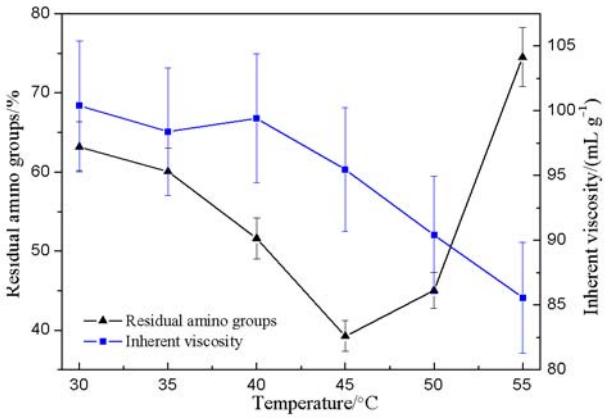

Fig. 10. Residual amino group content and inherent viscosity of gelatin solution crosslinked by NHS-C_6 at different temperatures.

to a shorter contact time with the adjacent molecules and lower viscosity.

Conclusions

A series of novel crosslinkers, *N*-hydroxysuccinimide-activated end-bit binary acid (NHS-C_4, -C_5, -C_6, -C_8, -C_{10}, -C_{14}), was synthesised and introduction of these crosslinkers changed the structure and conformation of gelatin. The content of residual free amino groups of the gelatin solution incorporated in different crosslinkers was compared. The denaturation temperature (T_d) of the crosslinked gelatin films obtained from DSC increased to a maximum of 290 ℃, 65 ℃ higher than that of native films and the different crosslinkers did not significantly affect T_d. In the SEM images, they exhibited a more homogeneous and smooth surface microstructure except when crosslinked by NHS-C_{14}, and the section structure influenced by different film-treatment conditions was recorded. The water contact angle images revealed the good hydrophobicity of the modified films. In addition, NHS-C_6 was used as a probe crosslinker, which exhibited the best crosslinking effect and the content of free -NH_2 was the lowest out of all the crosslinkers. The biodegradation results showed the better degradation-resistance and excellent stability of the films crosslinked by NHS-C_6. The best reaction conditions were achieved at 45 ℃ for 12 h when [NHS-C_6]/[-NH_2] = 2.5. In view of the ever-increasing use of gelatin, this study will play an important role in extending the existing NHS crosslinking technique and will broaden the application of gelatin films.

Acknowledgements. The authors wish to express their gratitude for the financial support received from 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 Programme for Scientific Research Innovation Team in Colleges and Universities of Shandong Province.

References

Andrianov, K. A., Fromberg, M. B., & Belkina, T. M. (1967). Kinetics of the esterification of trimethylolpropane with adipic and sebacic acid in the formation of oligomeric estero-acids. *Polymer Science U.S.S.R*, *9*, 2545–2549. DOI: 10.1016/0032-3950(67)90265-1.

Bakoš, D., Havlínová, D., & Mikula, M. (1993). The possible application of dissolution measurements to the study of composite films. *Chemical Papers*, *47*, 358–361.

Chang, S. T., Chen, L. C., Lin, S. B., & Chen, H. H. (2012). Nano-biomaterials application: Morphology and physical properties of bacterial cellulose/gelatin composites via crosslinking. *Food Hydrocolloids*, *27*, 137–144. DOI: 10.1016/j.foodhyd.2011.08.004.

Chen, Y. H., Zhang, M., Liu, W. T., & Li, G. Y. (2011). Properties of alkali-solubilized collagen solution crosslinked by *N*-hydroxysuccinimide activated adipic acid. *Korea-Australia Rheology Journal*, *23*, 41–48. DOI: 10.1007/s13367-011-0006-6.

Delhomme, C., Goh, S. L. M., Kühn, F. E., & Botz, D. W. (2012). Esterification of bio-based succinic acid in biphasic systems: Comparison of chemical and biological catalysts. *Journal of Molecular Catalysis B: Enzymatic*, *80*, 39–47. DOI: 10.1016/j.molcatb.2012.03.010.

Dudáš, J., Kotora, M., Bradáč, M., & Markoš, J. (2014). Design consideration of dimethyl succinate production process. *Chemical Papers*, *68*, 1667–1677. DOI: 10.2478/s11696-014-0580-4.

Fathima, N. N., Madhan, B., Rao, J. R., Nair, B. U., & Ramasami, T. (2004). Interaction of aldehydes with collagen: effect on thermal, enzymatic and conformational stability. *International Journal of Biological Macromolecules*, *34*, 241–247. DOI: 10.1016/j.ijbiomac.2004.05.004.

Grinberg, V. Y., & Tolstoguzov, V. B. (1972). Thermodynamic compatibility of gelatin with some D-glucans in aqueous media. *Carbohydrate Research*, *25*, 313–321. DOI: 10.1016/s0008-6215(00)81641-9.

Guan, L. B., Dan, W. H., Zeng, R., Mi, Z. J., Lin, H., Dan, N. H., Chen, C., Qu, J. J., & Ye, Y. C. (2006). Gelatin and its application in biomedical material. *Material Reviews*, *20*, 380–383.

Haroun, A. A., Ahmed, E. F., & El-Ghaffar, M. A. A. (2011). Preparation and antimicrobial activity of poly (vinylchloride)/gelatin/montmorillonite biocomposite films. *Journal of Materials Science: Materials in Medicine*, *22*, 2545–2553. DOI: 10.1007/s10856-011-4437-x.

Homenick, C. M., de Silveira, G., Sheardown, H., & Ardonov, A. (2011). Pluronics as crosslinking agents for collagen: novel amphiphilic hydrogels. *Polymer International*, *60*, 458–465. DOI: 10.1002/pi.2969.

Iwata, H., Matsuda, S., Mitsuhashi, K., Itoh, E., & Ikada, Y. (1998). A novel surgical glue composed of gelatin and *N*-hydroxysuccinimide activated poly (L-glutamic acid): Part 1. Synthesisof activated poly (L-glutamic acid) and its gelation with gelatin. *Biomaterials*, *19*, 1869–1876. DOI: 10.1016/s0142-9612(98)00095-7.

Kajiyama, T., Kobayashi, H., Taguchi, T., Saito, H., Kamatsu, Y., Kataoka, K., & Tanaka, J. (2004). Synthesis of activated poly (α,β-malic acid) using *N*-hydroxysuccinimide and its gelation with collagen as biomaterials. *Materials Science and Engineering: C*, *24*, 815–819. DOI: 10.1016/j.msec.2004.08.023.

Kozlov, P. V., & Burdygina, G. I. (1983). The structure and properties of solid gelatin and the principles of their modification. *Polymer*, *24*, 651–666. DOI: 10.1016/0032-3861(83)90001-0.

Brought to you by | New York University Bobst Library Technical Services
Authenticated
Download Date | 2/28/16 7:56 AM

Krištofič, M., Marcinčin, A., Ujhelyiová, A., & Murárová, A. (2000). Modification of PA 6 fibres with alkaline copolyamides. *Chemical Papers*, *54*, 53–58.

Lai, G., Du, Z. L., & Li, G. Y. (2007). The rheological behavior of collagen dispersion/poly(vinyl alcohol) blends. *Korea-Australia Rheology Journal*, *19*, 81–88.

Li, L. D. (2013). China Patent No. CN 202869916 U. Jinan, Shandong, China: State Intellectual Property Office of the People's Republic of China.

Ma, L., Gao, G. G., Mao, Z. G., Zhou, J., & Shen, J. C. (2004). Enhanced biological stability of collagen porous scaffolds by using amino acids as novel cross-linking bridges. *Biomaterials*, *25*, 2997–3004. DOI: 10.1016/j.biomaterials.2003.09.092.

Makedonopoulou, S., & Mavridis, I. M. (2001). The dimeric complex of cyclomaltoheptaose with 1,14-tetradecanedioic acid. Comparison with related complexes. *Carbohydrate Research*, *335*, 213–220. DOI: 10.1016/s0008-6215(01)00207-5.

Meng, X. J., Cui, Y. Z., Qiao, C. D., Xu, J., & Li, T. D. (2013). Interaction between binary quaternary ammonium compounds and gelatin. *Polymer Materials Science and Engineering*, *29*, 62–66.

Niu, G. G., Choi, J. S., Wang, Z., Skardal, A., Giegengack, M., & Soker, S. (2014). Heparin-modified gelatin scaffolds for human corneal endothelial cell transplantation. *Biomaterials*, *35*, 4005–4014. DOI: 10.1016/j.biomaterials.2014.01.033.

Ogawa, M., Portier, R. J., Moody, M. W., Bell, J., Schexnayder, M. A., & Losso, J. N. (2004). Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*). *Food Chemistry*, *88*, 495–501. DOI: 10.1016/j.foodchem.2004.02.006.

Park, H. S., Seo, J. A., Lee, H. Y., Kim, H. W., Wall, I. B., Gong, M. S., & Knowles, J. C. (2012). Synthesis of elastic biodegradable polyesters of ethylene glycol and butylene glycol from sebacic acid. *Acta Biomaterialia*, *8*, 2911–2918. DOI: 10.1016/j.actbio.2012.04.026.

Saito, H., Taguchi, T., Kobayashi, H., Kataoka, K., Tanaka, J., Murabayashi, S., & Mitamura, Y. (2004). Physicochemical properties of gelatin gels prepared using citric acid derivative. *Materials Science and Engineering: C*, *24*, 781–785. DOI: 10.1016/j.msec.2004.08.039.

Sloviková, A., Vojtová, L., & Jančař, J. (2008). Preparation and modification of collagen-based porous scaffold-for tissue engineering. *Chemical Papers*, *62*, 417–422. DOI: 10.2478/s11696-008-0045-8.

Sztuka, K., & Kołodziejska, I. (2009). The influence of hydrophobic substances on water vapor permeability of fish gelatin films modified with transglutaminase or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). *Food Hydrocolloids*, *23*, 1062–1064. DOI: 10.1016/j.foodhyd.2008.08.002.

Tsai, Y. T., Lin, H. M., & Lee, M. J. (2011). Kinetics of heterogeneous esterification of glutaric acid with methanol over Amberlyst 35. *Journal of the Taiwan Institute of Chemical Engineers*, *42*, 271–277. DOI: 10.1016/j.jtice.2010.07.010.

Van Slyke, D. D. (1911). A method for quantitative determination of aliphatic amino groups: applications to the study of proteolysis and proteolytic products. *The Journal of Biological Chemistry*, *9*, 185–204.

Velmurugan, P., Jonnalagadda, R. R., & Nair, B. U. (2013). Biochemical and biophysical characterization of EDC treated rattus type I collagen. *Process Biochemistry*, *48*, 1059–1064. DOI: 10.1016/j.procbio.2013.05.014.

Vijayakumar, V., & Subramanian, K. (2014). Diisocyanate mediated polyether modified gelatin carrier for controlled release. *Saudi Pharmaceutical Journal*, *22*, 43–51. DOI: 10.1016/j.jsps.2013.01.005.

Wissink, M. J. B., Beernink, R., Pieper, J. S., Poot, A. A., Engbers, G. H. M., Beugeling, T., van Aken, W. G., & Feijen, J. (2001). Immobilization of heparin to EDC/NHS-crosslinked collagen. Characterization and in vitro evaluation. *Biomaterials*, *22*, 151–163. DOI: 10.1016/s0142-9612(00)00164-2.

Xie, Y., Bai, F. Y., Li, J., Xing, Y. H., Wang, Z., Zhao, H. Y., Pu, Z. F., Ge, M. F., & Shi, Z. (2010). Synthesis, crystal structure and photoelectric property of two new coordination polymers constructed by longer-spanning suberic acid and 4,4'-bipyridine ligands. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *77*, 749–754. DOI: 10.1016/j.saa.2010.07.009.

Xu, J., Li, T. D., Tang, X. L., Qiao, C. D., & Jiang, Q. W. (2012). Effect of aggregation behavior of gelatin in aqueous solution on the grafting density of gelatin modified with glycidol. *Colloids and Surfaces B: Biointerfaces*, *95*, 201–207. DOI: 10.1016/j.colsurfb.2012.02.041.

Zeeman, R., Dijkstra, P. J., van Wachem, P. B., van Luyn, M. J. A., Hendriks, M., Cahalan, P. T., & Feijen, J. (1999). Successive epoxy and carbodiimide cross-linking of dermal sheep collagen. *Biomaterials*, *20*, 921–931. DOI: 10.1016/s0142-9612(98)00242-7.

Brought to you by | New York University Bobst Library Technical Services
Authenticated
Download Date | 2/28/16 7:56 AM