J Therm Anal Calorim (2011) 105:325–330
DOI 10.1007/s10973-011-1472-2

Di-carboxylic acid cross-linking interactions improves thermal stability and mechanical strength of reconstituted type I collagen

Part I. Oxalic acid

Tapas Mitra · G. Sailakshmi · A. Gnanamani · A. B. Mandal

Received: 13 October 2010 / Accepted: 9 March 2011 / Published online: 31 March 2011

Abstract This study emphasizes, cross-linking potential of a simple di-carboxylic acid, namely, oxalic acid with type I collagen for the preparation of collagen based biomaterial for clinical applications. Further the study discusses the characteristics features of the cross-linked material in comparison with the standard cross-linker. In addition, the study also demonstrates the role of ionic interactions in providing the thermal stability and tensile strength to the cross-linked biopolymer material. Type I collagen from rat tail tendon treated with oxalic acid at optimized concentrations provided a biopolymer material without changing the triple helical pattern of collagen (CD spectrum) and also with 6–7 fold increase in tensile strength than native collagen. FTIR spectral details demonstrate the ionic interactions between collagen and oxalic acid. Thermal stability analyses of oxalic acid cross-linked biopolymer revealed, high thermal stability compared to materials of glutaraldehyde cross-linked. The results of the study suggest oxalic acid as a suitable cross-linker for collagen and it cross-link with collagen through ionic interactions.

Keywords Type I collagen · Oxalic acid · Cross-linker · Thermal stability · Circular dichroism

T. Mitra · G. Sailakshmi · A. Gnanamani (✉)
Microbiology Division, Central Leather Research Institute (Council of Scientific and Industrial Research), Adyar, Chennai 600020, Tamil Nadu, India
e-mail: gnanamani3@gmail.com

A. B. Mandal
Chemical Laboratory, Central Leather Research Institute (Council of Scientific and Industrial Research), Adyar, Chennai 600020, Tamil Nadu, India

Introduction

Preparation of collagen based biopolymer materials for clinical applications was always been a great challenge, due to the problems encountered mainly with the cross-linkers and in additional with the thermal stability, mechanical strength, biocompatible, and biodegradable properties. Nevertheless, in body system, stabilization of synthesized collagen was established by natural cross-linkers (lysyl oxidase) under in situ condition, which provide proteolytic resistance and high mechanical (tensile strength) properties. But, during the course of extraction of collagen and reconstitution, the expected stability and strength could not be achieved. Thus it is often necessary to confer mechanical firmness and collagenase resistance (to the material of collagen based) by introduction of exogenous cross-linker into the molecular structure of collagen.

Till date exogenous cross-linking agents such as chromium [1], aldehydes [2], hexamethylene diisocyanate [3], carbodiimide [4], acylazides [5], citric acid, maleic acid derivatives [6], usnic acid [7], alginic acid [8], dialdehyde starch [9], and various other physical treatments like UV [10] and gamma irradiation [11] were used for cross-linking of collagen. In general all the said exogenous cross-linkers, cross-linked with collagen through; (i) covalent amide linkage (between activated –COOH functional group of cross-linker with ε-NH_2 group of collagen); (ii) covalent imine linkage (between –CHO functional group of cross-linker with ε-NH_2 group of collagen); (iii) co-ordinate bond formation (chromium cross-linking with collagen); (iv) H-bond formation (between polyphenolic –OH group with different type of amino acids of collagen molecule), etc. With all these bonding patterns, one can easily get more stable collagen based biopolymer materials. However, recent realization on demerits (non-biodegradable, toxicity,

and a weak mechanical property) of some of the cross-linkers (for example glutaraldehyde), demands new types of cross-linkers with all the expected properties. Moreover, in all the above said cross-linkers, ionic interactions with collagen were not discussed in reports.

In order to alleviate the said demerits, we attempted to have a suitable cross-linker for the preparation of collagen based biopolymer material with required properties. By chance, if the cross-linker contains two active sites, it may cross-link with ε-NH_2 group of two different collagen triple helices (like hand-shake), which may provide expected stability to the resultant biopolymer. The better choice was a di-carboxylic acid, which has two –COOH group and these two –COOH group able to interact ionically with two collagen helices as expected. Melina et al. [12] reported oxalic acid interacts with –NH_2 group of chitosan and formed a hydrogel. Results of his study suggested oxalic acid, a simplest dicarboxylic acid also can cross-linked with –NH_2 residue of amino group of collagen.

Oxalic acid, a chemical substance; found naturally occurring component in plants. Dark-green leafy foods were rich source of oxalic acid. Based on our knowledge and literature survey, till date no reports were available on the cross-linking interactions of collagen with oxalic acid.

Thus, keeping in mind the highest demand for collagen based biopolymer materials for wide applications (biomedical [13, 14], pharmaceutical [15, 16], food packaging [17, 18]), we attempted to prepare and characterize oxalic acid cross-linked collagen biopolymer and discussed in detail the hypothetical explanation for the thermal stability and mechanical strength of the resultant biopolymer. In addition, comparison on tensile strength, thermal stability was made with biopolymer material prepared using glutaraldehyde as cross-linker.

Materials and methods

Materials

Oxalic acid (OA) and glutaraldehyde were obtained from Sigma-Aldrich (USA). Type I collagen extracted from rat tail tendon was used as the collagen source. All other reagents were of Analytical Reagent grade and used without further purification.

Methods

Extraction of rat tail collagen and characterization (SDS-PAGE)

Rat tail collagen (RTC) fibers were teased out from tail of 6-month-old male albino rat (Wistar strain) and collagen was extracted according to Chandrasekaran et al. [19]. Collagen obtained from the above step was further subjected to SDS-PAGE analysis to assess the purity, type, and molecular profile. In brief, electrophoresis was carried out using 8% polyacrylamide gel. Followed by electrophoresis, gel was stained with coomassie blue and destained with the mixture of methanol and acetic acid. Molecular mass marker from Sigma (USA) was used to measure the molecular mass of the bands appeared.

Preparation of cross-linked biopolymer material

Different concentrations (0.05, 0.1, 0.2, 0.3, 0.4, and 0.5% w/v) of OA in solution form were prepared by dissolving the required quantities in 70 mM sodium phosphate buffer (pH 6.5) at room temperature.

About 0.5% type I collagen (dissolved in 0.005 M acetic acid) was mixed with different concentrations of OA (Table 1) and the homogenized solution obtained upon stirring for 30 min at 20 °C was incubated for 48 h at 4 °C. Followed by incubation, the reaction mixture was transferred to polypropylene plate (Tarson, India) and air-dried at 37 °C for 12 h. The polymer material in the form of sheets obtained from the above process was further designated as OA cross-linked collagen (OACC). Cross-linking of collagen with glutaraldehyde was also carried out for comparisons. Glutaraldehyde at respective concentration was mixed with 0.5% of collagen and sheet was prepared according to the procedure summarized above. In addition, a separate collagen sheet material without cross-linker (native) was also made accordingly and used for comparative study. The dried polymer sheets of both native and cross-linked biopolymer materials (OACC and GCC) were stored at 4 °C in airtight containers and used for the following analyses.

Characterization of biopolymer material

Infrared spectrum for Oxalic acid, native collagen and OACC samples were recorded using Spectrum One

Table 1 Preparation of OACC biopolymer material using various concentrations of oxalic acid

Collagen/mL	Oxalic acid concentration/%	Volume of oxalic acid/mL	Total volume of reaction mixture/mL
10	0.05	4	14
10	0.1	4	14
10	0.2	4	14
10	0.3	4	14
10	0.4	4	14
10	0.5	4	14

(Perkin-Elmer Co., USA model) instrument in the range of 4000–400 cm^{-1} with 20 scans. Thermal behavior, TG and DSC properties of the native as well as cross-linked biopolymer materials (GCC and OACC), evaluated by a TGA Q 50(V20.6 build 31) instrument. In brief, samples were heated from 25 to 800 °C under N_2 flow (40 and 60 mL min^{-1}) using a heating rate of 20 °C min^{-1}, and on a DSC Q 200(V 23.10 Build 79) calorimeter, using a heating rate of 10 °C min^{-1} from 0 to 300 °C under nitrogen (50 mL min^{-1}) atmosphere using standard mode. Tensile strength was measured using Universal testing machine (INSTRON model 1405). Experiments were carried out at constant cross-head speed of 5 mm min^{-1} with relative humidity of 65% at 20 °C. Before subjecting the samples to analysis, dumbbell shaped samples of uniform width and thickness were made and analyzed. With regard to circular dichroism (CD) analysis, type I collagen solution was incubated in the presence of 0.1% of oxalic acid at 4 °C for 24 h and recorded the spectrum at 25 °C using a Jasco 715 circular dichroism spectropolarimeter. A scan speed of 20 nm per min was used with an average of three scans per sample. A slit width of 1 nm and a time constant of 1 s were used. A 1 mm cell was used for the experiments. A reference spectrum containing phosphate buffer was also recorded. The CD spectra of the samples were obtained after subtracting the reference spectrum. Change in the conformation of collagen on addition of oxalic acid was recorded.

Results and discussion

Molecular profile

The electrophoretogram of type I collagen shown in Fig. 1a demonstrates presence of [(α_1) $(I)_2$ (α_2) $(I)_1$] as an intense band near 100 kDa followed by a faint band, corresponds to α_1 and α_2 chains respectively. In addition, a band observed near 200 kDa corresponds to β chain. The patterns of α and β isomers of collagen compares favorably with the standard values reported earlier [20]. Figure 1b depicts the physical appearance of OACC biopolymer material. Thickness of the resultant material was measured using screw gauge.

Optimization of OA concentration with collagen for the required mechanical strength and thermal stability studies revealed 0.1% of OA with 0.5% of collagen (w/v) provided the requisite properties as evidenced through the characteristic features.

Fourier transform infrared spectroscopy studies (FT-IR)

FT-IR studies were conducted to monitor the chemical modifications in collagen structure due to cross-linking with oxalic acid. Figure 2a–c illustrates the FT-IR spectral details of OA, native and OACC. When comparing the FT-IR spectral details of OA, native and OACC, a completely different spectrum for OACC was observed. The amide I, II, and III peaks of native collagen was located at 1658, 1553, and 1239 cm^{-1} and the primary –NH out-of-plane (o-o-p) bending observed as a broad peak at 655 cm^{-1}.

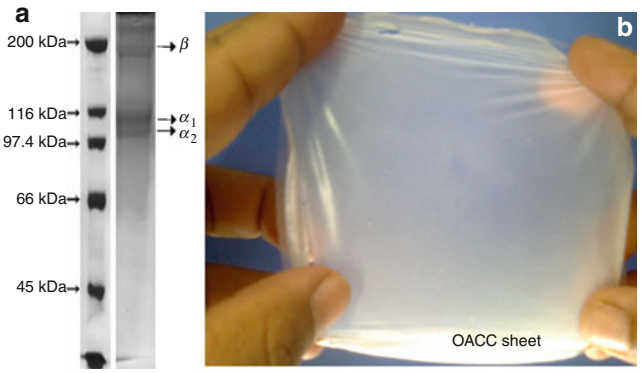


Fig. 1 **a** Molecular profile of type I collagen obtained from RTT. *Lane 1* molecular marker mass (Sigma, USA) and *lane 2* RTC. **b** Physical appearance of OACC biopolymer material

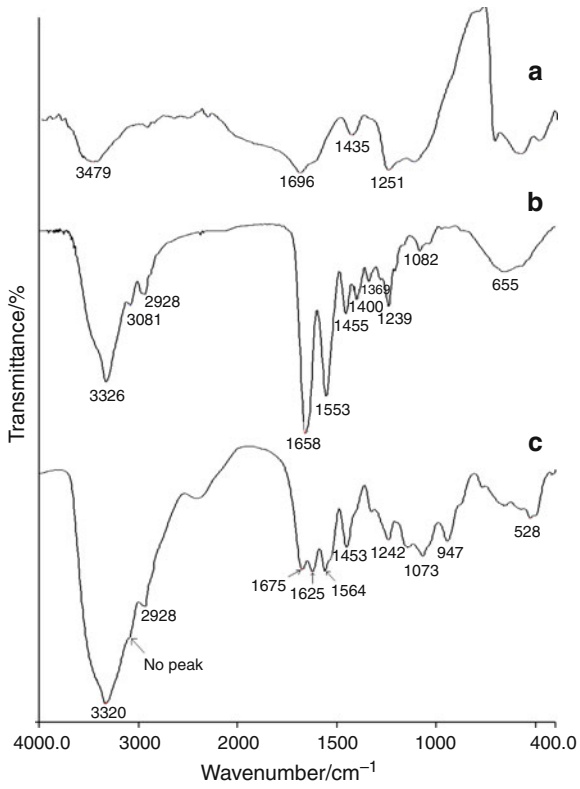


Fig. 2 FT-IR spectrum of (*a*) oxalic acid (OA); (*b*) Rat tail collagen (native); (*c*) Oxalic acid cross-linked collagen biopolymer material (OACC)

Table 2 FT-IR analysis of OA, native (RTC), and OACC

Wave number/ cm^{-1}	Assignment
655	1° amide δ(N–H) very broad band
1553	Strong 2° amide δ(N–H)
1658	3° amide v(C=O) strong
3081	Weaker band at about 3100 in 2° amide is attributed to a Fermi resonance overtone of 1550 band
3326	Strong 2° amide v(N–H)
1696	Broad (C=O) v of oxalic acid
1251	(C–O) v medium intensity of oxalic acid
3479	Very broad (O–H) v of oxalic acid

v stretching, δ bending

However, after cross-linking with oxalic acid, a sharp intense peak at 1658 disappears with the appearance of two new peaks at 1675 and 1625 cm^{-1} in OACC. This might be due to the ionic interaction between $–COO^-$ of oxalic acid (1625) and $–NH_3^+$ (1675) of collagen [21]. In addition, deep and sharp intense peak observed at 1553 cm^{-1} in native and the related overtone of 1553 at 3081 cm^{-1} was reduced without any overtone peaks in OACC. However, no change in the amide III was observed in OACC. Table 2 demonstrates the FT-IR peak assignment of OA, native and OACC.

Thermal analysis

The thermal stability of the native and OACC biopolymer material was evaluated by thermo gravimetric analysis (TG). As can be seen in Fig. 3a the decomposition patterns of the OA, native and OACC were presented in two steps; at temperatures below 100 °C, the mass loss can probably be attributed to the volatilization of low molecular mass compounds (e.g., adsorbed water) [22] and post-curing processes. The degradation of the OACC took place at temperatures higher than 290 °C with 25% mass loss. The main degradation step occurred at 400–500 °C, with a mass loss in the range of 50–55%, whereas in GCC 25 and 50% mass loss was observed at 215 and 353 °C and in native this was observed at 190 and 373 °C, respectively.

Differential scanning calorimetry (DSC) detection was used to study the thermal behavior of OA, native, and OACC. The higher transition temperatures indicate collagen had higher stability in a high temperature environment. The thermal stability also influences on the durability of the collagen based biopolymer materials. DSC studies recorded melting temperature differences among OA (103 °C), native (99 °C), OACC (137 °C), and GCC (151 °C) as shown Fig. 3b.

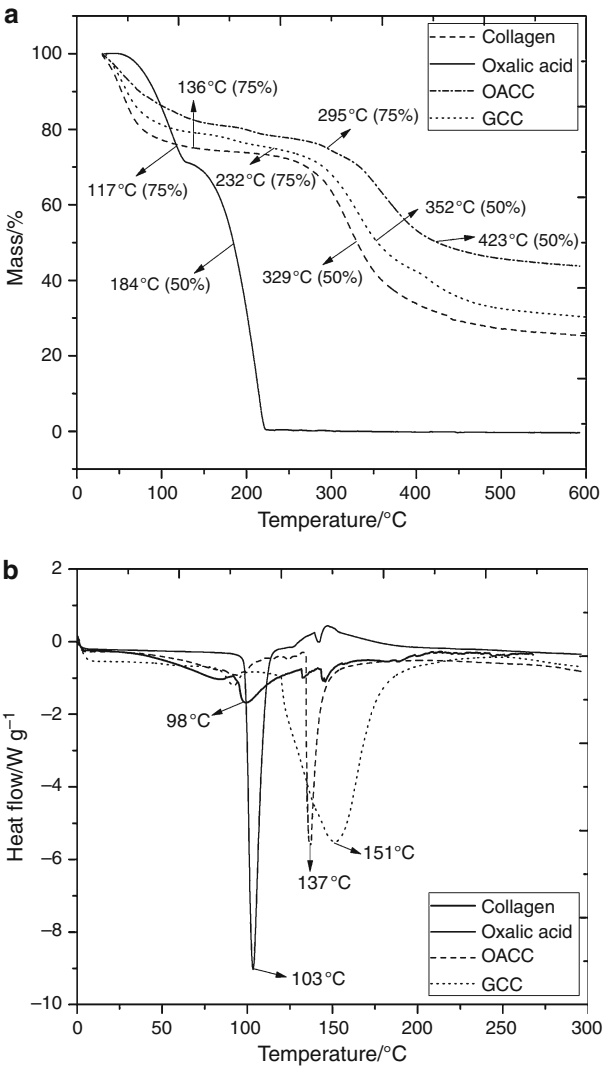


Fig. 3 **a** TG of oxalic acid (OA), native (in figure indicated as 'collagen') and oxalic acid cross-linked collagen (OACC), and GCC biopolymer materials. **b** DSC analysis of oxalic acid (OA), native (in figure indicated as 'collagen') and oxalic acid cross-linked collagen (OACC), and GCC

Tensile strength

The mechanical property of native collagen was studied to ensure that the sheet was intact during clinical applications. Results showed that OACC exhibit very high tensile strength 40.63 MPa than the native (6.8 MPa) and GCC (1.95). About 6–7 fold increase in tensile strength was observed after cross-linking with OA compared to native.

Circular dichroism

Collagen exhibits a unique CD spectrum with a small positive peak between 220 and 225 nm and a large negative at 197 nm [23, 24]. The CD spectrum of native

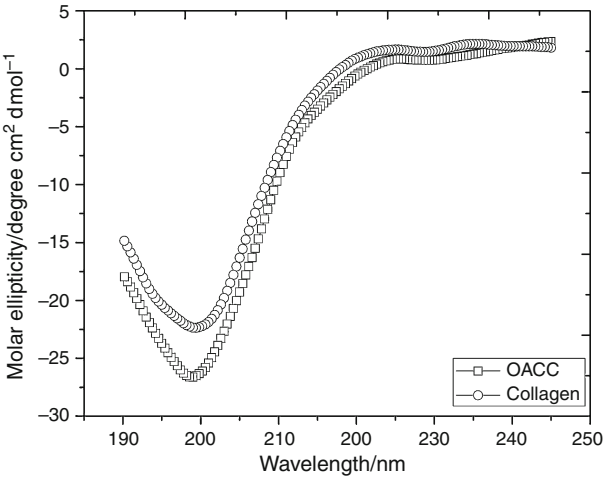
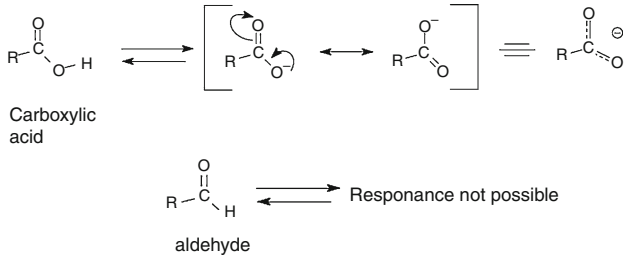


Fig. 4 Circular dichorism (CD) analysis of native collagen (rat tail tendon) and OACC

collagen and (0.1%) OACC were shown in Fig. 4. Since, the parameter Rpn denotes the ratio of positive peak intensity over negative peak intensity and the parameter can be used to differentiate triple helical conformation from other non-triple helical conformations [25], in the present study, the Rpn values for native collagen and OACC was 0.031 and 0.035, respectively. The Rpn values of collagen solution treated with oxalic acid did not change significantly. When compared to collagen there is a decrease in molar ellipticity at 220 nm and an increase at 197 nm for OACC. This may be due to aggregation of collagen molecule in the presence of OA without any change in the triple helical structure.

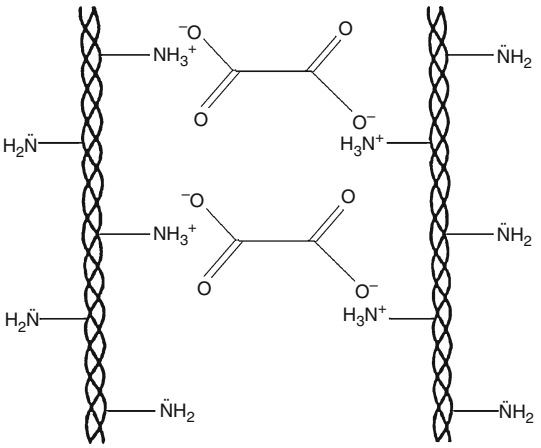
All these results emphasizes, OA cross-linked with collagen through ionic interaction as evidenced through FT-IR spectrum and the cross-linking provided a good mechanical strength and high thermal stability to the resultant biopolymer material.

To our knowledge till date no reports were available on crosslinking of collagen with oxalic acid. In general, amine group of any protein has high electron density, and therefore a powerful nucleophile, likely to react with electron deficient groups such as the carbon of a carbonyl group (–C=O), where the electronegative oxygen atom has pulled the bonding electrons towards itself. Similarly, in the case of collagen, the amine group in lysyl ε-NH_2 is a nucleophile group and ready to interact with electrophile compound and form a covalent bond through nucleophilic addition–elimination reaction, for example, cross-linking with glutaraldehyde. With reference to carboxylic acid, a mesomeric effect according to the reaction shown below, provide an increased electron density around the carbon, whereas in aldehyde, there was no mesomeric effect.



The reason for the formation of ionic interactions instead of covalent interactions observed in the present was hypothetically explained as;

When the electron density of central carbon atom in oxalic acid increases, either by means of increase/decrease the pH of the solution, the H^+ ion of –COOH group readily interact with the $–NH_2$ group of lysyl residue of collagen and transformed to $–NH_3^+$. The positive charge on the $–NH_3^+$ group removes all the nucleophile character of the amine and finally $–NH_3^+$ (collagen lysyl ε-NH_2) ionically interact with $–COO^-$ of oxalic acid and transformed to strong biopolymer material. The schematic reaction was summarized below for better understanding.



Characterization of resultant material with appreciable tensile strength and thermal stability implies, ionic interactions also play the major role in stabilization of collagen.

Conclusions

In this study the choice for the preparation of thermally stable biopolymer material was type I collagen of rat tail tendon and a simple dicarboxylic acid, oxalic acid (OA). The reason for the selection of type I collagen is due to its interaction with number of cells and its involvement in most of the human and animal diseases. Though numbers of applications are available for the different use of OA, its cross-linking potential with collagen has not yet in reports.

The cross-linking ability of OA with collagen was assessed using FT-IR, TG, DSC, and CD. The results obtained and the schematic representation of the reaction mechanism summarized suggests the suitability of OA as a cross-linking agent and the biopolymer material prepared upon crosslinking of collagen with OA, suitable for clinical applications without posing any toxicity to cells.

Acknowledgements All authors thank Department of Biotechnology, Ministry of Science and Technology, New Delhi, for the financial assistance provided in the form of project in vide sanction no. BT/PR10179/AAQ/03/385/2007.

References

1. Usha R, Ramasami T. Effect of crosslinking agents (basic chromium sulfate and formaldehyde) on the thermal and thermomechanical stability of rat tail tendon collagen fibre. Thermochim Acta. 2000;356(1–2):59–66.
2. Sheu M-T, Huang J-C, Yeh G-C, Ho H-O. Characterization of collagen gel solutions and collagen matrices for cell culture. Biomaterials. 2001;22(13):1713–9.
3. Miles CA, Avery NC, Rodin VV, Bailey AJ. The increase in denaturation temperature following cross-linking of collagen is caused by dehydration of the fibres. J Mol Biol. 2005;346(2):551–6.
4. Nam K, Kimura T, Kishida A. Controlling coupling reaction of EDC and NHS for preparation of collagen gels using ethanol/water co-solvents. Macromol Biosci. 2008;8:32–7.
5. Petite H, Rault I, Huc A, Menasche P, Herbage D. Use of the acyl azide method for cross-linking collagen-rich tissues such as pericardium. J Biomed Mater Res. 1990;24(2):179–87.
6. Saito H, Murabayashi S, Mitamura Y, Taguchi T. Characterization of alkali-treated collagen gels prepared by different cross-linkers. J Mater Sci Mater Med. 2008;19:1297–305.
7. Nunes PS, Bezerra MLS, Costa LP, Cardoso JC, Albuquerque RLC Jr, Rodrigues MO, Barin GB, Silva FAD, Jo AASA. Thermal characterization of usnic acid/collagen-based films. J Therm Anal Calorim. 2010;99:1011–4.
8. Mitra T, Sailakshmi G, Gnanamani A, Raja ST, Thiruselvi T, Mangala Gowri V, Selvaraj NV, Ramesh G, Mandal AB. Preparation and characterization of a thermostable and biodegradable biopolymers using natural cross-linker. Int J Biol Macromol. 2011;48(2):276–85.
9. Langmaier F, Mokrejs P, Mladek M. Heat-treated biodegradable films and foils of collagen hydrolysate crosslinked with dialdehyde starch. J Therm Anal Calorim. 2010;102:37–42.
10. Weadock KS, Miller EJ, Bellincampi LD, Zawadsky JP, Dunn MG. Physical crosslinking of collagen fibers: comparison of ultraviolet irradiation and dehydrothermal treatment. J Biomed Mater Res. 1995;29(11):1373–9.
11. Olde Damink LHH, Dijkstra PJ, van Luyn MJA, van Wachem PB, Nieuwenhuis P, Feijen J. Influence of ethylene oxide gas treatment on the in vitro degradation behavior of dermal sheep collagen. J Biomed Mater Res. 1995;29(2):149–55.
12. Melina H, Heuzey MC, Begin A. Viscoelastic properties of phosphoric and oxalic acid-based chitosan hydrogels. Rheol Acta. 2006;45:659–75.
13. Skotak M, Leonov AP, Gustavo L, Sandra N, Anuradha S. Biocompatible and biodegradable ultrafine fibrillar scaffold materials for tissue engineering by facile grafting of L-lactide onto chitosan. Biomacromolecules. 2008;9(7):1902–8.
14. Jaklenec A, Wan E, Murray ME, Mathiowitz E. Novel scaffolds fabricated from protein-loaded microspheres for tissue engineering. Biomaterials. 2008;29(2):185–92.
15. Ghaffari A, Navaee K, Oskoui M, Bayati K, Rafiee-Tehrani M. Preparation and characterization of free mixed-film of pectin/chitosan/Eudragit RS intended for sigmoidal drug delivery. Eur J Pharm Biopharm. 2007;67(1):175–86.
16. Wei X, Sun N, Wu B, Yin C, Wu W. Sigmoidal release of indomethacin from pectin matrix tablets: effect of in situ cross-linking by calcium cations. Int J Pharm. 2006;318(1–2):132–8.
17. Tharanathan RN. Biodegradable films and composite coatings: past, present and future. Trends Food Sci Technol. 2003;14(3):71–7.
18. Weber CJ, Haugaard V, Festersen R, Bertelsen G. Production and applications of biobased packaging materials for the food Industry. Food Addit Contam. 2002;19(4):172–7.
19. Chandrasekaran G, Torchia DA, Piez KA. Preparation of intact monomeric collagen from tail, aorta and skin and the structure of the nonhelical ends in solution. J Biol Chem. 1976;251:6062–7.
20. Lin YK, Liu DC. Comparison of physical–chemical properties of type I collagen from different species. Food Chem. 2006;99:244–51.
21. Pavia DL, Lampman GM, Kriz GS. Introduction to spectroscopy. 3rd ed. USA: Thomson Learning, Inc; 2001.
22. Puett D. DTA and heals of hydration of some polypeptides. Biopolymers. 1967;5(3):327–30.
23. Venugopal MG, Ramshaw JAM, Braswell E, Zhu D, Brodsky B. Electrostatic interactions in collagen-like triple-helical peptides. Biochemistry. 1994;33(25):7948–56.
24. Sacca B, Renner C, Moroder L. The chain register in heterotrimeric collagen peptides affects triple helix stability and folding kinetics. J Mol Biol. 2002;324(2):309–18.
25. Madhan B, Subramanian V, Rao JR, Nair BU, Ramasami T. Stabilization of collagen using plant polyphenol: role of catechin. Int J Biol Macromol. 2005;37(1–2):47–53.