Carbohydrate Polymers 102 (2014) 324–332

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Modification of collagen with a natural derived cross-linker, alginate dialdehyde

Yang Hu[a,b], Lan Liu[a,b], Zhipeng Gu[c], Weihua Dan[a,b], Nianhua Dan[a,b,*], Xixun Yu[c]

[a] Department of Biomass Chemistry and Engineering, Sichuan University, Chengdu, Sichuan 610065, China
[b] Research Center of Biomedical Engineering, Sichuan University, Chengdu, Sichuan 610065, China
[c] College of Polymer Science and Engineering, Sichuan University, Chengdu, Sichuan 610065, China

ARTICLE INFO

Article history:
Received 17 September 2013
Received in revised form 27 October 2013
Accepted 27 November 2013
Available online 4 December 2013

Keywords:
Collagen
Alginate dialdehyde
Modification
Cytocompatibility

ABSTRACT

The interaction between collagen and a natural derived cross-linker alginate dialdehyde (ADA) was investigated. Fourier transform infrared (FTIR) spectroscopy and the circular dichroism (CD) measurements indicate that the structure integrity of collagen is still maintained after the ADA treatment, while the differential scanning calorimetry (DSC) study suggests that ADA could promote collagen–ADA membrane's thermostability compared to pure collagen. And the atomic force microscopy (AFM) of cross-linked collagen reveals a denser network structure. Besides, the water contact angle test indicates that the hydrophilic property of collagen–ADA membrane is promoted, which is favorable for cell's attachment and proliferation. Meanwhile, the cytocompatibility results imply that not only no extra cytotoxicity is introduced into the collagen–ADA membrane after ADA treatment, but also collagen–ADA membrane facilitates cell's proliferation when the content of ADA is less than 20%. In conclusion, our study reveals that ADA stabilizes collagen as a cross-linker and preserves its triple helical structure.

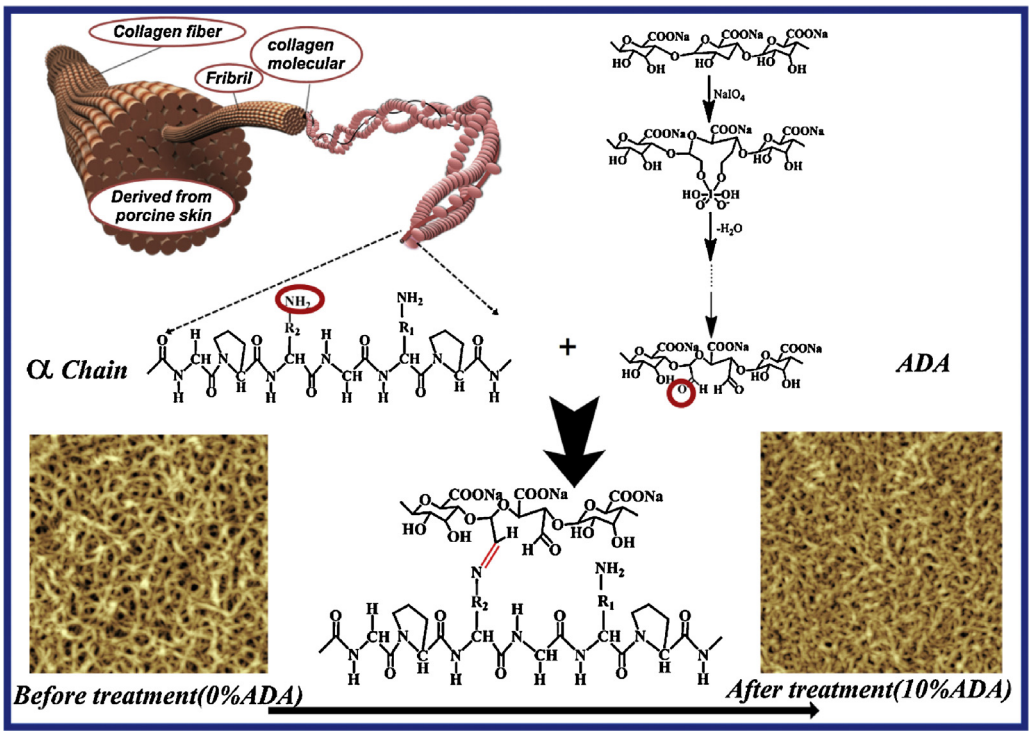
1. Introduction

Collagen is the most abundant protein in mammals, which plays an important role in the formation of tissues and organs, and influences cell expression in many cases (Hu, Liu, Dan, Dan, & Gu, 2013; Hu, Liu, Dan, Dan, Gu, & Yu, 2013; Tian, Chen, Ding, & Li, 2012; Zhang, Liu, & Li, 2009; Zuyderhoff & Dupont-Gillain, 2012). Nowadays, collagen has been explored extensively, in the yield of biomedical materials, such as sponges, membranes, gels and so on (Lungu et al., 2013; Motte & Kaufman, 2013; O'Brien, Haugh, Murphy, McKiernan, & Altenbuchner, 2011; Shepherd et al., 2013; Sundararaghavan, Monteiro, Firestein, & Shreiber, 2009), due to its superior biological properties (Motte & Kaufman, 2013; Safandowska & Pietrucha, 2013; Tian et al., 2012). However, reconstructed collagen-based scaffolds usually suffer from poor physicochemical properties (mechanical strength, thermostability, wettability, resistance to enzyme and so on), which limit their application accordingly. Hence, suitable chemical or physical modifications are necessary to enhance collagen-based scaffolds' corresponding physicochemical properties. Generally, various synthetic cross-linkers, such as glutaraldehyde (GA), hexamethylene-1,6-diaminocarboxysulphonate (HDACS), formaldehyde and carbodiimide (EDC) (Nagai et al., 2010; Powell & Drexler, 2011) have been widely used for the cross-linking of collagen-based scaffolds. Although it has been well reported that these synthetic cross-linkers exhibit superior performance in promoting collagen-based scaffold's corresponding physicochemical properties, the potential cytotoxic effect of these synthetic cross-linkers should not be ignored (Fratzl, 2008, chap. 12; He et al., 2011). Thus, it is necessary to develop naturally occurring cross-linkers for the modification of collagen-based scaffolds.

Alginate (ALG), ubiquitously found in brown algae, is a kind of anionic polysaccharide material, of which the typical structure is a block copolymer composed of two different repeating units, (1,4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers (Fan et al., 2013). Due to its superior biocompatibility, ALG has gained recent interest in the field of wound care and tissue engineering (Chang, Lee, Wu, Yang, & Chien, 2012; Shalumon et al., 2011; Wang et al., 2010). Note that, ALG could be oxidized by periodate and form multiple functional aldehyde groups, which is commonly called alginate dialdehyde (ADA), and this active group could react with the free amino groups in the same way as glutaraldehyde (Xu et al., 2013). Since there are a large amount of free amino groups within collagen molecules, we hypothesis that collagen could be cross-linked by ADA to some extent as is illustrated in Scheme 1. Meanwhile, the effect of ADA on the microstructure of collagen, i.e. from triple helixes to fibrils, remains largely unknown.

* Corresponding author at: Department of Biomass Chemistry and Engineering, Sichuan University, Chengdu, Sichuan 610065, China. Tel.: +86 28 85408988; fax: +86 28 85408988.
E-mail addresses: danweihua_scu@yeah.net (W. Dan), scuhuyang@126.com (N. Dan).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.11.050



Scheme 1. Schematic diagram showing the possible interactions between collagen molecule and alginate dialdehyde.

Thus, it is meaningful to further investigate the interaction between collagen and ADA in this work.

In this context, we have investigated the structure of type I collagen molecules and microfibrils in the presence of ADA by Fourier transform infrared (FTIR) spectroscopy, circular dichroism (CD), differential scanning calorimetry (DSC), scanning electron micrograph (SEM), atomic force microscopy (AFM) and contact angle analyzer. Furthermore, the cytocompatibility of collagen membranes after cross-linking by ADA has been also detected by using methyltetrazolium (MTT) in accordance with international standards (ISO10993-5). And the morphologies of cells seeded on the collagen membranes before and after cross-linking are obtained by confocal laser scanning microscopy (CLSM). Our aim is to further explore collagen–ADA interactions and understand the structure–property relations of ADA modified collagen membranes.

2. Materials and methods

2.1. Materials

The acid soluble collagen used in this study was self-prepared from porcine skin according to our previous report (Hu, Liu, Dan, Dan, & Gu, 2013; Hu, Liu, Dan, Dan, Gu, & Yu, 2013). The alginate dialdehyde (ADA) (the value of oxidation degree is 47.96 ± 2.67% approximately) was prepared according to our previously reported method (Xu et al., 2013). Other agents were used as received.

2.2. Preparation of membranes

Since the relatively limited solubility of collagen in weak acids, 5 mg/mL aqueous collagen solution was prepared by using 0.5 M acetic acid (HAc) as solvent. Subsequently, alginate dialdehyde (ADA) solution with the concentration of 0.1–1 mg/mL was incorporated into the collagen solutions under magnetic stirring. And the final content of ADA to collagen was 0%, 2%, 6%, 10% and 20%, respectively. After the deaeration process under mild ultrasonic (40 kHz, 120 W) for 3 min (He et al., 2011), the collagen–ADA membranes with thickness around 0.05 mm were obtained by casting the solution on a polytetrafluoroethylene (PTFE) plate with a diameter of 10 cm under 0.05 vacuum at 25 °C for 3 days. Specimens were stored in a refrigerated desiccator prior to use.

2.3. FTIR spectra measurements

Fourier transform infrared (FTIR) spectra were obtained from tablets containing 2 mg of collagen–ADA specimens in approximately 100 mg of potassium bromide (KBr) with an FTIR spectrophotometer (Spectrum One, PerkinElmer, Inc., Waltham, MA). The FTIR spectra were recorded in the range of 4000–400 cm^{-1} and at a resolution of 4 cm^{-1}. The spectra plots represented the average of 32 scans. All measurements were carried out in a dry atmosphere at room temperature (Hu, Liu, Dan, Dan, & Gu, 2013; Hu, Liu, Dan, Dan, Gu, & Yu, 2013).

2.4. Cross-link density

As is illustrated in our previous reports (Hu, Liu, Dan, Dan, & Gu, 2013; Hu, Liu, Dan, Dan, Gu, & Yu, 2013; Xu, Li, Yu, Gu, & Zhang, 2012), the amount of free amino groups, after heating with ninhydrin (NHN), is proportional to the optical absorbance of the solution. Hence the ninhydrin assay was used to detect the free-amino group content of collagen–ADA membranes. And the cross-linking density was calculated as the following equation:

$$\text{Cross} - \text{link density}(\%) = \frac{[(NH_{2\,\text{before}}) - (NH_{2\,\text{after}})]}{(NH_{2\,\text{before}})} \times 100 \qquad (1)$$

where $NH_{2\ \text{before}}$ is the amount of free amino groups in the collagen membrane before cross-linking and $NH_{2\ \text{after}}$ is the amount of free amino groups in the sample after cross-linking (Hu, Liu, Dan, Dan, & Gu, 2013; Hu, Liu, Dan, Dan, Gu, & Yu, 2013).

2.5. CD analysis

Collagen solutions (100 μg/mL) with varied content of alginate dialdehyde (ADA) were prepared in a sodium acetate buffer (1 mM, pH 4.0). The final contents of ADA (by dry weight) were 0%, 10% and 20%, respectively. All the solutions were kept at 4 °C overnight and equilibrated at room temperature for 1 h before testing. The CD measurement was performed on a J-810 CD spectropolarimeter (Jasco, J-810, Japan) in a wavelength range from 190 to 260 nm at 25 °C. The molar ellipticity was recorded at a speed of 20 nm/min and each sample was scanned 3 times.

2.6. DSC measurements

The differential scanning calorimetry (DSC) analysis was conducted on Netzsch DSC 200 PC in the temperature ranged from 25 to 180 °C. Approximately, 3 ± 0.1 mg collagen–ADA specimens were loaded in a DSC aluminum pan while an empty aluminum pan was used as the reference. Then the aluminum pan was placed in a Netzsch DSC operating with the Netzsch Proteus analysis software with a scanning rate of 5 °C min^{-1} in a N_2 flow of 60 mL min^{-1} (Hu, Liu, Dan, Dan, & Gu, 2013; Hu, Liu, Dan, Dan, Gu, & Yu, 2013). Thermal denaturation was recorded as the typical peak and two characteristic temperatures were measured corresponding to the peak (temperature of maximum power absorption during denaturation) and onset (temperature at which the tangent to the initial power versus temperature line crosses the baseline) temperature (Zeugolis et al., 2008). Besides the calorimetric enthalpy change (ΔH) was also calculated.

2.7. SEM/AFM observations

A scanning electron microscopy (SEM, Hitachi S3000N, Hitachi, Ltd., Japan) was applied to observe the collagen–ADA membranes' morphology both before and after cross-linking qualitatively (Chen, Yan, Yuan, Zhang, & Fan, 2011; Hu, Liu, Dan, Dan, & Gu, 2013; Hu, Liu, Dan, Dan, Gu, & Yu, 2013). All the specimens were sputter coated with aurum and imaged at accelerating voltage of 5 kV. Meanwhile, the atomic force microscopy (AFM) was used to evaluate the topographic change of collagen fibrils by introducing alginate dialdehyde (ADA). Briefly, collagen was firstly dissolved in 0.5 M acetic acid (HAc) with the concentration of 20 μg/mL. Then, different content of ADA (0%, 2%, 6%, 10%, 20%) was added into the collagen solutions, respectively. After a period of 12 h reaction, 20 μL of the mixture solution was carefully dropped onto a fresh mica substrate and dried overnight at room temperature (21 ± 1 °C). After that, the mica substrate was observed by atomic force microscope (AFM, SHIMADZU, SPM-9600, Japan) at room temperature in tapping mode.

2.8. Water contact angle tests

The hydrophilicity of the membrane was tested with a goniometer (dataphysics OCA-H200, Germany) by measuring the surface water contact angle (WCA) at room temperature (21 ± 1 °C) (Dettre and Johnson, 1965). In brief, 5 μL per drop of distilled water was carefully deposited onto the surface of the membrane, angles were measured on five different regions of each surface and averaged.

2.9. Cell culture and analysis

The cytocompatibility of collagen–ADA membranes were assessed in vitro using L929 fibroblasts with the method of colorimetric assay, as was described in our previous work (Hu, Liu, Dan, Dan, & Gu, 2013; Hu, Liu, Dan, Dan, Gu, & Yu, 2013; Qiu, Zhao, Wan, Zhao, & Chen, 2006). In brief, the collagen–ADA membranes were firstly cut into small pieces (1 cm × 1 cm) and sterilized by ethylene oxide vapor. Then specimens were placed into 24-well culture plate and cultured with cells with the concentration of 1×10^4 cells/well in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% (v/v) antibiotic solution at 37 °C for 1, 3 and 5 days. And the medium was changed every two days. At each time point, 20 μL 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide (MTT) was added into the culture plate and cultured at 37 °C for 4 h to form formazan crystals. Subsequently, 1.5 mL dimethylsulfoxide was added into the culture to dissolve the formazan crystals with stirring at room temperature. Then the optical density (OD) at 492 nm was measured with a microplate reader (Model 550, Bio Rad Corp. USA). The values were expressed as mean ± standard deviation ($n = 5$).

Meanwhile, confocal laser scanning microscopy (CLSM) was applied to further characterize the cell's growth on collagen–ADA membranes (Shalumon et al., 2011). Briefly, the cells were seeded into 24-well culture plates at a density of 2.5×10^4 cells/well, together with collagen–ADA membranes for 3 day. Then the samples were washed slightly with phosphate buffer solution (PBS, pH 7.4) and fixed by 4% paraformaldehyde in PBS for 30 min at room temperature. At the second step, the samples were permeabilized with PBS containing Triton X-100 for 10 min at 4 °C. After that, the samples were stained with 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI) in PBS for 10 min at 4 °C. Subsequently, the samples were washed twice and mounted under glass coverslips using glycerol/PBS. Finally, the fluorescent images were observed using a confocal laser scanning microscope (Leica TCS SP II, Leica).

2.10. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) on SPSS 10.0. Corresponding P-values less than 0.05 were considered significant.

3. Results and discussion

3.1. FTIR analysis

Fourier transform infrared (FTIR) spectra were applied to investigate the structure of collagen in the presence of ADA considering that the FTIR spectra of protein molecules can be correlated directly to their backbone conformation (Song et al., 2012). Fig. 1 shows the spectra of pure collagen and collagen–ADA complex. As is reported in the literatures (Andrews, Murali, Muralidharan, Madhulata, & Jayakumar, 2003), pure collagen with intact triple helix conformation can be characterized by its feature amide bands in IR spectra. The amide A and B bands are mainly associated with the stretching vibrations of N—H groups, which are centered at 3350 cm^{-1} and 3082 cm^{-1}, respectively (Hu et al., 2013a,b). The amide I band at 1650 cm^{-1} is assigned to the stretching vibrations of peptide C=O groups, while the amide II band at 1550 cm^{-1} is dominantly attributed to the N—H bending vibrations coupled to C—N stretching vibrations. Besides the amide III band at 1240 cm^{-1} mainly arises from the C—N stretching, N—H bending vibrations and wagging vibrations of CH_2 groups in the glycine backbone and proline side chains (Andrews et al., 2003; He et al., 2011). Fig. 1 suggests that the backbone of collagen in the presence of ADA does not change along with the increasing of ADA content, because the positions of main amide bands still maintain, especially the amide I band correlated to the helix structure of collagen (Andrews et al., 2003). Just the amide A and B bands become sharper and shifted to higher wavenumber, correlated to the broken of hydrogen bands in the side chains of collagen (Balakrishnan, Mohanty, Umashankar, & Jayakrishnan, 2005; He et al., 2011; Tian et al., 2012). Some researchers also reported

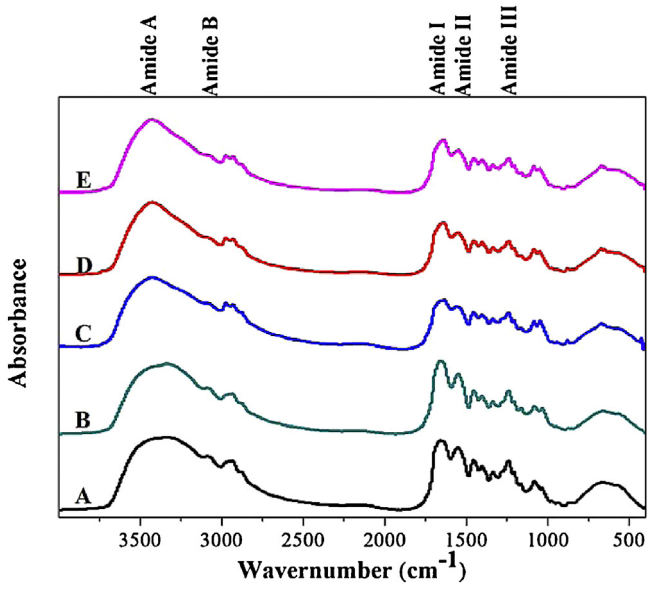


Fig. 1. FTIR spectra of collagen–ADA membranes with different ADA contents (A) 0%, (B) 2%, (C) 6%, (D) 10% and (E) 20%. FTIR absorption ratios of A_{III}/A_{1450} for collagen–ADA membranes (A, B, C, D and E) are 1.00, 0.99, 0.99, 0.98 and 0.98, respectively.

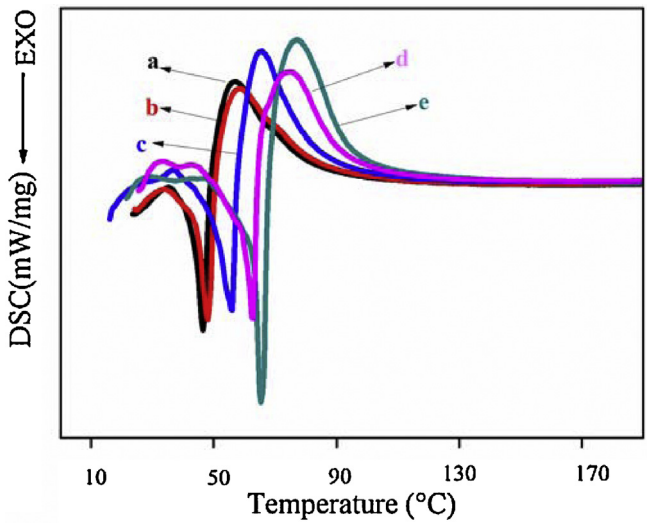


Fig. 3. DSC curves of collagen–ADA membranes with different ADA contents (a) 0%, (b) 2%, (c) 6%, (d) 10% and (e) 20%.

that protein could react with ADA between NH_2 and aldehyde group to form stable Schiff's base, where the corresponding hydrogen bands are replaced (Xu et al., 2012).

Meanwhile, since the absorbance peak at 1235 cm^{-1} is affected by changes in the triple helical structure of collagen and can therefore be used to quantify the denaturation of a collagen sample (O'Brien, Haugh, & Jaasma, 2009), the absorption ratios of Amide III to 1450 cm^{-1}, denoted as A_{III}/A_{1450} are also considered as a measurement of preservation of integrity of collagen–ADA membranes. Note that the ratio of the denatured collagen, gelatin is just about 0.6 according to the reports (Andrews et al., 2003; Hu et al., 2013a,b). The result of A_{III}/A_{1450} indicates that the triple helix conformation is not destructed by ADA, since the value of the ratios just decrease slightly from 1.0 for pure collagen to 0.98 for collagen–ADA membranes at most. It is acceptable that the integrity of collagen is the foundation for maintaining its superior biological properties. Here ADA acts as a cross-linker to firm collagen's triple structure but without destroying the backbone of collagen molecules, as is illustrated in Scheme 1.

3.2. CD analysis

To further determine whether the structure integrity of collagen in the presence of ADA was destroyed or not, circular dichroism (CD) measurements of collagen–ADA solutions with certain content were performed, as is shown in Fig. 2. Generally, the pure collagen exhibits two typical spectra in the range of 190–260 nm (the negative band is at ~198 nm and the positive band is at ~221 nm, respectively) (Fratzl, 2008; Hu, Liu, Dan, Dan, & Gu, 2013; Hu, Liu, Dan, Dan, Gu, & Yu, 2013). When the structure integrity or triple helix of collagen is completely destroyed, the spectrum at ~198 nm in CD measurement will shift into lower wavelength, and the spectrum at ~221 nm will disappear, which indicates the characteristics of a collagen triple helix. Further, partially denatured collagen exhibits a spectrum with lower intensity, especially at ~221 nm, as well as a lower value of R_{pn}, the ratio of the positive peak intensity to the negative peak intensity (He et al., 2012). On the contrary, the increase of R_{pn} indicates the aggregation of collagen molecules. Fig. 2 shows the changes of spectra of collagen–ADA solutions along with the content of ADA. The addition of ADA did not alter the CD spectra of collagen solutions significantly, indicating the interactions between collagen and ADA may occur dominantly on the approachable collagen molecular surface, and hence the backbone structure of collagen triple helix was still maintained after cross-linking. At a very high content of ADA (20%), the value of R_{pn} exhibits a slightly negligible increase (from 0.14 to 0.15) suggesting the aggregation of collagen molecules. As is described before, the aldehyde group within ADA could react with the amino group in collagen side chains to form more stable Schiff's base, whereas the collagen fibrillar could aggregate as is shown in Fig. 2, thus inducing the slightly increasing of R_{pn} value. Hence, we would like to draw the conclusion that the most maintained triple helix structure implies that ADA at certain content does not destroy the structure integrity of collagen, but stabilizes the collagen molecules

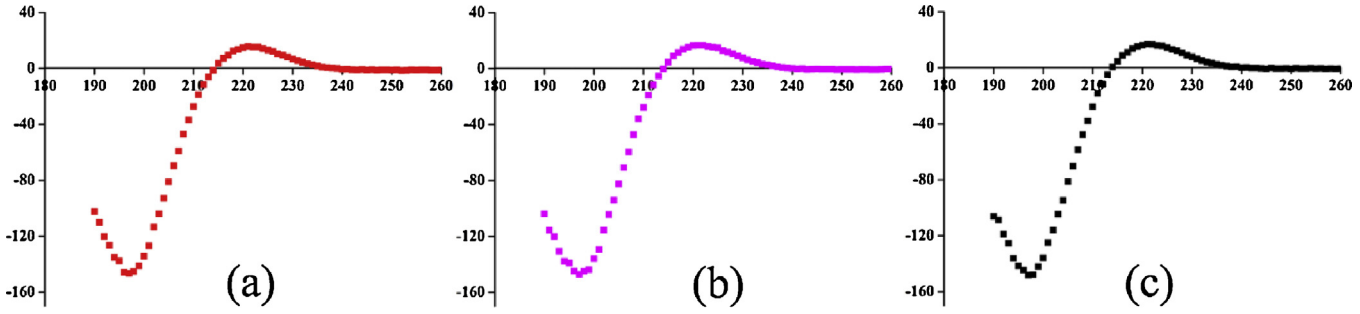


Fig. 2. CD spectra of the collagen solutions with different ADA contents (a) 0%, (b) 10% and (c) 20%.

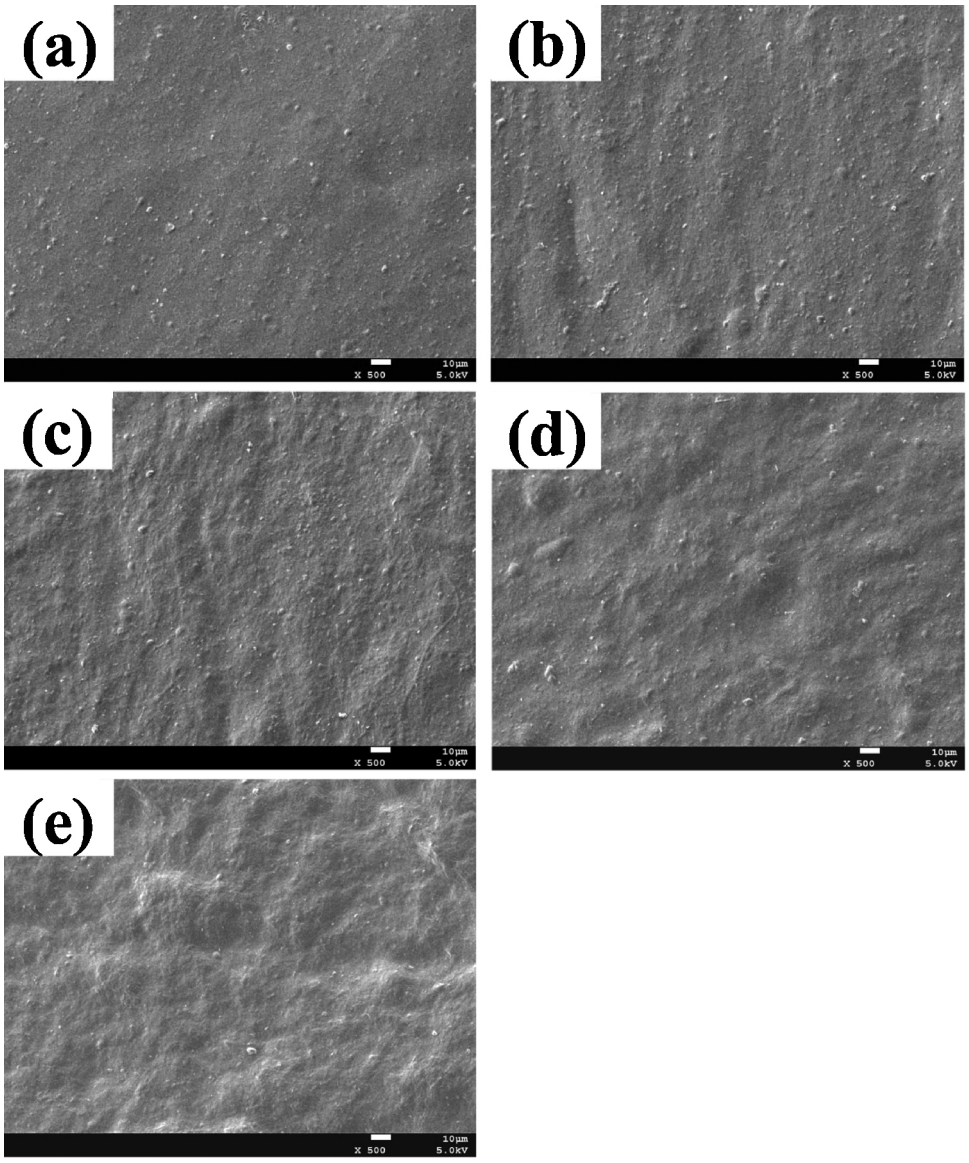


Fig. 4. SEM images of collagen–ADA membranes with different ADA contents (a) 0%, (b) 2%, (c) 6%, (d) 10% and (e) 20%.

by more stable bonds, which coincide with those reports for collagen molecules modified with aldehydes (Kim, Yaszemski, & Lu, 2009).

3.3. DSC measurement

Fig. 3 provides the thermal stability of collagen in the presence of alginate dialdehyde (ADA). When collagen–ADA membrane is heated, the helix-coil transition takes place, during which the triple helix melts and progressively dissociates into the three randomly coiled peptide α-chains (gelatin) (Zeugolis et al., 2008). Note that the endothermic peak is associated with the transformation from triple helical to random coil of collagen. It can be observed that the collagen–ADA membrane in the presence of ADA shows higher denaturation temperature (T_d) along with the increasing content of ADA to collagen. And the T_d is promoted by 20 °C approximately, when the content of ADA increases from 0% to 20%. Meanwhile, it is worthy to note that the calorimetric enthalpy (ΔH) of the transition denotes the energy required for the destruction of the hydrogen bonds, amido bonds and Van der Waals forces which preserve the collagen triple helixes. As is shown in the supplementary information (Table S1), the value of ΔH for collagen–ADA membrane at the ADA content of 20% reaches up to 1493.5 J/g, which is much higher than that for collagen–ADA membrane at the ADA content of 0% (about 787.4 J/g). The possible reason may be that ADA could react with the amino group (NH_2) of collagen side chains, as is revealed in the supplement data (Table S2), which could further form more stable Schiff's base as mentioned above. More to the point, the reaction is predominantly occurred between the ε-amino groups of lysine or hydroxylysine side groups of collagen and the available aldehyde (Balakrishnan et al., 2005). Besides, in this work, excessive content of ADA (20%) did not result in an apparently shift to higher temperature. When the content of ADA increased from 10% to 20%, the T_d and ΔH of corresponding collagen–ADA membranes were only promoted by 2.3 °C and 100.8 J/g, approximately. Here, it should be noticed that the improvement in T_d is favorable for maintaining the structure integrity of collagen which is crucial for the fabrication of collagen-based products (Fratzl, 2008).

3.4. SEM analysis

Considering that miscibility in a polymer membrane is crucial to specific interactions between the polymeric components (Dan et al., 2007), collagen–ADA membranes were observed by SEM

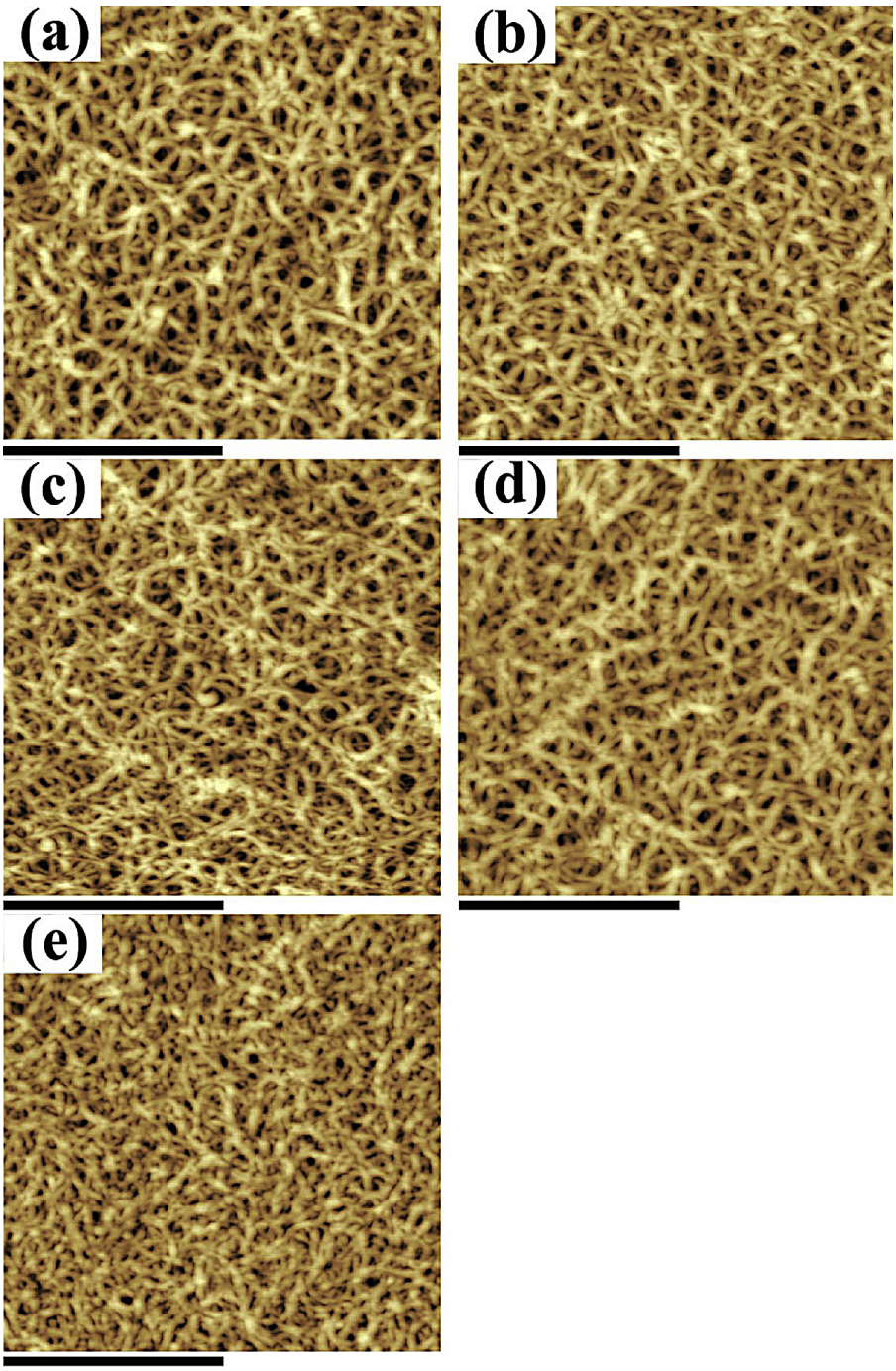


Fig. 5. AFM images of collagen on mica substrate in the presence of ADA. The contents of ADA are (a) 0%, (b) 2%, (c) 6%, (d) 10% and (e) 20%, respectively. (The scale bar is 500nm).

(Fig. 4). The structure present homogeneous macrostructures characterized by a relatively rough surface, whereas no apparent phase separation was detected (Silva et al., 2012), indicating the good miscibility between the two components (collagen and ADA). Also, the micro-roughness of collagen–ADA membrane at 20% of the cross-linker was observed to be promoted to some extent, which may be associated with the drastic interaction between collagen and ADA. And this phenomenon was in consistent with that of AFM images to a certain degree.

3.5. AFM analysis

To further explore the aggregation of collagen molecule in the presence of alginate dialdehyde (ADA), the corresponding morphological changes were observed by atomic force morphology (AFM). Fig. 5(a) shows the typical fibrillar structure of type I collagen, many curved molecules or microfibrils lie on the mica substrate and overlap with one another, which is similar to the previous report (Li, Mu, Cai, & Lin, 2009). The similar aggregation of collagen at 2%, 6%,

10% and 20% ADA was also observed, indicating that the presence of ADA does not destroy the packing structure of native collagen fibril. However, it is worthy to note that the collagen molecular or fibrillar seems to be crowded more intensively and exhibits meshy fibrous network topographies in Fig. 5(b–e) owing to the cross-linking effect induced by ADA, supporting the result of DSC analysis and the schematic illustration (Scheme 1).

3.6. Water contact angle tests

To explore the correlation between the microstructure of ADA cross-linked collagen and its surface property, water contact angles (WCA) on collagen–ADA membranes are given in Fig. 6 as a function of the ADA content. As is reported in the literatures (Dettre and Johnson, 1965; Fratzl, 2008; He et al., 2011), the value of WCA of scaffolds could reflect the hydrophobic-hydrophilic properties of scaffolds toward given liquid. Generally, a high value of WCA means more hydrophobic, whereas a low value of WCA always indicates more hydrophilic. It is obvious that the value of WCA decreases along with the increasing of ADA content, from ~90° for pure collagen membrane to ~70° for collagen–ADA membrane at the 20% cross-linker, respectively. The possible reason could be divided into two parts. On one hand, alginate dialdehyde, the derivative of alginate, has been performed the procedure of depolymerization of alginate to some degree, which is associated with the periodate oxidation to cleave the vicinal glycols in polysaccharides to form their dialdehyde derivatives. Then, the solubility of alginate dialdehyde (ADA) in water was found to be increasing after oxidation (Balakrishnan et al., 2005). Hence, the

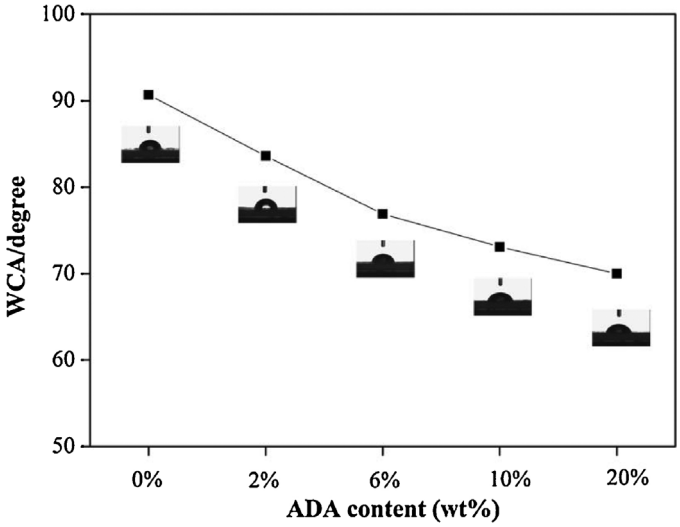


Fig. 6. Water contact angle (WCA) of collagen–ADA membranes. The symbols denote experimental points and solid line just serves to guide the eye.

value of WCA of collagen–ADA membranes was decreased by introduction of ADA, which means the hydrophilic of collagen–ADA membrane is promoted accordingly. On the other hand, the reaction between collagen and ADA is associated with the two groups (the ε-amino groups and available aldehyde) as mentioned above. Meanwhile, a certain amount of hydroxyl groups within ADA is brought into collagen side chains, which may further improve the hydrophilic of collagen–ADA membranes. Besides, it is worthy to

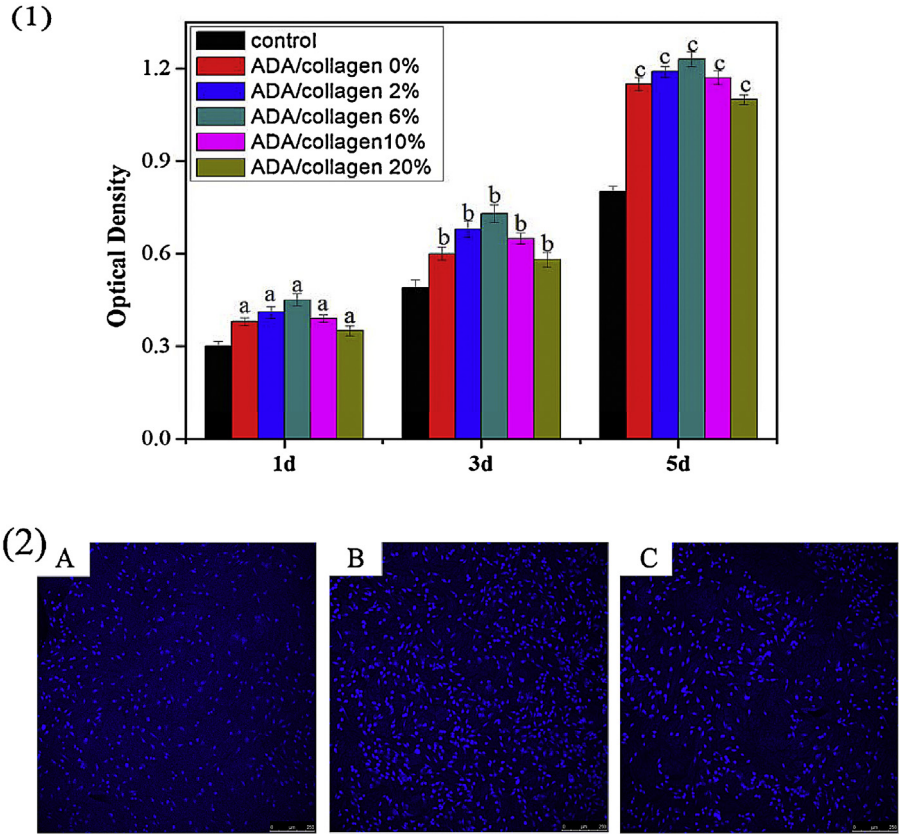


Fig. 7. (1) Proliferation of L929 fibroblasts cultured on the membranes ($n = 5$; asterisk (a–c) denotes the difference attained a statistically significant difference compared to the control group at different time period). (2) Nuclear staining images by DAPI using L929 fibroblasts on collagen–ADA membranes with different ADA content (a) 0%, (b) 10% and (c) 20%.

note that suitable hydrophilic property of collagen-based scaffolds is favorable for cell's migration, attachment and proliferation (Fratzl, 2008).

3.7. Cytocompatibility analysis

Since the biocompatibility of scaffolds is crucial for the applications in tissue engineering, to reconfirm the non-toxicity of collagen–ADA membranes, a preliminary cytotoxicity evaluation using human fibroblasts (L929) was performed in vitro. Fig. 7 illustrates the effect of varying contents of ADA on the proliferation of L929 fibroblasts. Neither the collagen–ADA membranes nor their extracts induced any morphological changes to the cells confirming their non-toxic nature compared to control groups (Fig. 7). Further, quantitative assessment of the cytotoxicity by MTT assay of fibroblasts after contact with the membranes all showed higher optical density than that of control groups at different time intervals (1d, 3d and 5d). On one hand, collagen is favorable to fibroblasts due to its specific biological properties, that is why the pure collagen membrane group showed higher optical density than control group. On the other hand, as was demonstrated in our previous work (Xu et al., 2012), ADA at certain content could promote the fibroblasts' proliferation and growth to some extent. Moreover, it is worthy to note that the optical density of collagen–ADA membrane decreased slightly when the content of ADA is larger than 10%. The possible reason may be that excessive unreacted ADA with free aldehyde group could react with proteins or polysaccharides on and inside the fibroblasts (Xu et al., 2012, 2013), hence it is important to clear away excessive ADA after the cross-linking process. However, collagen–ADA complex with certain ADA contents did not induce any extra cytotoxicity into this kind of scaffold.

4. Conclusion

In summary, we demonstrate the feasibility of ADA as a novel natural derived cross-linker in the fixation of collagen. Especially, this work focuses on the structure integrity of collagen before and after cross-linking process. The results suggest that the introduction of ADA could promote collagen-based scaffold's thermostability and hydrophilic property, which is favorable in the field of tissue engineering. Note that the ADA treatment does not destroy the triple helix conformation of collagen but result in the aggregation of collagen molecules. Meanwhile, the cytocompatibility studies imply that collagen–ADA membrane could improve cell's proliferation at a certain content of ADA, compared to the pure collagen group. Taken as a whole, these results may pave the way for developing new cross-linkers for collagen biomaterials.

Acknowledgement

The financial support of National Natural Science Foundation of China (contract grant number 21276164), Science and Technology Planning Project of China (contract grant number 2011BAC06B11) and the International Scientific and Technological Cooperation Project (contract grant number SH201108) are gratefully acknowledged.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.11.050.

References

Andrews, M. E., Murali, J., Muralidharan, C., Madhulata, W., & Jayakumar, R. (2003). Interaction of collagen with corilagin. *Colloid & Polymer Science*, *281*(8), 766–770.
Balakrishnan, B., Mohanty, M., Umashankar, P. R., & Jayakrishnan, A. (2005). Evaluation of an in situ forming hydrogel wound dressing based on oxidized alginate and gelatin. *Biomaterials*, *26*(32), 6335–6342.
Chang, J.-J., Lee, Y.-H., Wu, M.-H., Yang, M.-C., & Chien, C.-T. (2012). Preparation of electrospun alginate fibers with chitosan sheath. *Carbohydrate Polymers*, *87*(3), 2357–2361.
Chen, Y., Yan, L. D., Yuan, T., Zhang, Q. Y., & Fan, H. J. (2011). Asymmetric polyurethane membrane with in situ-generated nano-TiO(2) as wound dressing. *Journal of Applied Polymer Science*, *119*(3), 1532–1541.
Dan, W. H., Ye, Y. C., Zeng, R., Lin, H., Dan, N. H., Guan, L. B., et al. (2007). Miscibility studies on the blends of collagen/chitosan by dilute solution viscometry. *European Polymer Journal*, *43*(5), 2066–2071.
Dettre, R. H., & Johnson, R. E., Jr. (1965). Contact angle hysteresis. IV. Contact angle measurements on heterogeneous surfaces. *The Journal of Physical Chemistry*, *69*(5), 1507–1515.
Fan, L., Cao, M., Gao, S., Wang, T., Wu, H., Peng, M., et al. (2013). Preparation and characterization of sodium alginate modified with collagen peptides. *Carbohydrate Polymers*, *93*(2), 380–385.
Fratzl, P. (2008). *Collagen: Structure and mechanics*. New York: Springer Science + Business Media.
He, L., Cai, S., Wu, B., Mu, C., Zhang, G., & Lin, W. (2012). Trivalent chromium and aluminum affect the thermostability and conformation of collagen very differently. *Journal of Inorganic Biochemistry*, *117*, 124–130.
He, L., Mu, C., Shi, J., Zhang, Q., Shi, B., & Lin, W. (2011). Modification of collagen with a natural cross-linker, procyanidin. *International Journal of Biological Macromolecules*, *48*(2), 354–359.
Hu, Y., Liu, L., Dan, W., Dan, N., & Gu, Z. (2013). Evaluation of 1-ethyl-3-methylimidazolium acetate based ionic liquid systems as a suitable solvent for collagen. *Journal of Applied Polymer Science*, *130*(4), 2245–2256.
Hu, Y., Liu, L., Dan, W., Dan, N., Gu, Z., & Yu, X. (2013). Synergistic effect of carbodiimide and dehydrothermal crosslinking on acellular dermal matrix. *International Journal of Biological Macromolecules*, *55C*, 221–230.
Kim, J., Yaszemski, M. J., & Lu, L. (2009). Three-dimensional porous biodegradable polymeric scaffolds fabricated with biodegradable hydrogel porogens. *Tissue Engineering Part C: Methods*, *15*(4), 583–594.
Li, D., Mu, C., Cai, S., & Lin, W. (2009). Ultrasonic irradiation in the enzymatic extraction of collagen. *Ultrasonics Sonochemistry*, *16*(5), 605–609.
Lungu, A., Albu, M. G., Stancu, I. C., Florea, N. M., Vasile, E., & Iovu, H. (2013). Superporous collagen-sericin scaffolds. *Journal of Applied Polymer Science*, *127*(3), 2269–2279.
Motte, S., & Kaufman, L. J. (2013). Strain stiffening in collagen I networks. *Biopolymers*, *99*(1), 35–46.
Nagai, N., Kumasaka, N., Kawashima, T., Kaji, H., Nishizawa, M., & Abe, T. (2010). Preparation and characterization of collagen microspheres for sustained release of VEGF. *Journal of Materials Science: Materials in Medicine*, *21*(6), 1891–1898.
O'Brien, F. J., Haugh, M. G., & Jaasma, M. J. (2009). The effect of dehydrothermal treatment on the mechanical and structural properties of collagen-GAG scaffolds. *Journal of Biomedical Materials Research Part A*, *89A*(2), 363–369.
O'Brien, F. J., Haugh, M. G., Murphy, C. M., McKiernan, R. C., & Altenbuchner, C. (2011). Crosslinking and mechanical properties significantly influence cell attachment, proliferation, and migration within collagen glycosaminoglycan scaffolds. *Tissue Engineering Part A*, *17*(9/10), 1201–1208.
Powell, H. M., & Drexler, J. W. (2011). Dehydrothermal crosslinking of electrospun collagen. *Tissue Engineering Part C: Methods*, *17*(1), 9–17.
Qiu, K., Zhao, X. J., Wan, C. X., Zhao, C. S., & Chen, Y. W. (2006). Effect of strontium ions on the growth of ROS17/2.8 cells on porous calcium polyphosphate scaffolds. *Biomaterials*, *27*(8), 1277–1286.
Safandowska, M., & Pietrucha, K. (2013). Effect of fish collagen modification on its thermal and rheological properties. *International Journal of Biological Macromolecules*, *53*, 32–37.
Shalumon, K. T., Anulekha, K. H., Nair, S. V., Nair, S. V., Chennazhi, K. P., & Jayakumar, R. (2011). Sodium alginate/poly(vinyl alcohol)/nano ZnO composite nanofibers for antibacterial wound dressings. *International Journal of Biological Macromolecules*, *49*(3), 247–254.
Shepherd, J. H., Ghose, S., Kew, S. J., Moavenian, A., Best, S. M., & Cameron, R. E. (2013). Effect of fiber crosslinking on collagen-fiber reinforced collagen-chondroitin-6-sulfate materials for regenerating load-bearing soft tissues. *Journal of Biomedical Materials Research Part A*, *101A*(1), 176–184.
Silva, S. S., Santos, T. C., Cerqueira, M. T., Marques, A. P., Reys, L. L., Silva, T. H., et al. (2012). The use of ionic liquids in the processing of chitosan/silk hydrogels for biomedical applications. *Green Chemistry*, *14*(5), 1463–1470.
Song, W., Markel, D. C., Wang, S., Shi, T., Mao, G., & Ren, W. (2012). Electrospun polyvinyl alcohol–collagen–hydroxyapatite nanofibers: A biomimetic extracellular matrix for osteoblastic cells. *Nanotechnology*, *23*(11), 115101.
Sundararaghavan, H. G., Monteiro, G. A., Firestein, B. L., & Shreiber, D. I. (2009). Neurite growth in 3D collagen gels with gradients of mechanical properties. *Biotechnology and Bioengineering*, *102*(2), 632–643.
Tian, H., Chen, Y., Ding, C., & Li, G. (2012). Interaction study in homogeneous collagen/chondroitin sulfate blends by two-dimensional infrared spectroscopy. *Carbohydrate Polymers*, *89*(2), 542–550.

Wang, J., Fu, W., Zhang, D., Yu, X., Li, J., & Wan, C. (2010). Evaluation of novel alginate dialdehyde cross-linked chitosan/calcium polyphosphate composite scaffolds for meniscus tissue engineering. *Carbohydrate Polymers*, *79*(3), 705–710.

Xu, Y., Huang, C., Li, L., Yu, X., Wang, X., Peng, H., et al. (2013). In vitro enzymatic degradation of a biological tissue fixed by alginate dialdehyde. *Carbohydrate Polymers*, *95*(1), 148–154.

Xu, Y., Li, L., Yu, X., Gu, Z., & Zhang, X. (2012). Feasibility study of a novel crosslinking reagent (alginate dialdehyde) for biological tissue fixation. *Carbohydrate Polymers*, *87*(2), 1589–1595.

Zeugolis, D. I., Khew, S. T., Yew, E. S., Ekaputra, A. K., Tong, Y. W., Yung, L. Y., et al. (2008). Electro-spinning of pure collagen nano-fibres – just an expensive way to make gelatin? *Biomaterials*, *29*(15), 2293–2305.

Zhang, M., Liu, W., & Li, G. (2009). Isolation and characterisation of collagens from the skin of largefin longbarbel catfish (*Mystus macropterus*). *Food Chemistry*, *115*(3), 826–831.

Zuyderhoff, E. M., & Dupont-Gillain, C. C. (2012). Nano-organized collagen layers obtained by adsorption on phase-separated polymer thin films. *Langmuir*, *28*(4), 2007–2014.