

The effects of different cross-linking conditions of genipin on type I collagen scaffolds: an in vitro evaluation

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Abstract The purpose of this paper is to analyze the properties of fabricating rat tail type I collagen scaffolds cross-linked with genipin under different conditions. The porous genipin cross-linked scaffolds are obtained through a two step freeze-drying process. To find out the optimal cross-link condition, we used different genipin concentrations and various cross-linked temperatures to prepare the scaffolds in this study. The morphologies of the scaffolds were characterized by scanning electron microscope, and the mechanical properties of the scaffolds were evaluated under dynamic compression. Additionally, the cross-linking degree was assessed by ninhydrin assay. To investigate the swelling ratio and the in vitro degradation of the collagen scaffold, the tests were also carried out by immersion of the scaffolds in a PBS solution or digestion in a type I collagenase respectively. The morphologies of the non-cross-linked scaffolds presented a lattice-like structure while the cross-linked ones displayed a sheet-like framework. The morphology of the genipin cross-linked scaffolds could be significantly changed by either increasing genipin concentration or the temperature. The

swelling ratio of each cross-linked scaffold was much lower than that of the control (non-cross-linked). The ninhydrin assay demonstrated that the higher temperature and genipin concentration could obviously increase the cross-linking efficiency. The in vitro degradation studies indicated that genipin cross-linking can effectively elevate the biostability of the scaffolds. The biocompatibility and cytotoxicity of the scaffolds was evaluated by culturing rat chondrocytes on the scaffold in vitro and by MTT. The results of MTT and the fact that the chondrocytes adhered well to the scaffolds demonstrated that genipin cross-linked scaffolds possessed an excellent biocompatibility and low cytotoxicity. Based on these results, 0.3 % genipin concentrations and 37 °C cross-linked temperatures are recommended.

Keywords Cross-link · Genipin · Type I collagen · Scaffolds

Introduction

Collagen is one of the most important and abundant extracellular matrix proteins present in the mammalian body. It plays a considerable role in many supporting and connecting tissues. To date, it has been used for biomaterial applications in a wide variety of forms, mainly as 3D hydrogels, foams, PEM films and collagen-based 2D films or 3D scaffolds (Kopecek 2007; Zhang et al. 2005; Chaubaroux et al.

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2012; Bi et al. 2011). Until now, about 28 genetically distinct forms have been known to us. Among these, type I collagen obtains the most attention due to its abundance, excellent biocompatibility, and ubiquity. It mainly originates in bone, ligaments and tendons, skin, etc. Collagen and/or collagen-based gel, films, and scaffolds prepared from a commercially available collagen solution have been widely used as biomaterials in tissue engineering, drug delivery, and wound healing. However, many challenges have emerged for the researchers and clinicians. For example, most applications of these scaffolds require them to undergo physically or pathologically mechanical stresses from the body. Therefore it is crucial that being able to maintain and control the structural–functional integrity. Additionally, the ideal scaffolds used for tissue engineering must not only have high porosity with a large surface area/volume ratio to provide a greater space for cells adhesion, proliferation, and allow the exchange of nutrition and waste, but they must also have low cytotoxicity, distinguished biocompatibility, and biodegradability. But the degradation of collagen scaffolds *in vivo* cannot be controlled as well, especially due to the presence of collagenase *in vivo*. Collagen molecules have a triple-helical structure with three extended protein chains wrapping around one another and the presence of 4-hydroxyproline resulting from a post-translational modification of peptide-bound prolyl residues provides a distinctive marker for these molecules (Grant 2007). It is possible that these drawbacks can be overcome by covalently cross-linked collagen fibrils within and between the constituent collagen molecules.

To our knowledge, there are numerous cross-linking methods to obtain a relatively stable collagen construction. Irradiation with ultraviolet (UV) light has been used to cross-link collagen due to its safety and simplicity (Cornwell et al. 2007; Dong et al. 2005; Menter et al. 2001), but the potential of UV-mediated DNA degradation limited the usage of cross-linking cellular tissues and tissue equivalents. Besides this, UV cross-linking also caused degradation of collagen fibers which influenced attachment, migration, and proliferation of cells populating the fibers (Weadock et al. 1995). Dehydrothermal, another physical method, is used to cross-link extruded collagen fibers and increase their wet ultimate tensile strength (Weadock et al. 1995). But it has limited use in load-bearing implants due

to the potential decrease the denaturation temperature of DHT cross-linked fibers significantly (Weadock et al. 1996). In light of the low cross-linking degree yielded by these physical methods, seeking a safe and effective chemical treatment is still the dominant priority. Although a great deal of chemical agents such as formaldehyde, glutaraldehyde, and epoxy compound have been used in the cross-linking of collagen scaffolds (Koide and Daito 1997; Weadock et al. 1983–1984; Vizárová et al. 1995), the greatest concern is cytotoxicity.

In recent years, many researchers have paid more attention to the plant-derived agents such as hesperidin, proanthocyanidin, epigallocatechin gallate, and genipin. Most of them are mainly used in dental tissue to stabilize the collagen matrix (Bedran-Russo et al. 2011; Castellan et al. 2010; Kato et al. 2010; Hiraishi et al. 2011). However, the genipin, a compound extracted from the Gardenia fruit (Chiono et al. 2008), is a versatile agent and has been employed to cross-link gelatin (Liang et al. 2003; Chen et al. 2005), chitosan (Kuo and Lin 2006), and collagen or collagen-based biomedical scaffolds (Bi et al. 2011; Mathew et al. 2013; Yan et al. 2010). In addition to that, genipin can spontaneously react with silk fibroin and silk sericin (Yoo et al. 2011). In a recent study (Aramwit et al. 2013), the authors fabricated genipin cross-linked sericin/PVA scaffolds to assess the effect of healing full-thickness wounds and gained satisfactory results. Most important is the reason that genipin is superior to other chemical cross-linking reagents is its low cytotoxicity. The enhanced mechanical strength and resistance against *in vitro* enzymatic degradation is the result of genipin cross-linking. It is comparable to the glutaraldehyde, and while it shows some cytotoxic, it is about 5,000–10,000 times less toxic (Sung et al. 1998; Cauich-Rodriguez et al. 1996). Interestingly, genipin also possesses sporicidal properties (Reich and Akkus 2013; Reich et al. 2013), which may be an additional benefit to the fabrication of biomedical scaffolds.

Although the application of genipin as a cross-linking reagent for natural or artificial scaffolds seems prospective, a possibility has not been established as properties of scaffolds can be greatly influenced by different cross-linking conditions such as genipin concentrations and solvents, cross-linking temperatures and times, PH value, etc. In a recent study, the authors demonstrated that the reaction of genipin

cross-linking could be promoted by dense CO₂ (Fernandes-Silva et al. 2013). In the present study, we evaluated the effects of two variables, genipin concentrations and cross-linking temperatures, on the properties of type I collagen scaffolds. To find the optimal cross-linking conditions, the morphology of the scaffolds, swelling ratio, in vitro degradation rate, mechanical strength, the cross-linking degree and the biocompatibility of these scaffolds were investigated and compared.

Materials and methods

Collagen extraction and scaffold preparation

SD rat tail tendons were first immersed in 0.1 % benzalkonium bromide solutions for 15 min and cut in small pieces. About 20 g of this materials mixed with 0.05 M Tris/HCl and 1 M NaCl. The mixture was placed in 4 °C refrigerator and stirred regularly for 72 h. Supernatants and impurities were removed before centrifugation, and the residue was washed with distilled water (three times) after centrifugation. Washed residue was stirred with 0.5 M acetic acid for 72 h. After this acetic solubilization step, the mixture was centrifuged at 4 °C and 10,000g for 25 min. The supernatant was purified through salt precipitation and dialysis against distilled water for 2 days. Dialyzed collagen extract was freeze-dried into a sponge and stored frozen until further use. Pre-scaffolds were produced by dissolving the collagen sponge in 0.5 M acetic acid preparing solutions with collagen concentrations 8 mg/ml. The solution was stirred overnight, filtered, and neutralized using 5 M NaOH solutions. When the PH values of the solutions were about 7.0, they were transferred into 48-well microplates (800 µl/well) and freeze-dried. A slight amount of solution was left for SDS-PAGE analysis.

Sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE)

We prepared the collagen solution for the sodium dodecylsulfate/polyacrylamide gel electrophoresis analysis. The standard substance of rat tail type I collagen (Sigma, USA) was used for control. The electrophoresis buffer was prepared by adding 3 g of Tris, 14.4 g of glycine and 1 g of SDS to 1 l of

Table 1 Cross-linking conditions

Group	Cross-linking concentration	Temperature	Cross-linking time
A	0.1 %	4 °C	24 h
B	0.3 %	4 °C	24 h
C	0.5 %	4 °C	24 h
D	0.1 %	20 °C	24 h
E	0.3 %	20 °C	24 h
F	0.5 %	20 °C	24 h
G	0.1 %	37 °C	24 h
H	0.3 %	37 °C	24 h
I	0.5 %	37 °C	24 h
J	0	0	0

distilled water. Both the samples and standard substances were heated for 4 min at 100 °C. An aliquot (20 µl) of each sample was applied to each well. Gels (Sangon Biotech, China) were subjected to electrophoresis at 50 mA using a Mini-Protean II Cell (Bio-Rad, USA). Next the gels were stained with Coomassie Blue staining solution (0.1 g Coomassie Blue R-250, 45 ml methanol, 10 ml acetic acid and 45 ml distilled water) for 30 min at 60 °C. The excess stain was removed with several washes of de-staining solvent (methanol: acetic acid: distilled water = 1:1:8). The gel was analyzed with the BioDoc-IT gelatum imaging system (UVP, USA).

Scaffolds cross-linked with genipin

Scaffolds were divided into ten groups (Table 1) and cross-linked by immersion in 10 ml of PBS containing different concentrations (0.1, 0.3 and 0.5 wt%) of genipin (Challenge Bioproducts) for 24 h at different temperatures (4, 20, 37 °C). After being cross-linked, scaffolds of different groups were washed with distilled water to remove any residual genipin that might still be present. Afterwards, the 3D scaffolds were freeze-dried again.

Scanning electron microscopy (SEM)

The morphology of the scaffolds prepared was observed by a JEOL JSM-6360LV scanning electron microscope. The matrices were fixed by mutual conductive adhesive tape on copper stubs and covered with gold using a sputter coater. The same procedure

was followed for the cells/scaffold construct after the cell seeding studies.

Mechanical properties analysis

Scaffolds of different groups were made into cylinder samples of 10 mm in diameter and 5 mm in height ($n = 4$). The mechanical test was carried out using a material testing machine (Instron 5540, USA) by compression in the vertical direction at a deformation rate of 1.5 mm/min until failure at 20 °C. The compressive strength was calculated by $Q = F_{\max}/S$, where F_{\max} is the maximum load on the load-deformation curve and S is the cross-sectional area of each sample. The non-cross-linked scaffolds were set as the controls.

Determination of the swelling ratio

The swelling ratio test was carried out according to the procedure of the Yan method (Yan et al. 2010). Firstly, dry scaffolds were weighed (w_0) and then hydrated in PBS for 3 h at room temperature. After carefully removing the excess surface water with filter paper, the wet scaffolds were weighed (w) again. The swelling ratio of the scaffolds was defined as the wet weight increase ($w - w_0$) to the initial weight (w_0). The non-cross-linked collagen sponges were set as the controls.

Determination of cross-linking degree

The cross-linking degree (CD) of the different groups was determined by ninhydrin (NHN) assay according to the procedure of the Mi method (Mi et al. 2001). The scaffolds were weighed and a 7 mg sample from each different group was heated to 100 °C in a water bath with 4 ml NHN solution for 20 min. The solution was then cooled down to 20 °C, diluted with 5 ml 50 % isopropanol, and the optical absorbance of the solution at 570 nm was measured with a spectrophotometer (TU-1810, China) using glycine at various concentrations (1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml) as standard. The equation was used for testing the cross-linking degree of the sample as follows:

$$\text{CD} = \frac{(\text{NHN reactive eamine})_{\text{fresh}} - (\text{NHN reactive eamine})_{\text{fixed}}}{(\text{NHN reactive eamine})_{\text{fresh}}} \times 100\%$$

The 'fresh' element means the mole fraction of free NH_2 in non-cross-linked samples while 'fixed'

indicate the mole fraction of free NH_2 remaining in cross-linked samples.

In vitro collagenase degradation

The biodegradability of the type I collagen scaffolds was determined by incubating each sample in 2 ml PBS (pH 7.4) containing 200 μg collagenase type I (sigma, USA) at 4 °C for 12 h. Afterwards, the reaction was discontinued by adding 200 μl 0.2 M ethylenediaminetetraacetic acid (EDTA) and cooling the commixture in an ice bath immediately. The supernatant of the mixture was hydrolyzed in 6 M HCl at 110 °C for 24 h. The UV spectroscopy absorbance of hydroxyproline was examined by a spectrophotometer (TU-1810, China) according to the Woessner method (Woessner 1961). The biodegradation degree is defined as the proportion of hydroxyproline content in the cross-linked samples to that in non-cross-linked ones.

Cytotoxicity analysis

The cytotoxicity of samples was assessed by the MTT colorimetric assay. Scaffolds were sterilized by gamma irradiation and immersed in low glucose Dulbecco's modified Eagle's medium (L-DMEM) at 37 °C for 48 h. L-929 mouse fibroblast cells were seeded in 96 well plates at a density of 5.0×10^3 cells/well in 0.1 ml L-DMEM supplemented with 10 % fetal bovine serum (FBS), cultured at 37 °C and 5 vol% CO_2 for 24 h and then treated by leaching liquor of samples. The cells were treated with a normal culture media as control group. After 1, 3, and 7 days of culture, the medium was then removed and the cells were washed once with PBS, then 20 μl of 0.5 % MTT solution was added to each well followed by incubation for 4 h at 37 °C and 5 vol% CO_2 . Subsequently, the MTT solution was removed and 150 μl DMSO was added to each well. The 96 well plates were placed on a shaker for 10 min and the optical density (OD) value of each well was measured at 490 nm using an ELISA reader (Bio-Rad, Model II, USA).

Isolation and culture of chondrocytes

The primary chondrocytes were isolated from the joint cartilage and xiphoid process of SD rat (3 weeks) by

enzymatic digestion. First the cartilage was collected and cut into small pieces. Then the pieces were subjected to collagenase type II (1 mg/ml) (Sigma) digestion at 37 °C for 14 h on a shaker. The obtained chondrocytes were cultured in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F-12 (DMEM/F12, Hyclone, USA) supplemented with 10 % fetal bovine serum (FBS, Hyclone, USA) and 1 % antibiotic. Cultures were maintained in an incubator at 37 °C and 5 vol% CO₂. The culture medium was changed every 3 days. The second passage chondrocytes and the scaffolds cross-linked by genipin were used in present study. The scaffolds were sterilized with gamma irradiation. Briefly, the scaffolds were pre-wetted in the culture medium overnight and then put into the 24-well culture plate before cell seeding. Afterwards, the scaffolds were gently compressed by forceps to squeeze the redundant culture medium. Then, 200 µl of the chondrocyte suspension were seeded on per scaffold (3 × 10⁵ cells/scaffold). The scaffolds were transferred to another 24-well culture plate after 4 h of cell adhesion. The culture medium in the plate was changed for the first 24 h and changed every 3 days after the first change. Cultures were maintained in an incubator with 5 vol% CO₂ at 37 °C.

Adhesion and morphology of chondrocytes

Cell adhesion, morphology, and average distribution on the scaffolds were observed by SEM analysis. After 1 and 3 days of culture, the cell-scaffold constructs were washed in PBS and fixed in 2.5 % glutaraldehyde (in PBS). Afterwards, the samples were rinsed three times in PBS and subjected to a series of increasing ethanol conditions (from 30 to 100 %), 10–15 min each, to allow dehydration of the samples. Last, the samples were allowed to air dry and sputter coated with gold and analyzed with the SEM.

Statistical analyses

Statistical analyses were performed using SPSS software, version 16.0 (SPSS Inc, USA). The data are presented as mean ± SD, and the levels were compared by the one-way analysis of variance (ANOVA). *P* values <0.05 were considered as statistically significant.

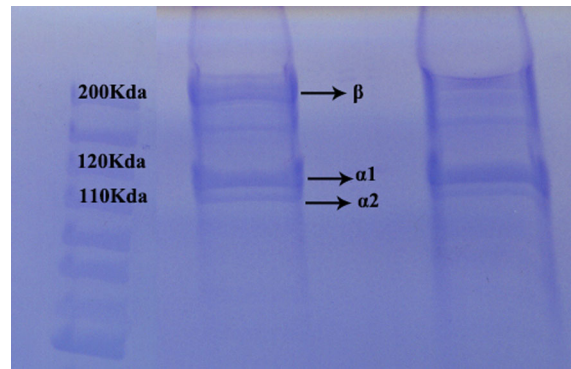


Fig. 1 Representative SDS-PAGE blot showing collagen with the corresponding $\alpha 1$, $\alpha 2$, and β subunits. The *left* is marker and the *right* is control

Results

Characters of extracted collagen and SDS-PAGE analysis

The purified collagen solution obtained from rat tails was semi-transparent and viscous. The porous scaffolds were fabricated by a freeze-dried process. In order to characterize the collagen, the SDS-PAGE was employed. SDS-PAGE analysis indicates that the extracted and purified collagen from rat tail tendons is mainly of type I, which was characterized by the presence of two alpha chains ($\alpha 1$, $\alpha 2$) and a beta component (Fig. 1). Their molecular weights were approximately 120, 110 and 210 kDa, which resembles control.

Scaffolds cross-linking with genipin

The collagen scaffolds cross-linked with genipin produced blue pigment. The color appearance of collagen scaffolds changes with the different genipin cross-linking conditions. As shown in Fig. 2, it seems that the higher concentration of GP and cross-linking temperature increases the intensity of blue.

Morphological characterization

The cross-section of the cross-linked structures was analyzed by scanning electron microscopy (SEM). Figure 3 represents the microscopic images of the cross-linked and the non-cross-linked collagen scaffold. All scaffolds presented a three-dimensional interconnected porous structure. The fiber- and lattice-like structure of non-cross-linked collagen



Fig. 2 Collagen scaffolds cross-linked with different genipin concentrations and temperature. The *white one* is a non-cross-linked scaffold

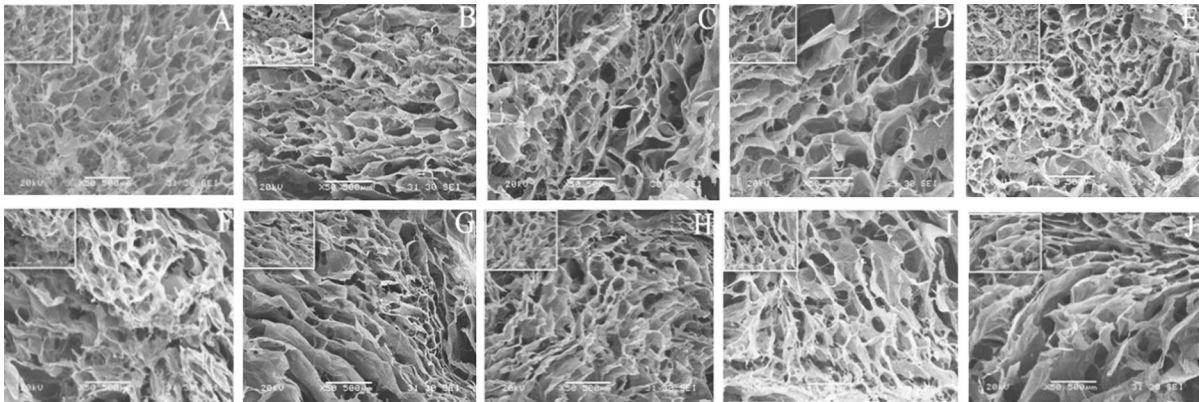


Fig. 3 The cross-section SEM images of non-cross-linked and genipin cross-linked collagen scaffolds. **a** Represents the morphology of the non-cross-linked scaffold (control) and **b–j** display the morphology of the genipin cross-linked scaffolds

scaffold with pore size ranged from 100 to 250 μm is presented in Fig. 3a. However, the morphologies of the genipin cross-linked scaffolds undergo a sheet-like structural transition Fig. 3b–l. Although the pore sizes have not been changed markedly, it seems that the sheet-like framework closely aligned with the higher genipin concentration and cross-linking temperature. In addition, the fibers of non-cross-linked collagen scaffold are totally not seen in all cross-linked groups.

Mechanical properties

The mechanical properties of the scaffolds are evaluated by compression model. As shown in Fig. 4, the compressive strength augments greatly with the increased genipin concentration and cross-linking temperature compared with control ($P < 0.05$).

Swelling ratio

The swelling ratio of collagen scaffolds with different genipin concentration and cross-linking temperature is

respectively. (**b**—0.1 % 4 °C, **c**—0.1 % 20 °C, **d**—0.1 % 37 °C, **e**—0.3 % 4 °C, **f**—0.3 % 20 °C, **g**—0.3 % 37 °C, **h**—0.5 % 4 °C, **i**—0.5 % 20 °C, **j**—0.5 % 37 °C)

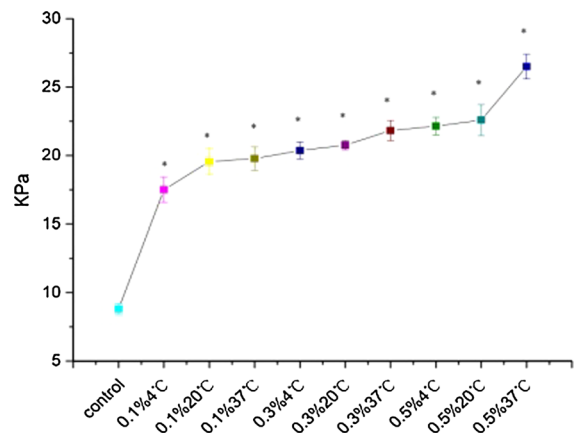


Fig. 4 The compressive strength increased gradually with an increasing genipin concentration and cross-linking temperature ($*P < 0.05$)

shown in Fig. 5. The swelling ratio of each cross-linked scaffold was much lower than that of the control (non-cross-linked) ($P < 0.05$). The swelling ratios of the scaffolds which in 0.3 % 37 °C, 0.5 % 20 °C and

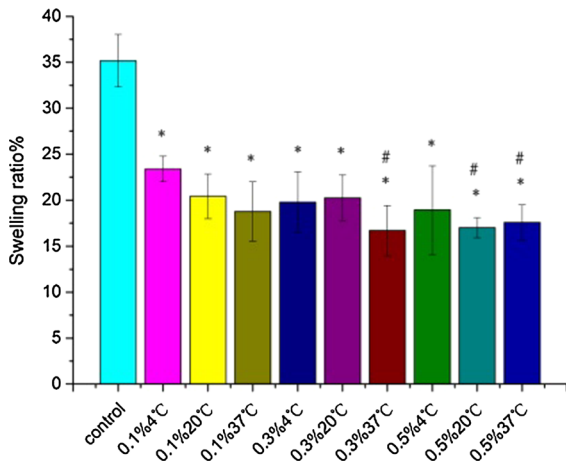


Fig. 5 The swelling ratio decreased with increasing genipin concentration, as well as cross-linking temperature. Asterisk indicates statistical significance when compared with control. Hash indicates statistical significance between the 0.1 % 4 °C group and 0.3 % 37 °C, 0.5 % 20 °C, 0.5 % 37 °C group

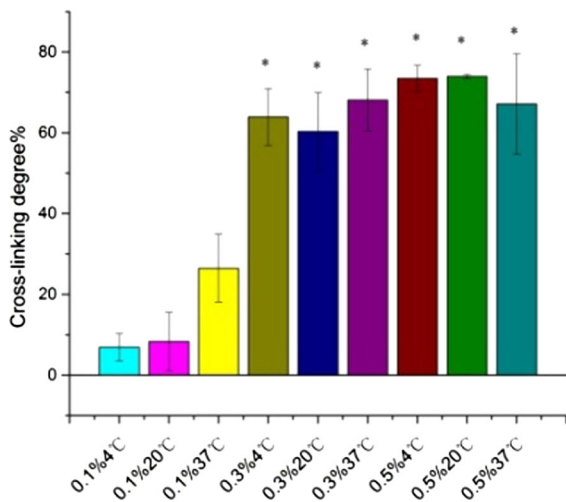


Fig. 6 The cross-linking degree of collagen scaffolds being cross-linked via different genipin concentrations and different temperatures. Asterisk indicates statistical significance when compared with 0.1 % genipin cross-linked collagen scaffold ($P < 0.05$)

0.5 % 37 °C groups were significantly lower than that of in 0.1 % 4 °C ($P < 0.05$).

Cross-linking degree

Figure 6 displays the cross-linking degree of the collagen scaffolds. The cross-linking degree ranged from 6.90 to 26.48 % when cross-linked by 0.1 %

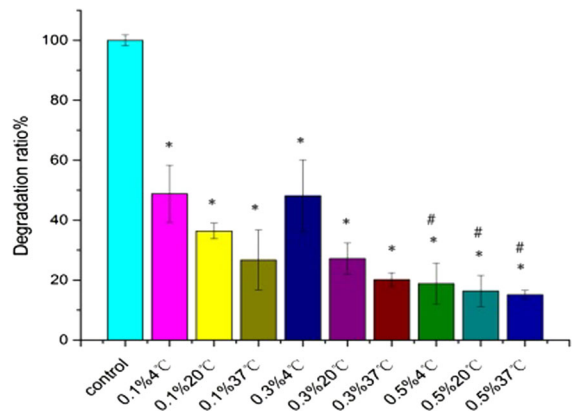


Fig. 7 The in vitro biodegradability of collagen scaffolds treated with or without genipin after digestion in 100 µg/ml collagenase type I for 12 h. Asterisk indicates statistical significance when compared with control. Hash indicates no statistical significance between the 0.3 % 37 °C and 0.5 % groups

genipin. The cross-linking degree of scaffolds which in 0.3 and 0.5 % groups were obviously higher than that of in 0.1 % groups ($P < 0.05$).

Collagenase degradation

The enzymatic degradation properties of the collagen scaffolds with or without cross-linking treatment are shown in Fig. 7. After being treated with genipin for 24 h, the anti-degradation ability of collagen scaffolds increased remarkably ($P < 0.05$). This is especially true in the 0.3 % 37 °C and 0.5 % groups. Moreover, there is no statistical significance between the last four groups.

Cytotoxicity

The cytotoxicity of collagen scaffolds treated with genipin was assessed by the MTT colorimetric assay. As shown in Fig. 8, there is low cytotoxicity to L929 fibroblasts in 0.1 % groups and 0.5 % 37 °C group at day 1 ($P < 0.05$). However, there is no statistical significance when compared with the control on days 3 and 7.

Adhesion and morphology of chondrocytes

Cell morphology and adherence is evaluated on the cross-linked collagen scaffolds, after cell seeding, by SEM. As shown in Fig. 9a, the chondrocytes maintain

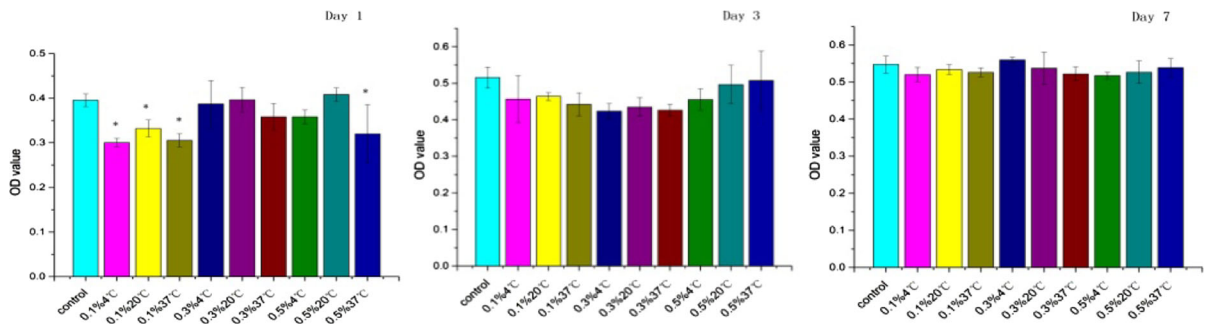


Fig. 8 Low cytotoxicity is found in 0.1 % groups and 0.5 % 37 °C group at day 1 ($P < 0.05$). There is no statistical significance when compared with the control on days 3 and 7

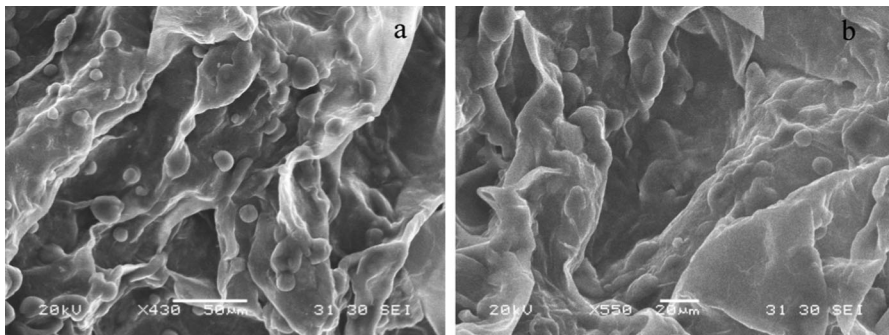


Fig. 9 SEM images of chondrocytes cultured in genipin cross-linked scaffolds for 1 and 3 days

a round shape in day 1 indicating that they just adhere to the scaffold. At day 3, the cells have adhered to the scaffold and secreted little extracellular matrix (Fig. 9b). Due to the cells adhere the inside rather than the surface of scaffold, the cells still present a quasi-circular shape.

Discussions

It is just because of its inherent biological characteristics, such as biodegradability and weak antigenicity, make type I collagen an optimal resource for the biomedical applications (Lee et al. 2001). Although it has promising features, the weakly mechanical properties need to be improved. Based on the knowledge of collagen fibrils have been shown to be strengthened by covalent cross-link of the constituent collagen molecules. There is need to find a safe and cost-effective cross-linking agent to resolve this difficult problem. Due to its safety, low cytotoxicity, and excellent cross-linking effectiveness,

genipin has gained much attention for constructing biomedical scaffolds. Thus optimal cross-linking conditions should be investigated to yield the ideal genipin cross-linking scaffolds.

In this study, we investigated the effect of genipin concentration and cross-linking temperature on the physical properties and biological potential of type I collagen scaffolds. Firstly, we successfully extracted the collagen from rat tail tendons. Following the detection of the collagen by SDS-PAGE analysis, we fabricated the scaffolds cross-linked by genipin under different conditions. Genipin can spontaneously react with the amine groups on amino acids or proteins to form dark blue pigments (Sung et al. 2000). The change of color in collagen scaffolds cross-linked with genipin indicates microstructure changes during cross-linking. The reaction between genipin and an amino acid in collagen molecule can form a monomer and a further radical reaction will cause dimerization. The mixtures of polymers formed from these reactions are the reason of blue pigment (Touyama et al. 1994). In our study, the intensity of blue increased with the

higher concentration of genipin and cross-linking temperature. Maybe it can be an extrinsic indicator of reaction between genipin and an amino acid in collagen molecule.

To observe the influence of cross-linking on the spatial configuration of collagen scaffolds, further investigations were performed. By SEM observation, an interesting phenomenon could be revealed. With an increasing genipin concentration and cross-linking temperature, the morphologies of scaffolds underwent a fiber-like and lattice-like to a sheet-like structural transition. The sheet-like framework closely aligned with the higher genipin concentration and cross-linking temperature while the pore size has not been changed markedly.

In term of the rationale that the mechanical strength of a porous scaffold relies mainly on its pore size (Kon and de Visser 1981), the mechanical strength of cross-linked scaffolds in this study should be improved lightly. However, based on the knowledge of cross-linking is an oxidation reaction between active chemical groups that could enhance the mechanical strength by forming chemical bonds between the polymers (Lund et al. 2008). The mechanical strength of scaffolds cross-linked by genipin in this study was increased apparently compared to control. Our results indicated that the compressive strength increased gradually with an increasing genipin concentration and cross-linking temperature. The results obtained in this work are in coincidence with collagen/chitosan scaffolds produced by the same technique in other works reported in the literature (Bi et al. 2011).

The swelling ratio of scaffold plays an important role in maintaining the structural stability when implanted these scaffolds into *in vivo*. Similarly, the mechanical properties of porous scaffolds may be influenced by the swelling ratio. Because collagen is hydrophilic materials and its poor mechanical properties will lead to the collapse of the porous structure when it resided in hydrated environment. Thus, the swelling ratio is a substantial index used to assess the structural stability of collagen scaffold. It is well known that the swelling property of the scaffolds would be decreased by the cross-linking treatment. The explanation for this is that the hydrophilic groups have been consumed by the genipin during the cross-link reaction. In our case, the swelling ratio of the genipin cross-linked collagen scaffolds was lower than that of the corresponding control. This is especially

true in the 0.3 % 37 °C, 0.5 % 20 °C, 0.5 % 37 °C groups. The higher genipin concentration and cross-linking temperature result in the lower swelling ratio.

The cross-linking degree is another important index for the evaluation of scaffolds. As biodegradation of scaffolds has a close relationship with the cross-linking degree. In this work, the cross-linking degree of scaffolds was assessed by the NHN assay. The percentage of free amino groups remaining in the scaffold after cross-linking could reflect the degree of the cross-linking reaction. Our results demonstrated that the cross-linking degree of the collagen scaffolds increased with an increasing genipin concentration and cross-linking temperature. However, it seemed that the genipin concentration rather than temperature played a crucial role in the increased the cross-linking degree in 0.3 and 0.5 % group. We deduced that the higher genipin concentration results in more and more amino group cross-linking which led to a higher cross-linking degree.

In order to investigate the degradation rate of these scaffolds, we incubated each sample in a solution of collagenase type I. As *in vitro* the collagenase could not last long action time in the environment of common temperature or 37 °C, we selected 4 °C as our experimental temperature. Since the main components of type I collagen are glycine, proline and hydroxyproline, we can evaluate the degradation rate of scaffolds by detecting the ratio of hydroxyproline content in the degraded matrix to that of non-cross-linked matrices. Our data indicates that after being treated with genipin for 24 h, the anti-degradation ability of collagen scaffolds increased remarkably. In the 0.3 % 37 °C and 0.5 % groups, this trend is evident. In the 0.1 % 4 °C and 0.3 % 4 °C groups, however, the degradation rate is higher than other cross-linking groups.

Therefore 4 °C is not optimal cross-linking temperature except for the presence of higher genipin concentration. To our knowledge that collagenase induces collagen degradation by cleaving the peptide bonds. The intramolecular and intermolecular network of collagen cross-linked by genipin prohibits this action of collagenase. Moreover, the available amino groups can be consumed by genipin at a higher concentration. Therefore, the 0.5 % groups display significant enhancement in biostability compared to other groups.

The cytotoxicity of genipin has been a controversial issue in recent years. Although some studies argued that genipin was safe enough to living cells, this

compound was also reported to induce cell death even at a small dose (Kim et al. 2005; Lima et al. 2009). It is difficult to obtain a consensus partly due to the different genipin concentration and cells using in these studies. Some authors recommend the dose of genipin using in tissue engineering to be controlled within 0.5 mM (Wang et al. 2011). In a recent study, however, the authors certified that there was no cytotoxicity when cross-linking collagen–cellulose nano-composites at 4 mM genipin concentration (Mathew et al. 2013). Additionally, different cells such as L929 fibroblasts, osteoblasts, chondrocytes, Balb/c3T3 were also used in these researches. The cell line probably possessed the stronger toxin immunity compared to the primary cells. The results would be more complicated in terms of these factors. Our results suggest that genipin have a mildly acute cytotoxicity to L929 fibroblasts for 24 h culture. But toxicity is neither dose dependent nor time dependent.

Lastly, we evaluated the chondrocyte morphology and the adherence to the cross-linked collagen scaffolds by SEM. The rat chondrocytes were able to adhere to the scaffold and secrete an extracellular matrix. The results suggested that collagen scaffolds cross-linked by genipin possessed excellent biocompatibility.

Conclusions

The development of cross-linked three-dimensional scaffolds of type I collagen extracted from rat tails was carried out under different conditions. Based on our data, the optimization of process conditions demonstrated that successful cross-linking with genipin could be achieved at 0.3 % genipin concentrations and 37 °C. The scaffolds possessed exceptional swelling ratios, biodegradation degrees, cross-linking degrees, compressive strength and lower cytotoxicity under this condition. Since biomaterials cross-linked by genipin can be influenced by different conditions, further studies are needed to confirm the optimal combination among these conditions.

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