Carbohydrate Polymers 93 (2013) 635–643

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

How can genipin assist gelatin/carbohydrate chitosan scaffolds to act as replacements of load-bearing soft tissues?

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ARTICLE INFO

Article history:
Received 25 September 2012
Received in revised form
25 November 2012
Accepted 30 November 2012
Available online 29 December 2012

Keywords:
Gelatin
Carbohydrate chitosan
Genipin
Scaffold
Soft tissue engineering

ABSTRACT

In the engineering of soft tissues, designing the scaffolds with adequate strength and controllable biodegradation properties are essential. To fulfill such design criteria gelatin/carbohydrate chitosan (G/CC) scaffolds have gained much attention in engineering replacement of soft tissues. In this research, the influence of different cross-linking methods and parameters with genipin on the physico-chemical properties of G/CC scaffolds was investigated in detail. The scaffolds with different concentrations of G and CC were prepared and cross-linked trough two different methods, and the impact of cross-linker concentration beside of cross-linking time and temperature were fully analyzed. The obtained results suggested that the 1% genipin-cross-linked G60/CC40 scaffolds, prepared at room temperature for 24 h through scaffold cross-linking method, could be a promising replacement in missing segments of load-bearing soft tissues. This article describes a systematic study of genipin cross-linking effects on G/CC scaffolds.

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1. Introduction

The ultimate purpose of tissue engineering is exact duplication of native tissue function. However, interim tissue engineering approaches do not necessarily need to obtain high degrees of clinical success. Thus, we need to set up key tissue parameters, which are necessary to produce load-bearing replacements (Yan et al., 2010). For this purpose, tissue engineers tend to use three-dimensional (3D) porous scaffolds which play a crucial role in tissue engineering, as they not only provide temporary templates for the formation of new tissue and guidance of cell ingrowths, but also provide adequate stable porous structures for nutrients and oxygen transport (Bi et al., 2011). During these days, sport engineers are seriously dealing with defects of load-bearing soft tissues such as meniscus, cartilage, disc, which pose critical clinical problems. It is generally regarded that these soft tissues have very limited potential to heal spontaneously, whether the damage is due to degenerative alterations, pathological conditions, or trauma such as sport injuries. Many diverse complex surgeries have been applied to cure disease resulted by these causes. In some cases, at short time period it seems that satisfactory cure have been occurred but the repaired tissue has particular difference in mechanical properties in load-bearing situations with native soft tissue and therefore these methods efficiency in long time periods are rarely satisfactory (Guilak, Awad, Fermor, Leddy, & Gimble, 2004; Nehrer, Spector, & Minas, 1999). Tissue engineering approaches assist sport engineering problems because it has been proved one of the most promising alternative therapies for soft tissue defects (Iwasa, Engebretsen, Shima, & Ochi, 2009; Lee, Grodzinsky, Hsu, & Spector, 2003; Sherwood et al., 2002).The compressive strength and stiffness of scaffolds in soft tissue engineering plays an important role in successfulness of tissue engineering ultimate goal (Mao, Yin, & Yao, 2003). In this strategy, various scaffolds have been prepared from naturally derived and/or biodegradable synthetic polymers (Bi et al., 2011; Buma, Ramrattan, van Tienen, & Veth, 2004; Freymann et al., 2011; Heijkants et al., 2004; Klompmaker et al., 1993; Lien, Li, & Huang, 2008; Mandal, Park, Gil, & Kaplan, 2011; Nettles, Elder, & Gilbert, 2002; Sherwood et al., 2002; Sommerlath & Gillquist, 1993; Xia et al., 2004; Yan et al., 2010, 2011; Wang et al., 2010). A wide range of polymers is limited by the lack of suitable mechanical properties, degradation behavior, controlled porosity and biocompatibility (Chiono et al., 2008; Thein-Han, Saikhun, Pholpramoo, Misra, & Kitiyanant, 2009; Xia

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0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.11.099

et al., 2004) because of that finding the most adequate materials are essential.

Carbohydrate chitosan (CC) and gelatin (G) based scaffolds can be considered as potential candidates for soft load-bearing tissue regeneration such as meniscus and cartilage due to controllable mechanical property, degradability and biocompatibility (Bi et al., 2011; Cheng et al., 2003; Chiono et al., 2008; Huang, Onyeri, Siewe, Moshfeghian, & Madihally, 2005; Jiankang et al., 2009; Mao, Zhao, Yin, & Yao, 2003; Thein-Han et al., 2009; Yang et al., 2010). Due to the hydrogel characteristics of these polymers, a significant amount of fluid can be retained in their structure. Hence, they can produce high compressive modulus comparable with native load-bearing soft tissues. These materials can be used for the treatment or repair of articular cartilage and meniscus (Minuth WW, 1998; Stella, D'Amore, Wagner, & Sacks, 2010).Generally, CC is a naturally derived polysaccharide obtained from *N*-deacetylation of chitin, which is the copolymer of D-glucosamine and *N*-acetyl-D-glucosamine (Huang et al., 2005). This biopolymer is normally soluble in the medium with pH lower than 7. However, in dilute acids (pH less than 6) the free amino groups on CC skeleton are protonated resulting in soluble molecules. The high charge density in dilute acidic solution allows CC to form complex by ion interaction with lots of water soluble polyanionic species (Yang et al., 2010). CC does not have a remarkable mechanical property so for improving the mechanical and biological properties blending with other polymers is widely investigated (Cheng et al., 2003). As an important alternative, G can be blended by CC, this polymer is basically denatured from collagen obtained by breaking its triple helix structure mostly by hydrolysis (Burugapalli, Koul, & Dinda, 2004). If G has been used in pure form, it is known to be hemostatic as seen from the presence of an acute in vivo inflammatory response and increased neutrophil activity. Note that these characteristics may be attributed to the anionic nature of G. This charge may be balanced by blending it with cationic molecules like CC. Moreover, the hydrophilicity of G due to its amino and carboxyl groups helps in improving water retention and oxygen and nutrient transfer throughout the scaffold architecture. (Chiono et al., 2008; Thein-Han et al., 2009; Yan et al., 2010).

The main limitation of G for the production of tissue substitutes arises from its rapid dissolution in aqueous environments. Hence, chemical and physical cross-linking methods have also been used to increase the stability of these materials in aqueous media. Physical cross-linking methods for biopolymers have been widely studied (Kartha, Bello, & Harker, 1967) and it seems that the main advantage of these methods is that they do not cause potential harm, whereas their main drawback arises from the difficulty to obtain the desired cross-linking degree. Thus, researchers prefer to use chemical cross-linkers (Kuijpers et al., 2000). However, the main limitation in the use of chemical cross-linkers arises from the presence of some un-reacted molecules inside the scaffolds and from the risk of toxic by-products by reaction between the substrate and the cross-linking agent during in vivo biodegradation. Covalently cross-linked hydrogels may also be formed after reaction with various chemicals such as epoxides, glutaraldehyde, cyclodextrin and diglycidyl ethers, which are usually cytotoxic (Butler, Ng, & Pudney, 2003). For this reason, much interest has been recently addressed toward naturally derived cross-linking agents, with low toxicity. Hence, genipin can be a specific natural and suitable cross-linking reagent. Genipin naturally derived from its parent compound, geniposide, traditionally used as one of the basic elements of Chinese medicine and it can be isolated from the fruits of Gardenia jasminoides Ellis (Butler et al., 2003; Mi et al., 2003). The fruits Gardenia jasminoides plants have long been used in Chinese medicine for their anti-inflammatory, antiphlogistic, choleretic, diuretic, and haemostatic features. It has recently provoked interest for its ability to cross-link CC and special proteins such as G containing residues of primary amine groups (Lien et al., 2008; Yan et al., 2010). Several studies have been carried out to compare genipin with commonly used cross-linkers, which assessed genipin lower cytotoxicity and higher biocompatibility (Sung, Huang, Huang, Tsai, & Chiu, 1998). Sung, Huang, Huang, Tsai, & Chiu, 1998) reported that genipin-fixed tissues showed a significant mechanical strength and resistance against enzymatic degradation compared to the fixed tissues with other cross-linkers.

Table 1
The nominal composition of the prepared scaffolds.

Sample	G %	CC %	Sample	G %	CC %
3%(G0/CC100)	0	100	5%(G0/CC100)	0	100
3%(G20/CC80)	20	80	5%(G20/CC80)	20	80
3%(G40/CC60)	40	60	5%(G40/CC60)	40	60
3%(G60/CC40)	60	40	5%(G60/CC40)	60	40
3%(G80/CC20)	80	20	5%(G80/CC20)	80	20
3%(G100/CC0)	100	0	5%(G100/CC0)	100	0

To cut the long story short, the composites of G and CC have been reported to have improved mechanical, physical and chemical properties compared with those of single components (Gómez-Estaca, Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010; Rivero, García, & Pinotti, 2009; Sionkowska, Wisniewski, Skopinska, Kennedy, & Wess, 2004), for instance Chiono et al. (2008) prepared composite films of G/CC and reported significant improvements for biomedical applications. This was attributed to the formation of polyelectrolyte complexes (PECS) through electrostatic interactions operating between the ammonium groups of CC and the negatively charged side-chain groups in G molecules.

In this study, a combination of two different cross-linking methods under different conditions was studied in detail. The objectives were synthesis and evaluation of natural green materials for load-bearing soft tissue replacement and scaffold optimization. Hence, various groups of porous G/CC scaffolds based on different ingredient concentrations (CC/G: 100/0, 80/20, 60/40, 40/60, 20/80, 0/100) were prepared. The efficacy of cross-linking with genipin in media simulating physiological conditions was evaluated by means of water uptake, water retention and biodegradation measurements. In addition, the concentration of polymers, cross-linking methods and parameters were the principle factors regarding the vastly investigated mechanical properties. The investigation of different concentrations helped us to find the optimal component supporting soft load-bearing tissues.

2. Materials and methods

2.1. Material

G microbiology grade was supplied from Merck company. CC derived from crab shell (with 86% degree of deacetylation (DD) and ~200 kDa), genipin (G4796 ≥98% (HPLC), powder) and acetic acid were purchased from Sigma–Aldrich Company. All commercially available solvents and reagents were of analytical grade and used without further purification.

2.2. Synthesis of G/CC scaffolds

In a typical experiment, CC was dissolved in 1% (v/v) acetic acid solution in demineralized water and its acid-insoluble fraction was removed by filtration. Also, G was dissolved in demineralized water at 50 °C, obtaining 3% (w/w) and 5% (w/w) solutions. Then, the G/CC solutions with different concentrations of basic polymers were obtained (Table 1). Each mixture was kept at 50 °C under moderate stirring until the gels started to form, as suggested by visual inspection. Increase of mixture viscosity, which decreases the effective rotation rate of the magnetic stirrer, could be another

claim for gel formation. The obtained final gels were cooled to reach room temperature, then casted on a Petri-dish and refrigerated at 4 °C for 2 h, frozen at −20 °C for 24 h and lyophilized in a vacuum freeze-dryer for 48 h.

2.3. Cross-linking of the scaffolds

2.3.1. Scaffold cross-linking method

The scaffolds were divided into different groups. A group of them was cross-linked by immersion in 30% (v/v) ethanol containing 0.5%, 1%, 1.5%, 2% (w/v) of genipin for 24 h at 25 °C. In another group, 3%(G60/CC40) and 5%(G60/CC40) scaffolds were cross-linked by immersion in 30% (v/v) ethanol containing 1% (w/v) genipin for different periods (12 h, 24 h, 36 h) at different temperatures (4 °C, 25 °C, 37 °C). The uncross-linked samples were considered as control samples. At the end of cross-linking process, the scaffolds from different groups were washed with distilled water and immersed in a saturated solution of glycine. When the color of the solution no longer changed, the scaffolds were removed and neutralized with 0.1 M NaOH for 1 h. The excess base was removed by repeated washing with distilled water until the pH returned to the physiologic range (7.0–7.4). Neutralized samples were freeze-dried for the subsequent experiments.

2.3.2. Mixing cross-linking method

The G/CC solutions were mixed directly with the cross-linker solution containing 1.0% (w/v) genipin, and uniform dark blue mixed solutions were approached in about 2 min with vigorous stirring (Lien et al., 2008). The mixed solutions were cool downed to reach room temperature 25 °C. The cross-linking reaction was done for 48 h at room temperature. The procedure that complicate the cross-linking mechanism is induction of the polymerization by oxygen radical of genipin that happens in a heterocyclic compound formation which gives the solution an specific blue color (Butler et al., 2003; Chiono et al., 2008). The cross-linked samples were then freeze-dried to obtain porous scaffolds. After cross-linking process, the scaffolds were washed with distilled water and immersed in a saturated solution of glycine. When the color of solution no longer changed, the scaffolds were removed and neutralized with 0.1 M NaOH for 1 h. The excess base was removed by repeated washing with distilled water until the pH returned to the physiologic range (7.0–7.4). Then, the scaffolds were put in a freezer at −20 °C for 3 h and moved into a vacuum freeze-dryer for 36 h.

2.4. Characterization

2.4.1. Micro-structural analysis

The morphology of the samples was evaluated using scanning electron microscope (SEM). Different types of prepared scaffolds were mounted onto aluminum stubs, and the surfaces and fracture sections of liquid N_2 frozen samples were coated with a thin layer of Gold (Au) by sputtering (EMITECH K450X, England). Then, the morphology and microstructure of the samples were observed on a SEM (Philips XL30) that operated at the acceleration voltage of 15 kV.

2.4.2. Pore size and porosity measurement

The porosity was estimated by liquid displacement methods (Zhang & Ma, 1999). The pore sizes of scaffolds were determined by a mercury intrusion porosimeter (AutoPore IV 9500, Micromeritics, USA) (Nettles et al., 2002) with a fling pressure changing from 3.4 to 413.7 MPa. The effective size of the pores was calculated as the mean diameters of scaffold pores. At least 25 pores were considered from different areas of the same sample. The values were expressed as the mean ± standard error.

2.4.3. Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR)

The load-bearing groups of G/CC scaffold cross-linked with genipin were examined by FTIR with Bomem MB 100 spectrometer. For IR analysis, in first 1 mg of the powder samples were carefully mixed with 300 mg of KBr (infrared grade) and palletized under vacuum. Then, the pellets were analyzed in the range 400–4000 cm^{-1} at the scan speed of 23 scan/min with 4^{-1} resolution.

2.4.4. Mechanical testing measurement

The mechanical behavior (vertical compression) of scaffolds was investigated by conducting compression strength test according to ASTM F 451-86. The samples were cut to cylindrical shapes with 1 cm^2 area and the thickness was measured with an electric digital caliper (Mozafari et al., 2010a). E (Young Modulus) of the scaffolds were tested by Roel-Amstel (load cell: 25 kN; resolution: 1 N) at a deformation rate of 1 mm/min until failure. The slope of linear region in the stress–strain curve gave the elastic modulus values (Huang et al., 2005).

2.4.5. Density measurement

The density of scaffolds was calculated from the ratio of the freeze-dried scaffolds weight to their volume, according to the following equation (1):

$$\rho = \frac{4 \times w}{\pi \times D^2 \times H} \quad (1)$$

where D is the diameter, H the thickness and W the weight of scaffold, respectively (Mao, Zhao et al., 2003).

2.4.6. Water uptake and water retention tests

Water uptake and water retention ability of the scaffolds were determined as below. The dried scaffolds were weighed (D), immersed in phosphate buffered saline (PBS) pH = 7.4 at room temperature for 30 min, gently blotted with filter paper to remove the excess water, and weighed (W) to determine water uptake. To measure the water retention capability, the wet scaffolds were transferred to centrifuge tubes with filter paper at the bottom, centrifuged at 500 rpm for 3 min and weighed immediately (C). The percentage of water absorption (W_A) and water retention (W_R) of the scaffolds at equilibrium were calculated using Eqs. (2) and (3) (Mao, Zhao et al., 2003; Thein-Han et al., 2009):

$$W_A = \frac{w - D}{D} \times 100 \quad (2)$$

$$W_R = \frac{C - D}{D} \times 100 \quad (3)$$

2.5. Statistical analysis

All the experiments were performed in fifth replicate. The results were given as means ± standard error (SE). Statistical analysis was performed by using One-way ANOVA and Tukey's test with significance reported when $P < 0.05$. Also for investigation of group normalizing, Kolmogorov–Smirnov test was used.

3. Results and discussions

3.1. Microstructure of the scaffolds

Visually observation, the G scaffolds were colorless but by further addition of CC concentration the color of blended samples approached to yellow. Also, genipin cross-linked samples became purple, and by increasing the genipin concentration the samples approached to dark blue.

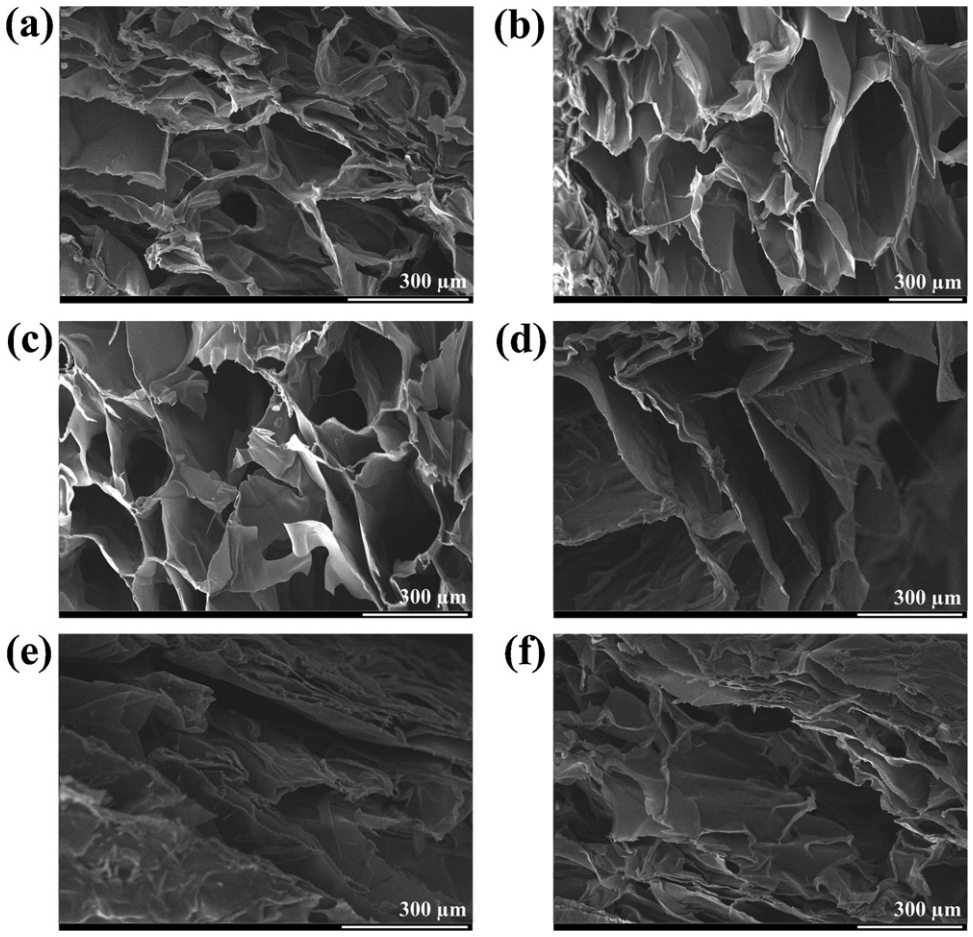

Fig. 1. Typical SEM micrographs describing the cross-sectional morphology of the scaffolds. (a) sample 3%(G100/CC0), (b) 3%(G80/CC20), (c) 3%(G60/CC40), (d) 3%(G40/CC60), (e) 3%(200/CC80), and (f) 3%(G0/CC100).

As an important note, the architecture of the scaffolds plays a key role that can trigger biological response and eventually determine the success of tissue-engineered constructs (Zhang et al., 2011). Fig. 1 shows the typical SEM micrographs describing the cross-sectional morphology of G, CC and different G/CC scaffolds. Representative microstructures of pure G, CC and blended scaffolds indicated the proper pore size and interconnected pore structure of blended samples, and all the scaffolds possessed a three-dimensional (3D) interconnected macroporous structure. The structure of G/CC scaffolds with pore size ranged from 150 to 350 μm is presented in Fig. 1. It can be also seen that the G/CC scaffolds with CC percentages more than 40% has sheet-like structures and their pore size decreased as shown in Fig. 1(d)–(f). The explanation for this observation might be related to the semi-crystalline nature of CC which tends to form membrane structures (Ma, Mao, Shen, Hu, & Han, 2003). In addition, during the cross-linking process, the scaffolds with higher CC contents seems to have more cross-linking points, so the small sheets would be linked together to form larger sheets and inducing the formation of larger pores. It is also noticed that honeycomb-like ordered structures with large pore walls and pore size ranged from 150 to 200 μm were observed in the scaffolds. There were no obvious changes in the pore size and morphology of G60/CC40 scaffolds after cross-linking with different concentrations of genipin.

In contrast, the scaffolds containing lower amounts of CC would form small pieces of sheets which lead to the slightly increase of pore size. Since G possesses higher mechanical strength than CC, G60/CC40 scaffold can retain its original structure after cross-linking. For this reason no collapse or fusion of the pores was observed in the cross-linked scaffolds.

It could be also concluded that the amount of the pore walls and surface area were thin enough for possible cell attachment. Although the optimal scaffold pore size for successful tissue healing is controversy, it seems that the scaffold pore sizes of around 100–400 μm are adequate in a wide range of tissue engineering application (Cooper, Lu, Ko, Freeman, & Laurencin, 2005; Lee, 2005; Mozafari, Rabiee, Azami, & Maleknia, 2010; Zhang et al., 2011). In addition, a minimum pore size of 150 μm is proposed for hard tissue and 200–250 μm for soft tissue ingrowth (Lee, 2005).

3.2. Chemical bonding reactions

Fig. 2 shows the FTIR spectra, in the 400–4000 cm^{-1} spectral range, of plain G, plain CC and G/CC blended scaffolds. The FTIR spectrum of CC exhibits characteristic bands of peaks at around 3480 cm^{-1}, corresponding to the stretching vibration of N—H, while the bands around 1660 cm^{-1} and 1578 cm^{-1} are the characteristic peaks of amide I and amide II (—CH_3—C=O group, denoting the presence of acetyl group), respectively. This confirms that CC is partially in deacetylated form. In addition, the peaks around 2917 cm^{-1} and 2874 cm^{-1} are assigned to —CH_2 and —CH_3 groups (aliphatic group). The sharp peaks around 1420 cm^{-1} are assigned to the CH_3 symmetrical deformation mode, and 1155 cm^{-1}, 1097 cm^{-1} and 1043 cm^{-1} correspond to indicate the C—O stretching vibrations. The amino group has a characteristic absorption band in the region

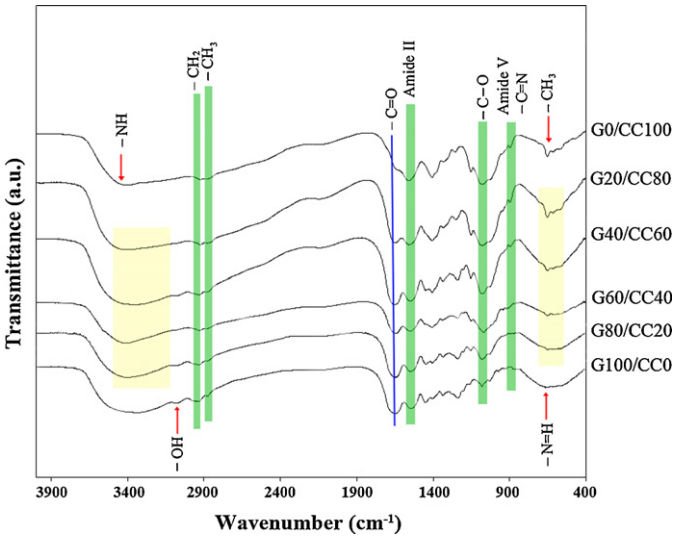

Fig. 2. The FTIR spectrum of different samples.

of 3400–3500 cm^{-1}, which is masked by the absorption band due to —OH group.

The FTIR spectrum of CC exhibits characteristic bands of peaks at around 1640 cm^{-1} and 1542 cm^{-1}corresponding to carbonyl and amino bands, respectively. The G spectrum also shows strong absorption bands at around 3200–3500, 1640, and 1578, 1234 and 628 cm^{-1},which are due to —N—H stretching, —C=O stretching, and amides II, amide V, —C=N torsion and —N=H bending, respectively. The mentioned characteristic bands are in agreement with the reported data previously by Mozafari et al. Similarly, all the mentioned characteristic bands were presented in the FTIR spectrum for the composite samples (G80/CC20, G60/CC40, G40/CC60, G20/CC80), and the spectra depicted similar characteristic peaks of the parent molecules.

As it can be seen in Fig. 2, the intensity of peaks around 920 and 610 cm^{-1} increased by further addition of CC into the G matrix. These characteristic peaks were absent in the FTIR spectrum of plain G sample (Hamlekhan et al., 2010; Hamlekhan, Moztarzadeh, Mozafari, Azami, & Nezafati, 2011). Incorporation of G also led to small modifications such as shifting of both amino and carboxyl bands in the spectrum of CC.

As it can be seen in Fig. 3, and according to the location of absorption bands, the —C=O and amino II absorption bands around 1660 cm^{-1} and 1590 cm^{-1}, respectively, shifted to lower wavenumbers as the G content increased. These small but visible changes would suggest a chemical interaction between CC and G molecular chains, and intermolecular hydrogen bonding between CC and G molecules in the polyelectrolyte complex formation. This may also be related to the formation of an interpenetrating polymeric network of CC and G without any significant change in chemical property.

As the absorption band of genipin-cross-linked amide groups around 1600 cm^{-1} merges with the strong absorption band of amide I in CC and G, it is certainly difficult to clarify the cross-linking reaction. The absorbance band at 1567 cm^{-1} indicated that genipin reacted with CC and G via a nucleophilic attack by the amino groups the olefinic carbon atoms of deoxyloganin aglycone followed by the opening of the dihydropyran ring (Mi, Sung, & Shyu, 2000). Subsequently, the absorbance band at 1644 cm^{-1} suggested that the amine groups on CC molecules were partly converted into the genipin-cross-linked heterocyclic amines. This band also contributed to the formation of amide bonding through a nucleophilic attack by the amine groups at the ester group of genipin. According to Mi et al. (2003), CC has a higher reaction tendency to be

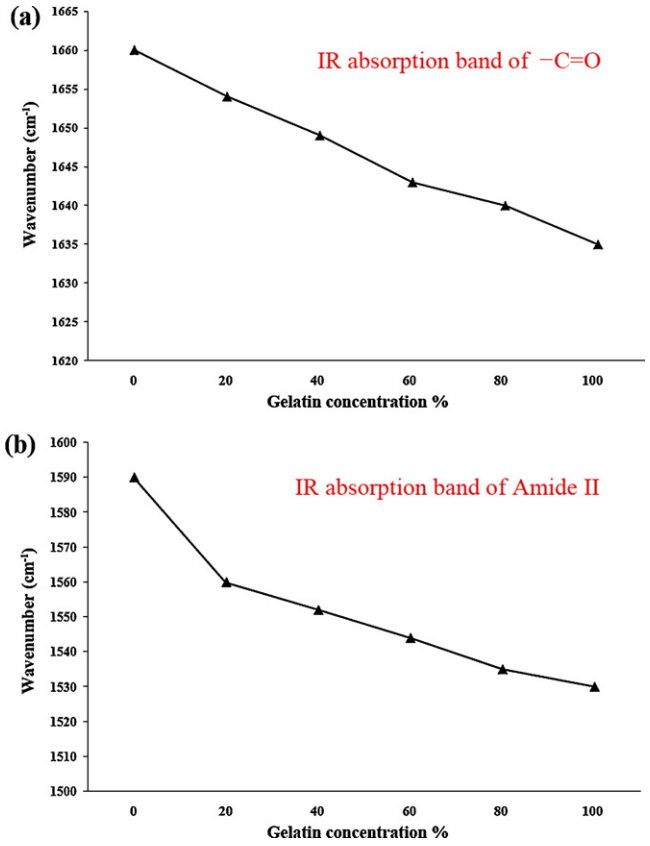

Fig. 3. The location of FTIR absorption bands versus concentration of G in the scaffolds. (a) IR absorbance of —C=O. (b) IR absorbance of amide II.

cross-linked with genipin, as compared with its G counterpart. It is clear that genipin only reacts with primary amine groups of lysine and arginine residues on G but it reacts with every glucosamine unit on CC. This indicated that CC could react with genipin to assemble more sufficient and extensive cross-link bridges (Lien et al., 2008; Yan et al., 2010).

3.3. Mechanical behavior (vertical compression)

3.3.1. Effect of G, CC and genipin concentration

Generally, an ideal scaffold for tissue engineering should be highly porous with adequate mechanical properties. As an important class of organs, load-bearing soft tissues have special mechanical properties for example the compressive modulus of meniscus are low and in the range 50–400 kPa (Yan et al., 2011). For this purpose, the prepared samples were tested to determine the effects of G, CC and genipin concentrations on the mechanical behavior of the samples. As shown in Fig. 4, compressive modulus increased greatly when the G concentration increased from 0 to 100%. The young modulus of plain G, plain CC and G/CC scaffolds suggested that polymer (especially G concentration) and cross-linker concentration were significantly responsible for the increase of compression modulus. The obtained results demonstrated that the cross-linked samples with 1% (w/v) genipin represented the highest compressive modulus among samples with same initial conditions. In addition, by increasing the concentration of genipin form 0.5% to 1% the compressive modulus gradually increased and reached a peak at a concentration of 1%, and from 1 to 2% significantly decreased.

It is obvious that the mechanical property of porous scaffolds depend mainly on the pore size, hence smaller pores can play efficient role for enhancing the compressive modulus of G/CC

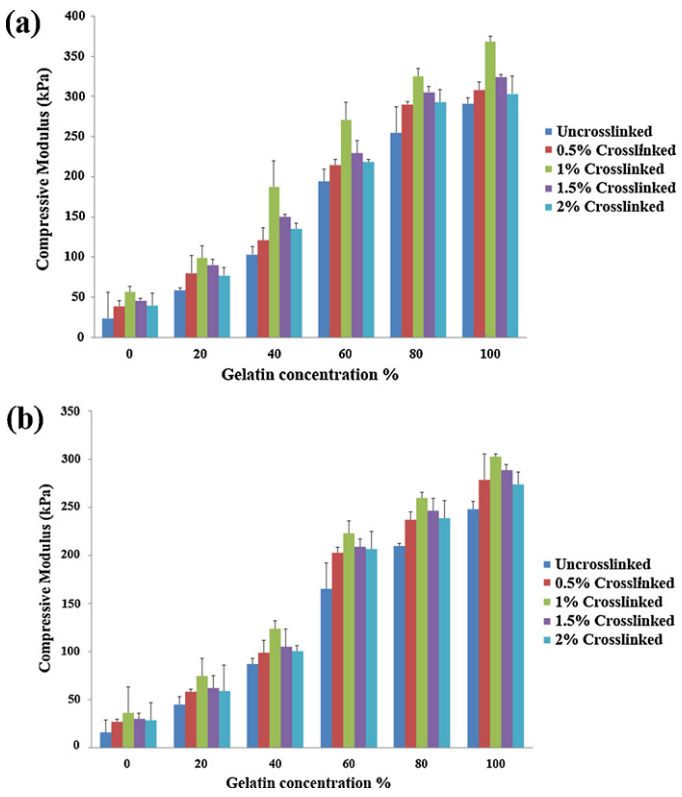

Fig. 4. Effects of G, CC and genipin concentrations on the mechanical behavior of different samples. (a) Compressive modulus of the scaffolds with 5% polymer concentration versus G concentration. (b) Compressive modulus of the scaffolds with 3% polymer concentration versus G concentration.

scaffolds. It is reported that mechanical property of porous scaffolds could be improved by forming chemical bonds through the cross-linking reaction between active chemical groups (Lund et al., 2008). Thus, two contradictory effects were taking place during the cross-linking process. The cross-linking reaction increased the mechanical strength of the scaffolds due to the formation of chemical bonds. On the other hand, the mechanical strength was decreased due to the gradually enhancement of scaffold pore size. When the cross-linking reagent was at lower concentrations (from 0.5 to 1.0%), the enhancement of mechanical strength was stronger than the inhibition caused by the increasing pore sizes. As a result, the final compressive strength demonstrated an increase. However, when the concentration of genipin reached 1.5% or more, the pore sizes might be increased and the inhibitive effect of cross-linking was enhanced, which finally resulted in diminished mechanical strength. Table 2 gives the data obtained from mechanical compressive tests of the scaffolds compared with natural load-bearing soft tissue (Mandal et al., 2011; Levental, Georges, & Janmey, 2007; Tran et al., 2010).

Table 2
Mechanical properties of the scaffolds (prepared by scaffold cross-linking method with specific condition) compared with natural load-bearing soft tissues.

Sample	Compressive modulus (kPa)	Pore size (μm)	Porosity %
Natural load-bearing soft tissues (Buma et al., 2004; Iwasa et al., 2009)	150–350	200–250	80–90
3%(G60/CC40)	223 ± 8	200	90 ± 1.2
5%(G60/CC40)	270 ± 12	200	88 ± 1.5
3%(G40/CC60)	187 ± 9	150	88 ± 0.7
5%(G80/CC20)	325 ± 15	220	87 ± 1.3
3%(G80/CC20)	260 ± 4	250	91 ± 0.5

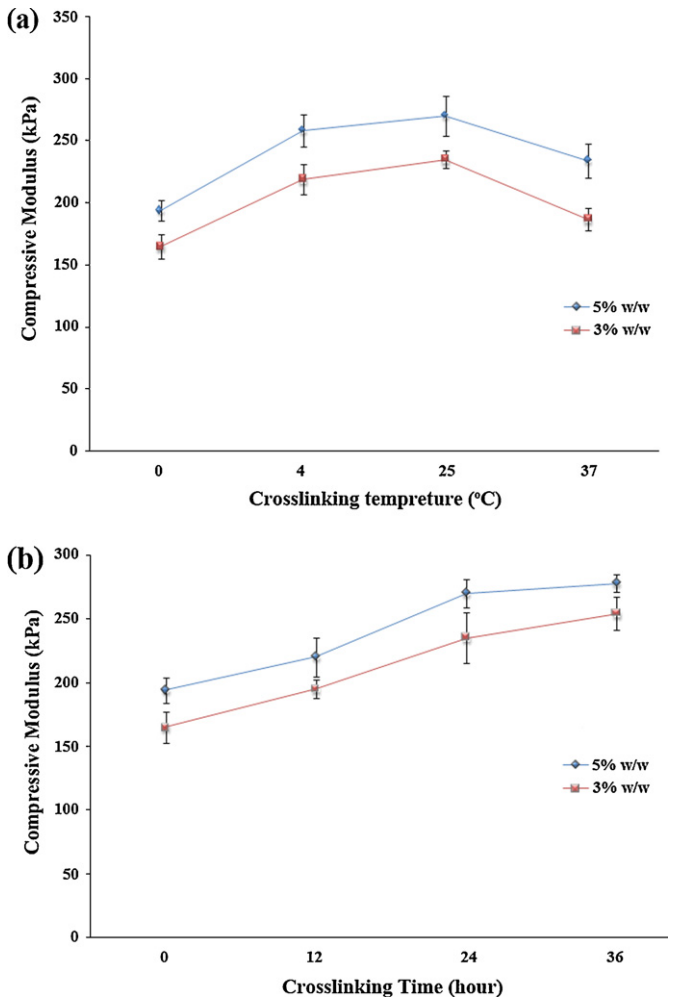

Fig. 5. Effects of cross-linking time and temperature on the mechanical behavior of different samples. (a) Compressive modulus versus cross-linking time. (b) Compressive modulus versus cross-linking temperature.

3.3.2. *Effect of cross-linking condition and method*

As it can be seen in Fig. 5(a), the compressive modulus of 3%(G60/CC40) and 5%(G60/CC40) samples under the cross-linking temperature of 25 °C was significantly higher than that at either 4 or 37 °C. There were no magnificent differences of compressive modulus between 4 and 37 °C. Interestingly by increasing the cross-linking time the compressive modulus showed only a slight tendency to increase, as shown in Fig. 5(b). In addition, the compressive modulus increased slightly after 24 h, in another word after this period of time the cross-linking process has been completed.

Direct observations indicated that the average pore size was enlarged with increasing of the cross-linking temperature. Also, the enhancement of cross-linking temperature contributes to the cross-linking reaction. According to Yang et al. (2010), the changes of mechanical strength caused by cross-linking temperature could also be explained as follows: under lower temperatures, the negative effect of increasing pore size on the mechanical strength is lower than the positive effect of cross-linking, resulting in an increase in the compressive strength from 4 to 25 °C but at the temperatures around 37 °C the pore size is greatly enlarged, which caused a decline in the compressive strength. In contrast to the effects of genipin concentration and cross-linking temperature, the

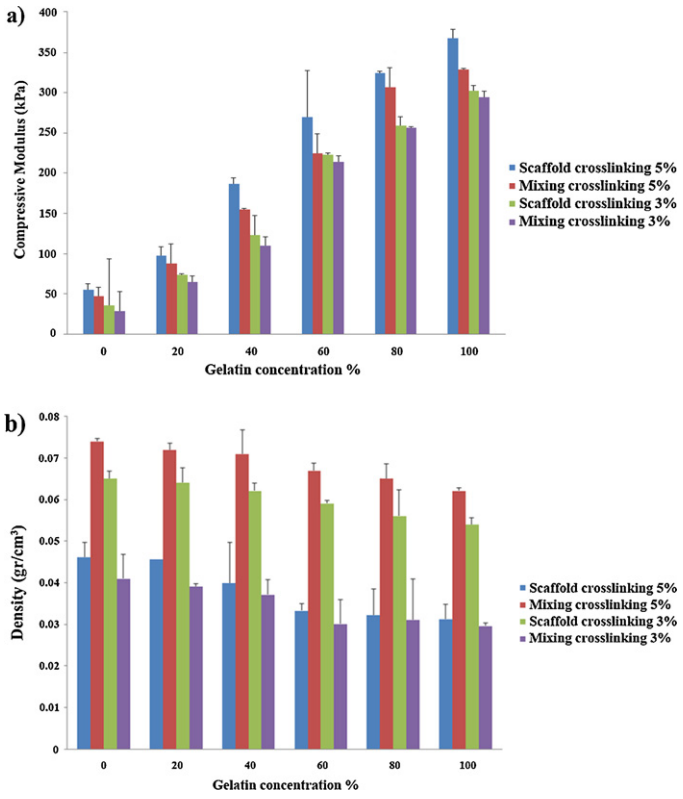

Fig. 6. (a) Effects of cross-linking methods on the mechanical behavior of different scaffolds. (b) Effects of G and CC concentrations and cross-linking methods on the density of different scaffolds.

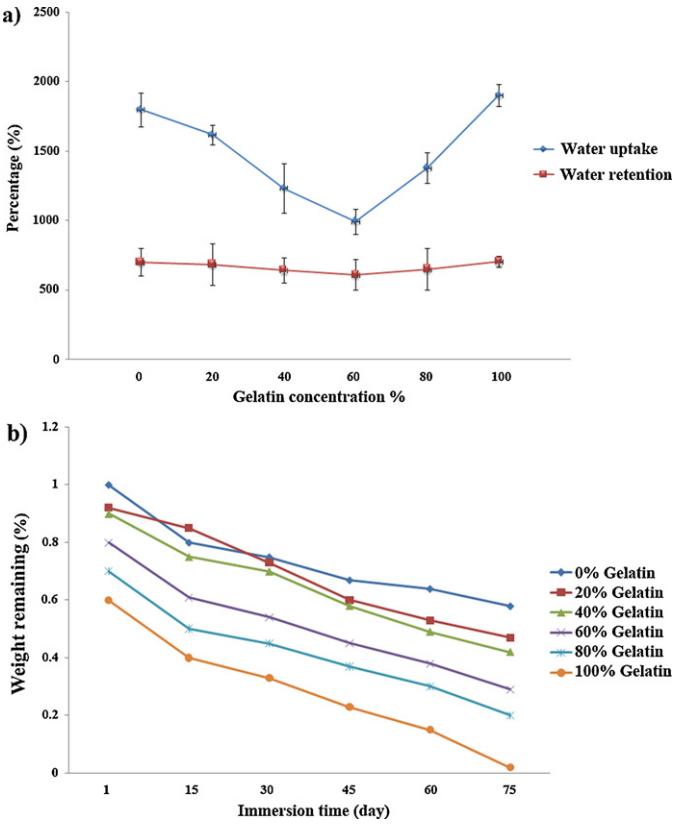

Fig. 7. (a) Water uptake and water retention behavior of different samples cross-linked through scaffold cross-linking method in 25 °C for 24 h and 1% genipin concentration versus G concentration. (b) Weight loss behavior of different samples cross-linked through scaffold cross-linking method in 25 °C for 24 h and 1% genipin concentration versus different time intervals.

influence of cross-linking time on the mechanical strength corresponded with our presumption that the mechanical strength would increase with a prolonged cross-linking time. Under certain conditions, i.e., the same concentration and temperature, prolonging the cross-linking time is helpful to make the reaction sufficient. Moreover, increasing the cross-linking time seemed to have little influence on the pore sizes of the scaffolds (Bi et al., 2011). Thus, the compressive strength showed a tendency to increase with increased cross-linking time.

The difference between young modulus of the prepared samples cross-linked with two different methods is shown in Fig. 6(a). According to this figure, the scaffolds cross-linked with scaffold cross-linking method showed higher compressive modulus.

3.4. Density measurement

The concentration of G/CC solution has a crucial influence on the density of final scaffolds. As shown in Fig. 6(b), by increasing the concentration of G in the hydrogel combination, a slight decrease in the density of scaffolds was resulted. As the graph illustrated slip of this decrease was tangible between 20 and 60%, which could be aimed by the specific physical bonds between G and CC. For different volume of G/CC solutions, the lower concentration of the solution cause less density, larger pore size, and smaller compressive strength (Mao, Zhao et al., 2003).

3.5. Water uptake and water retention tests

Water uptake and water retention abilities of cross-linked G/CC scaffolds are shown in Fig. 7(a). As it can be seen in this figure, the water uptake of different samples with various concentrations has significant difference. The addition of G into the CC samples decreases the degree of water absorption. However, there is no significant difference between water retention ability of CC, G and their blended scaffolds. Here, a principal rule can be obtained in which the scaffolds containing larger amount of CC observed water more than their weight. Water uptake and water retention ability of the scaffolds measured after applying suitable condition for cross-linking (1% concentration of genipin in 25 °C for 24 h) and scaffold cross-linking method.

3.6. In vitro biodegradation properties

An ideal scaffold for load-bearing soft tissues should have an adequate degradation behavior which allow ingrowths of new tissues and thereafter allow remodeling of these tissues in load-bearing conditions. In a study, van Tienen et al. (2002) investigated the in vitro degradation of two slightly different polymers, and observed that both polymers had lost about 50% of their initial mass during 24 weeks. They suggested that the biological remodeling of new tissues should be able to match with the degradation rate of the polymers, which shows the importance of degradation speed. To investigate the degradation behavior of G/CC scaffolds cross-linked by scaffold cross-linking method, they were incubated in lysozyme containing PBS solution, the data shown in Fig. 7(b). The scaffolds in PBS showed a reduction in weight, probably due to the loss of G part. However, the presence of lysozyme slightly decreased the weight of samples, suggesting degradation of CC in the blended scaffolds (Huang et al., 2005). At the end of 6 weeks, the net weight of CC and G/CC scaffolds in the presence of lysozyme were comparable. As it can be seen, by increasing the G concentration a significant increase in weight loss has been observed. Finally, the samples were cross-linked in an optimized condition in which the concentration

of genipin was 1% (at 25 °C for 24 h). In another word, it could be concluded that further addition of the G concentration caused an increase in the rate of biodegradation behavior (Huang et al., 2005).

4. Conclusion

Here, a systematic study was conducted to investigate the possibility of using genipin as a green cross-linker to modify G/CC scaffolds for soft tissue applications. The cross-linked scaffolds possessed different porous structures depending on the ratios of G and CC in the scaffolds. The cross-linked porous scaffolds were fabricated with significantly controllable mechanical properties and suitable characteristics for specific use in load-bearing musculoskeletal tissues. The experiments also suggested that the mechanical property of scaffolds could be effectively adjusted by the ratio of the components, cross-linker concentration and cross-linking method. This detail study demonstrated that the optimized hybrid scaffolds could be suitable candidates for use in load-bearing soft tissue engineering.

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