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Effects of different cross-linking conditions on the properties of genipin-cross-linked chitosan/collagen scaffolds for cartilage tissue engineering

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Abstract A cross-linking reagent is required to improve mechanical strength and degradation properties of biopolymers for tissue engineering. To find the optimal preparative method, we prepared diverse genipin-cross-linked chitosan/collagen scaffolds using different genipin concentrations and various cross-linking temperatures and cross-linking times. The compressive strength increased with the increasing of genipin concentration from 0.1 to 1.0%, but when concentration exceeded 1.0%, the compressive strength decreased. Similarly, the compressive strength increased with the increasing of temperature from 4 to 20°C, but when temperature reached 37°C, the compressive strength decreased. Showing a different trend from the above two factors, the effect of cross-linking time on the compressive strength had a single increasing tendency. The other results also demonstrated that the pore size, degradation rate and swelling ratio changed significantly with different cross-linking conditions. Based on our study, 1.0% genipin concentration, 20°C cross-linking temperature and longer cross-linking time are recommended.

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1 Introduction

Articular cartilage is an important component of the musculoskeletal system and often suffers injuries (caused by trauma, disease, and age-related degeneration) that may result in unbearable pain and dysfunction [1, 2]. Due to the presence of relatively few cells with low mitotic activity, a low supply of progenitor cells and a lack of vascularization, the adult cartilage tissue has limited self-repair capability [3]. To overcome these biological limitations, many surgical procedures such as drilling, abrasion, and microfracture of the subchondral bone have been applied [4, 5]. Although the repaired tissue seems in some cases satisfactory and can decrease pain and morbidity in the short term, the repaired tissue differs in its mechanical properties in comparison with native articular cartilage, and therefore the long-term repairing effects of such methods are barely satisfactory [6, 7]. Other techniques for cartilage repair have included the transplantation of allograft or autograft cartilage in an effort to restore tissue function. However, the uncertain long-term efficacy and significant morbidity with the donor site of autograft cartilage in the joint limit the application of these techniques [7, 8].

Tissue engineering is a promising therapeutic approach that combines cells, biomaterials, and environmental factors to induce differentiation signals into surgically transplantable formats to promote tissue repair and/or functional restoration. The ultimate goal of tissue engineering is to regenerate the native architecture and function of the targeted tissue. The introduction of tissue engineering techniques in the reconstruction and regeneration of cartilage has shown great potential [7, 9]. In tissue engineering techniques, the regeneration of any tissue is achieved by culturing isolated cells on biocompatible and biodegradable

materials as scaffolds onto which cells are seeded and induced to proliferate [10]. A large number of studies have shown the importance of selecting appropriate biomaterials as scaffolds to support cell adhesion and proliferation [11–13].

Chitosan has recently received much attention in the field of biomaterial research because of its special properties, its inexpensive, abundant supply, and its wide range of uses, from the food industry to the biomedical and pharmaceutical fields [14]. Chitosan is a partially deacetylated derivative of chitin, which is the primary structural polymer in arthropod exoskeletons, shells of crustaceans, or the cuticles of insects [15]. As a semi-crystalline polysaccharide, it is normally insoluble in aqueous solutions above pH 7. But in dilute acids (pH 6), its free amine groups are protonated and the molecule becomes soluble. This pH-dependent solubility provides a convenient mechanism for processing under mild conditions [16]. Chitosan is proven to be nontoxic, biodegradable, and biocompatible, and has structural similarities to glycosaminoglycans (GAG), which are an important structural component of the cartilage extracellular matrix (ECM) [17, 18]. As the most abundant protein in cartilage tissue, collagen has been a popular component for use together with chitosan in recent cartilage repair studies [19]. However, some major drawbacks of the current scaffolds made of collagen and/or chitosan are their limited mechanical strength and and their rapid biodegradation.

In order to improve the mechanical strength of polymers, chemical cross-linking reagents, such as glutaraldehyde, formaldehyde, and epoxy compounds, have been introduced into collagen or chitosan-based scaffolds [20–22]. However, all these chemical reagents are highly cytotoxic and may impair the biocompatibility of the cross-linked biomaterials [23, 24]. For these reasons, much interest is now directed toward naturally derived cross-linking reagents with low toxicity.

Genipin is a hydrolytic product of geniposide that can be isolated from the fruits of *Gardenia jasminoides Ellis* [25]. It can spontaneously react with the amine groups on amino acids or proteins to form dark blue pigments [26]. The cytotoxicity of genipin is significantly lower than that of chemical cross-linking reagents, and it was reported that genipin is about 5000–10,000 times less cytotoxic than glutaraldehyde [27]. Additionally, the biocompatibility of materials cross-linked by genipin is superior to those cross-linked by glutaraldehyde or epoxy compounds [28]. Most importantly, the genipin-cross-linked tissue had a comparable mechanical strength and resistance against in vitro enzymatic degradation to the glutaraldehyde-cross-linked tissue [28]. Although the application of genipin as a cross-linking reagent for chitosan-based scaffolds seems prospective, the optimal method has not been established since properties of scaffolds can be greatly affected by different cross-linking conditions.

In the present study, we prepared diverse genipin-cross-linked collagen/chitosan scaffolds with different genipin concentrations and various cross-linking temperatures and cross-linking times. In order to find the optimal preparative method, the pore sizes, porosity rate, cross-linking index, swelling ratio, degradation rate, compressive strength, and biocompatibility of these scaffolds were investigated and compared.

2 Materials and Methods

2.1 Preparation of genipin-cross-linked collagen/chitosan scaffolds

Both a 2% (w/v) solution of collagen type II (Sigma, USA) and chitosan (87.4% degree of deacetylation, 4.66×10^5 Da molecular weight purchased from Vanson HaloSource, USA) were prepared in 0.2 M acetic acid. The two solutions were then mixed at a ratio of 1: 3(v/v). These blends were frozen at −20°C and lyophilized for 12 h in a freeze dryer (Alpha1-2, christ, Germany) to make pre-scaffolds.

Pre-scaffolds of uniform size (6 mm in diameter, 3 mm in thickness) were divided into several groups (as in Table 1) and cross-linked by immersion in 10 ml of 40% (v/v) ethanol containing different concentrations (0.1%, 0.5%, 1.0%, 1.5%, 2.0%) of genipin (Wako Pure Chemical Industries, Ltd., Japan) for different time periods (6 h, 12 h, 18 h, 24 h) at different temperatures (4°C, 20°C, 37°C). In order to observe the effects of each single factor (genipin concentration, cross-linking time or cross-linking temperature) on the properties of the scaffolds, two conditions (e.g. 0.1% concentration, 6 h or 4°C) were kept constant while the third condition was altered to determine

Table 1 Division conditions

Groups	Genipin concentration	Cross-linking time	Cross-linking temperature
A	0.1%	6 h	4°C
B	0.5%	6 h	4°C
C	1.0%	6 h	4°C
D	1.5%	6 h	4°C
E	2.0%	6 h	4°C
F	0.1%	12 h	4°C
G	0.1%	18 h	4°C
H	0.1%	24 h	4°C
I	0.1%	6 h	20°C
J	0.1%	6 h	37°C
K	0	0	0

the effects of that condition. After being cross-linked, scaffolds of 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 Na_2HPO_4 (pH 9.1) for 1 h. The excess base was removed by repeated washing with distilled water until the matrix pH returned to the physiologic range (7.0–7.4), and then the matrix was freeze-dried for the subsequent experiments.

2.2 Mechanical test

Scaffolds of different groups were made into cylinder samples of 10 mm in diameter and 20 mm in height ($n = 5$). The mechanical test was performed when the specimens were completely dried. The mechanical test was analyzed in a materials testing machine (MTS-858, MTS System Inc, USA) by compression in the vertical direction at a deformation rate of 5 mm/min until failure at 20°C. The compressive strength was calculated by $S = F_{max}/A$, where F_{max} is the maximum load on the load-deformation curve and A is the cross-sectional area of each sample.

2.3 Observation of porosity and pore size

Scaffolds of different groups were mounted onto aluminum stubs, followed by coating with gold–palladium. A scanning electron microscope (SEM, S-3000N, Hitachi, Japan) was used to perform the morphological analysis. The porosity was estimated by liquid displacement methods [29]. The pore sizes of scaffolds were determined by a mercury intrusion porosimeter (AutoPore IV 9500, Micromeritics, USA) as reference [30] with a fling pressure changing from 3.4 to 413.7 MPa.

2.4 In vitro degradation

The in vitro degradation was determined in 25 ml PBS containing 37.5 μg of lysozyme (Sigma-Aldrich, Canada) at 37°C. The concentration of lysozyme was chosen to correspond to the concentration in human serum [15]. Briefly, scaffolds ($n = 5$) with a known dry weight of each group were sterilized by ethylene oxide and incubated in the lysozyme solution with gentle mechanical agitation. The lysozyme solution was refreshed daily to ensure continuous enzyme activity. After 7, 14, 21 and 28 days, scaffolds were removed from the solution, rinsed with distilled water, dried under a vacuum and weighed. The extent of in vitro degradation was calculated according to the following equation: $D = [(W_0 - W_t)/W_0] \times 100\%$, where D is the degradation rate, W_0 is the initial dried weight of the scaffold and W_t is the dried weight of the scaffold after degradation.

2.5 Determination of the cross-linking degree (CD)

CD was determined by the ninhydrin (NHN) assay [31]. NHN solution was prepared as follows: Solution A: 25 ml deionized H_2O containing 1.05 g citric acid, 0.4 g NaOH and 0.04 g $SnCl_2 \cdot 2H_2O$; Solution B: 25 ml ethylene glycol monomethyl containing 1 g NHN. The two solutions were mixed with stirring for 45 min. The final NHN solution was stored in a dark bottle. Before determination, the scaffolds were weighed and a 5 mg sample from each different groups 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 using a spectrophometer (UV-210, Hitachi, Japan). After heating with NHN, the amount of free amino groups in the test sample was proportional to the optical absorbance of the solution. The concentration of free NH_2 groups in the sample was determined from a standard curve of the glycine concentration versus absorbance. The measured concentration was divided by the sample weight and multiplied by the sample molecular weight to obtain the mole NH_2/mole sample. The CD of the sample was calculated using the following equation: $CD = [(\text{NHN reactive amine})_{fresh} - (\text{NHN reactive amine})_{fixed}]/(\text{NHN reactive amine})_{fresh} \times 100\%$, where 'fresh' is the mole fraction of free NH_2 in non-cross-linked samples and 'fixed' is the mole fraction of free NH_2 remaining in cross-linked samples ($n = 5$).

2.6 Determination of the swelling ratio

The swelling ratio was determined by immersion in phosphate buffered saline (PBS) (pH 7.4) at 20°C with gentle shaking as reference [32]. Twenty-four hours later, the weight of the swollen scaffold was measured and the swelling ratio was calculated with the following equation: $E_{sw} = [(W_{sw} - W_0)/W_0] \times 100\%$, where E_{sw} is the swelling ratio of the scaffold, W_0 is the initial dried weight of the scaffold and W_{sw} is the weight of the swollen scaffold ($n = 5$).

2.7 Cell isolation

Bone marrow-derived cells (BDCs) and adipose tissue-derived cells (ADCs) were used to observe the biocompatibility of these scaffolds. BDCs were obtained from the os longum of female white New Zealand rabbits aged between 8 and 12 months, weighting between 2.5 and 3.3 kg (all animal procedures used in the current study

were approved by our institutional animal care committee) as reference [33]. Briefly, the rabbits were anesthetized with an intramuscular administration of ketamine (50 mg/kg) and xylazine (10 mg/kg). Under general anesthesia, the bone marrow was aspirated from the tibia using a 10 ml syringe containing 0.1 ml heparin (3000 U/ml saline solution), with a 16-gauge needle. The marrow aspirates were suspended in PBS, centrifuged, and resuspended in a control medium of Dulbecco's modified Eagle's medium (DMEM; Sigma, USA) supplemented with 1% antibiotics/antimycotics (final concentration: penicillin 100 units/ml, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B) and 10% PBS. The cells were plated at a concentration of 2×10^6 nucleated cell/cm^2 in flasks. The non-adhenrent cells were removed by changing the medium. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 containing 10% fetal bovine serum (FBS; HyClone, USA) and 1% penicillin/streptomycin. The medium was changed every 3 days. Cell subcultures of the third passage were used in the experiments. The ADCs were prepared as described previously with modifications [34]. The adipose tissue of female white New Zealand rabbits (between 8 and 12 months) was washed two times with PBS. After centrifugation at 1500 rpm for 5 min, the resultant adipose tissue was digested for 30 min at 37°C with 15 ml of 0.075% collagenase type II (Sigma, USA) and then 15 ml of DMEM containing 10% FBS. The fiber fragments and tissue debris were separated by 100 μm nylon filters. The filtered cells were suspended in DMEM containing 10% FBS and then plated in a tissue culture flask. The non-adherent cells were removed by replacing the medium at 72 h. The culture medium (DMEM with 10% FBS, 100 U/ml penicillin, and 100 g/ml streptomycin) was changed every 3–4 days during the culture. Adherent ADCs were rinsed thoroughly with PBS and detached with 0.05% trypsin- EDTA. Cell subcultures of the third passage were used in the experiments.

2.8 Cytotoxicity assay

In order to evaluate the cytotoxicity of the scaffold, BMCs or ADCs were seeded at a density of 3×10^4 cells/well in 12-well plates in stationary conditions. Prior to cell culture experiments, 12-well plates were placed under UV light for 30 min for sterilization. Scaffolds of different genipin concentrations (0, 0.1%, 0.5%, 1%, 1.5% and 2%), having 6 mm diameter and 3 mm thickness, were sterilized by gamma radiation at 25 KGy for 1 h. Then scaffolds were immersed in conditioning medium for 1.5 h prior to cell seeding. The cells were kept in a 37°C, 5% CO_2 environment. The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed for different time periods: 1, 3, 5, 7 and 14 days as follows. The cell-seeded scaffolds ($n = 5$) were rinsed in 0.15 M PBS and immersed in a mixture consisting of serum-free cell culture medium and MTT reagent at a 5:1 ratio. The incubation was performed for 3 h at 37°C in a humidified atmosphere containing 5% CO_2. After this, 100 μl of cells were transferred to 96-well plates and the optical absorbance values were determined at 490 nm. Cells cultured without scaffolds were used as a control.

2.9 Cell adhesion and morphology by SEM

Cell adhesion, morphology, and average distribution were observed by SEM analysis. The cell-scaffold constructs were washed in 0.15 M PBS and fixed in 2.5% glutaraldehyde (in PBS). The samples were then 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. The samples were allowed to air dry afterward, and then they were sputter coated with gold and analyzed with the S-3000N SEM.

2.10 Statistical analyses

Statistical analyses were performed using SPSS software, version 12.0 (SPSS Inc, USA). The data are presented as means ± SD, and the levels were compared by the non-parametric Mann–Whitney U test or the Student's t-test. P values less than 0.05 were considered as significant.

3 Results

3.1 Mechanical testing

As shown in Fig. 1a, under the initial condition, the compressive strength increased greatly when the genipin concentration increased from 0.1 to 1.0% ($P < 0.05$), but when the genipin concentration was between 1.0 and 2.0%, the compressive strength decreased significantly ($P < 0.05$). Under the initial condition, the compressive strength significantly increased from 6 to 24 h ($P < 0.05$) (Fig. 1b). The compressive strength varied with temperature. The strength at 20°C was significantly higher than that at either 4 or 37°C ($P < 0.05$). There were no significant differences of compressive strength between 4 and 37°C ($P > 0.05$) (Fig. 1c).

3.2 The porosity and pore size

All the scaffolds showed a three-dimensional (3D) porous structure. It was observed that pores inside the scaffolds were interconnected in irregular patterns and the pore size varied over a large range under different cross-linking

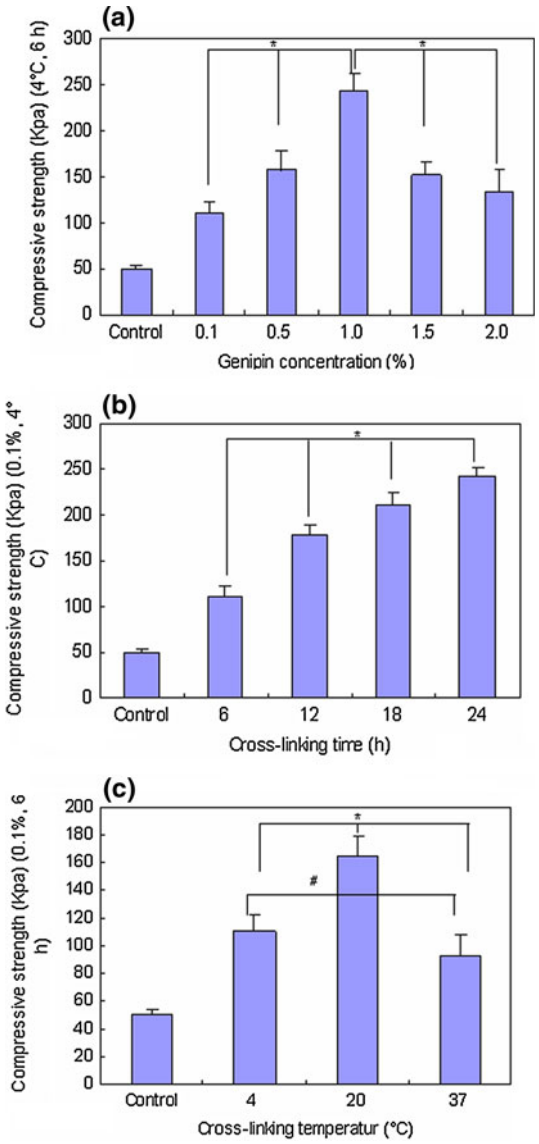

Fig. 1 **a** shows that with an increasing genipin concentration, the compressive strength increased gradually and reached a peak at a concentration of 1.0% (* $P < 0.05$). From 1.0 to 2.0%, the compressive strength decreased greatly (* $P < 0.05$). By contrast, with a prolonged cross-linking time the compressive strength showed only a single tendency to increase. The compressive strength under the cross-linking temperature of 20°C was significantly higher than that at either 4 or 37°C (* $P < 0.05$). **b**. There were no significant differences of compressive strength between 4 and 37°C ($^{\#}$ $P > 0.05$). **c** Values represent the mean ($n = 5$) ± SD. * $P < 0.05$ non-parametric Mann–Whitney U test and the Student's t-test

conditions. Both the genipin concentration and cross-linking temperature greatly affected the final porous structures of the scaffolds (Fig. 2). The pore size of the scaffold increased gradually with increasing genipin concentration ($P < 0.05$) (Fig. 3a). Similarly, the pore size of the scaffold also increased with increasing cross-linking temperature ($P < 0.05$) (Fig. 3c). However, the pore size did not change much with increasing cross-linking time ($P > 0.05$) (Fig. 3b). The porosity rate showed the same changing tendency as the pore size. With increasing genipin concentration, the porosity rate of the scaffold increased gradually ($P < 0.05$) (Fig. 4a). Similarly, the porosity rate also increased with increasing cross-linking temperature ($P < 0.05$) (Fig. 4c). However, the porosity rate did not change much with increasing cross-linking time ($P > 0.05$) (Fig. 4b).

3.3 In vitro degradation

The degradation rates of all cross-linked scaffolds were significantly lower than that of the control (uncross-linked scaffold) at different defined conditions ($P < 0.05$). An accelerated mass loss was observed at the 0.1% genipin concentration from 7 to 28 days ($P < 0.05$). Using a concentration of 0.5% or 1.0%, there was no significant mass loss from 7 to 28 days ($P > 0.05$). By contrast, with concentrations of 1.5% and 2.0%, the degradation did not change much from 7 to 21 days ($P > 0.05$), but both degradations showed a sudden increase from 21 to 28 days ($P < 0.05$) (Fig. 5a). Under the initial condition, the degradation rate decreased with an increasing cross-linking time ($P < 0.05$) (Fig. 5b). The degradation rate also decreased with an increase of the cross-linking temperature ($P < 0.05$) (Fig. 5c).

3.4 Determination of the cross-linking degree (CD)

All of the cross-linked scaffolds turned dark-bluish. The color deepened with the increase of genipin concentration and the cross-linking time. As shown in Fig. 6a, under the initial condition, the cross-linking degree increased gradually when the genipin concentration increased from 0.1 to 1.0% ($P < 0.05$), but not from 1.0 to 2.0% genipin solution ($P > 0.05$). Figure 6c also shows that the cross-linking degree changed greatly with the cross-linking temperature. The cross-linking degree of either 20 or 37°C was significantly higher than that of 4°C ($P < 0.05$). Also, the cross-linking degree of 37°C was significantly higher than that of 20°C ($P < 0.05$). Under the initial condition, the cross-linking degree increased with the prolongation of the cross-linking time ($P < 0.05$) (Fig. 6b).

3.5 Swelling ratio

The swelling ratio of each cross-linked scaffold was much lower than that of the control (uncross-linked) ($P < 0.05$). The swelling ratios of the scaffolds significantly decreased with the increase of both cross-linking time and temperature ($P < 0.05$). The swelling ratio also decreased with increasing genipin concentrations from 0.1 to 1.0%

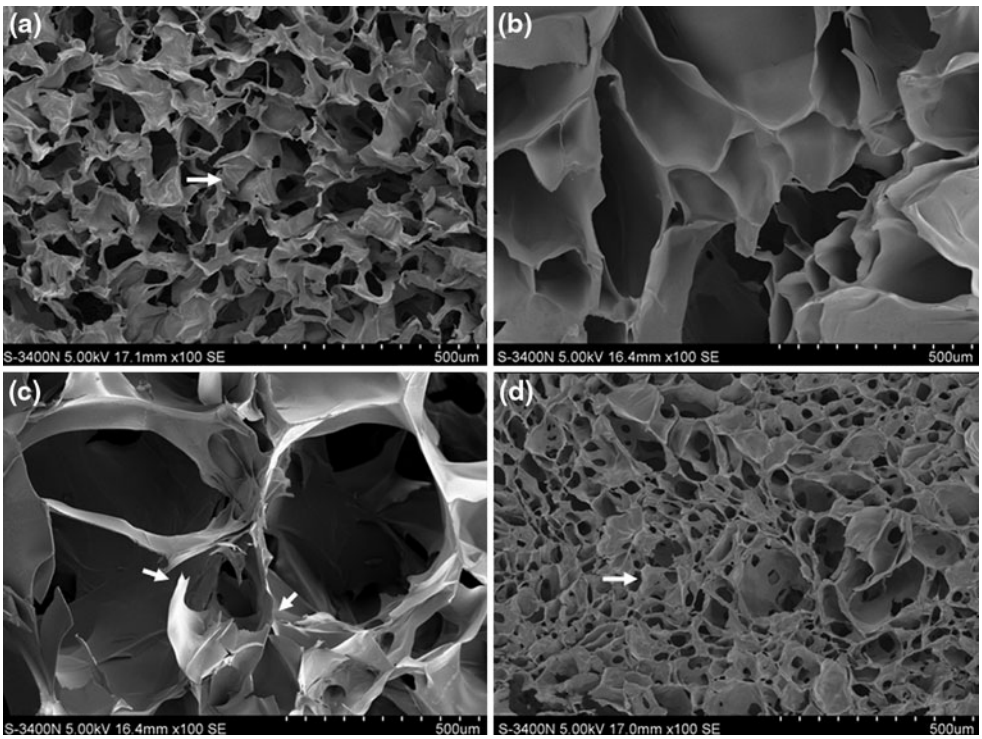

Fig. 2 All of the scaffolds showed a three-dimensional (3D) and porous structure. The pore wall of the uncross-linked scaffold was paper-like (arrow) with a smaller pore size and porosity (**a**). With an increasing genipin concentration (**b**) and cross-linking temperature (**c**), the pore size became larger. At a temperature of 37°C, the pores of the scaffold became broken and many fissures were formed on the surface of the pore walls (arrow, **c**). The pore size showed no changes with an increase of cross-linking time, and 12 h later the pore wall became thicker (arrow, **d**)

($P < 0.05$), but from 1.0 to 2.0%, the swelling ratio changed only a little ($P > 0.05$) (Fig. 7).

3.6 Cytotoxicity assay

MTT was performed to assess the cell viability at different defined time periods. The scaffolds showed a slight inhibitory effect on the viability of BDCs, and this effect was not independent of the genipin concentration. From 5 to 14 days, the MTT results of all the scaffold groups were lower than that of the control group (without scaffold) ($P < 0.05$) (Fig. 8a). On the contrary, it can be observed that the ADCs were able to remain viable within the scaffolds during the entire experimental time, although it seemed that the MTT of the scaffold groups was a little lower than that of the control group (without scaffolds) at the 1 and 3 days time points ($P > 0.05$). There were no differences in the values among the different scaffold groups from 1 to 14 days ($P > 0.05$) (Fig. 8b).

3.7 Cell adhesion and morphology by SEM

SEM analysis revealed that ADCs were well adhered onto the scaffold surface and appeared to be morphologically normal throughout the entire experiment. From the SEM picture obtained at 1 week it was clear that cells adhered to the scaffolds and spread along its structure, forming multilayers. Cell morphology was still clearly fibroblastic-like, with extensive cell-to-cell interactions (Fig. 9b). On the contrary, BDCs could seldom be found on the surface of the scaffolds (Fig. 9a).

4 Discussion

An ideal scaffold for cartilage tissue engineering should maintain sufficient mechanical strength and a stable 3D structure during the in vitro and in vivo growth and remodeling process. Many factors, including the concentration of components and the cross-linking reagents, cross-linking time, composition of the solvent mixture, and cross-linking temperatures as well as freezing temperatures, affected the final mechanical strength and the porous structure of scaffolds made of polymers [35–37]. Thus, in order to yield ideal polymer scaffolds, the optimal cross-linking condition should be investigated.

Based on our knowledge of cross-linking, we proposed a hypothesis that the compressive strength of the polymer material should increase with the development of the cross-linking reagent. But in this study, we found that the

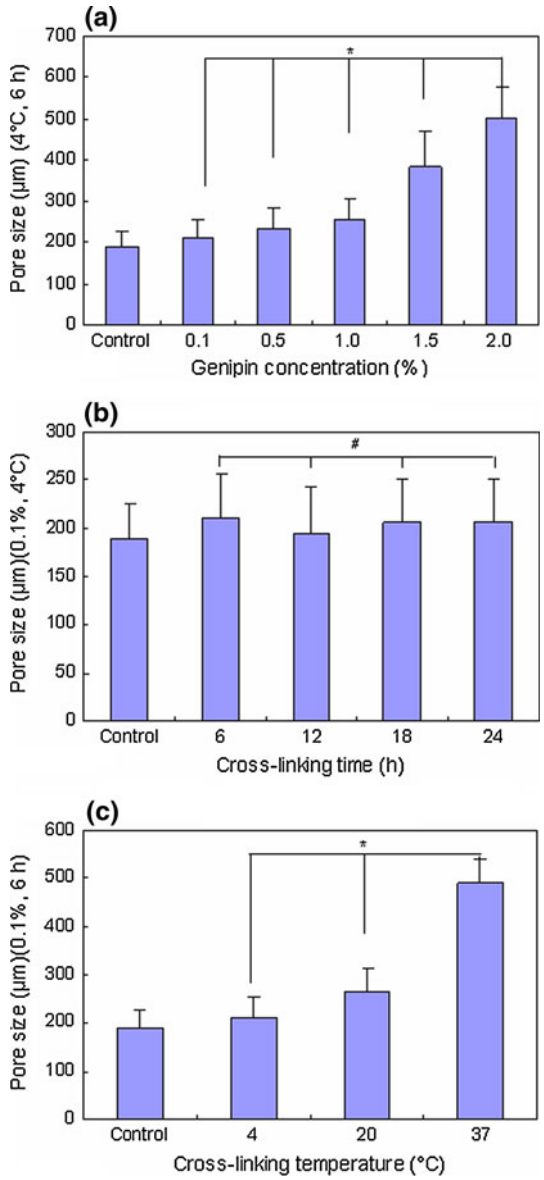

Fig. 3 The pore size of the scaffolds increased gradually with an increase of both the genipin concentration (**a**) and the cross-linking temperature (**c**) (* $P < 0.05$). However, the pore size hardly changed with increasing cross-linking time (# $P > 0.05$) (**b**). Values represent the mean (n = 5) ± SD. * $P < 0.05$ non-parametric Mann–Whitney *U* test

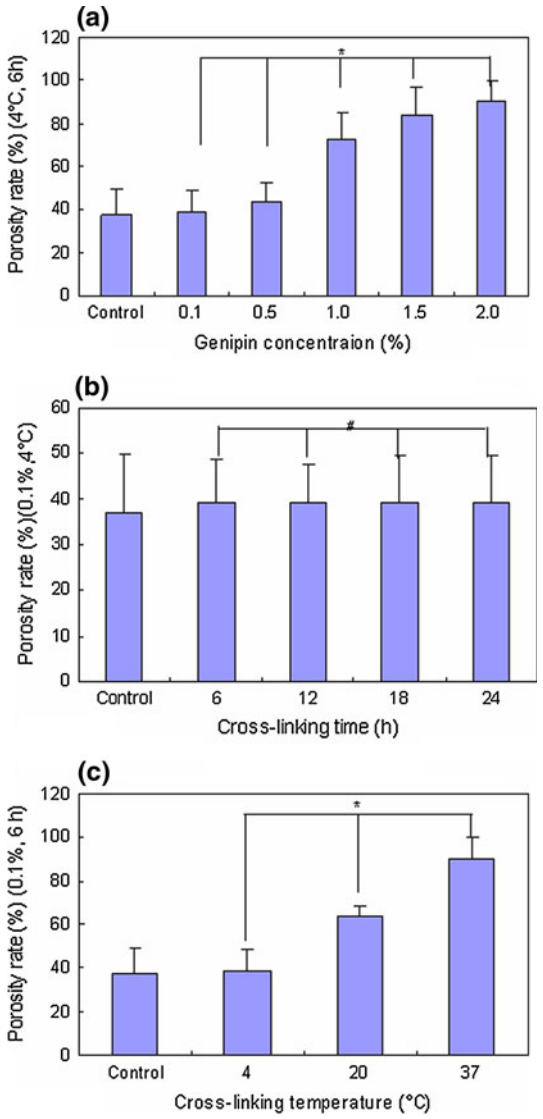

Fig. 4 The porosity rate increased gradually with an increase of both the genipin concentration (**a**) and the cross-linking temperature (**c**) (* $P < 0.05$). However, the porosity rate hardly changed with increasing cross-linking time (# $P > 0.05$) (**b**). Values represent the mean (n = 5) ± SD. * $P < 0.05$ non-parametric Mann–Whitney *U* test

cross-linking reagent genipin caused unexpected effects on the compressive strength of chitosan/collagen scaffolds. Mechanical tests showed that with concentrations between 0.1 and 1.0%, the compressive strength of the scaffold increased gradually. However, at the concentrations from 1.0 to 2.0%, the compressive strength decreased significantly.

To explain the discrepancy between the results of the mechanical test and our presumption, further investigations were performed. By SEM observation and pore size measurement, an interesting phenomenon was revealed: with an increasing genipin concentration, the average pore size of the scaffold also increased. It has been reported that the mechanical strength of a porous scaffold depends mainly on its pore size, and smaller pores are helpful to enhance the biomechanical strength of engineered constructs [36]. It is also well known that cross-linking is an oxidation reaction between active chemical groups that could improve the mechanical strength by forming chemical bonds between the polymers [38]. Thus, two contradictory effects were taking place during the cross-link process: on the one hand, the cross-linking reaction increased the mechanical strength of the scaffold due to the formation of chemical bonds; on the other hand, the mechanical strength was decreased due

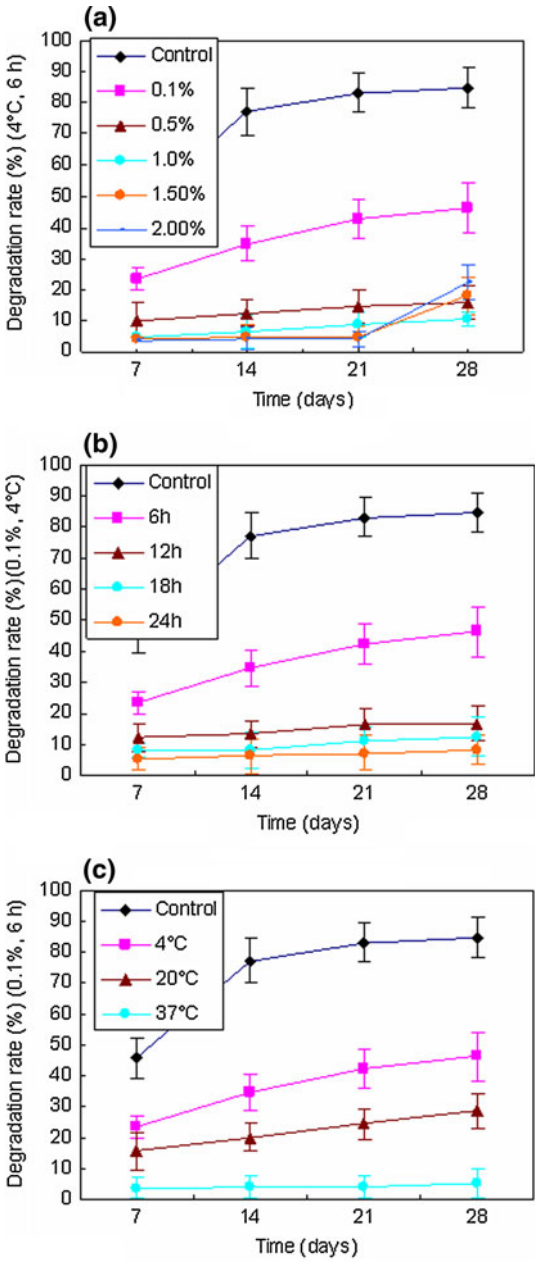

Fig. 5 An accelerated mass loss was observed at a genipin concentration of 0.1% from 7 to 28 days ($P < 0.05$). At concentrations of 0.5% and 1.0%, there was no significant mass loss from 7 to 28 days ($P > 0.05$). At concentrations between 1.5 and 2.0%, the degradation hardly changed from 7 to 21 days and suddenly increased from 21 to 28 days ($P < 0.05$) (**a**). Under the initial condition, the degradation rate decreased with an increasing cross-linking time and cross-linking temperature ($P < 0.05$) (**b–c**). The degradation rate of all cross-linked scaffolds was significantly lower than that of the uncross-linked one ($P < 0.05$). Values represent the mean ($n = 5$) ± SD. * $P < 0.05$ non-parametric Mann–Whitney U test

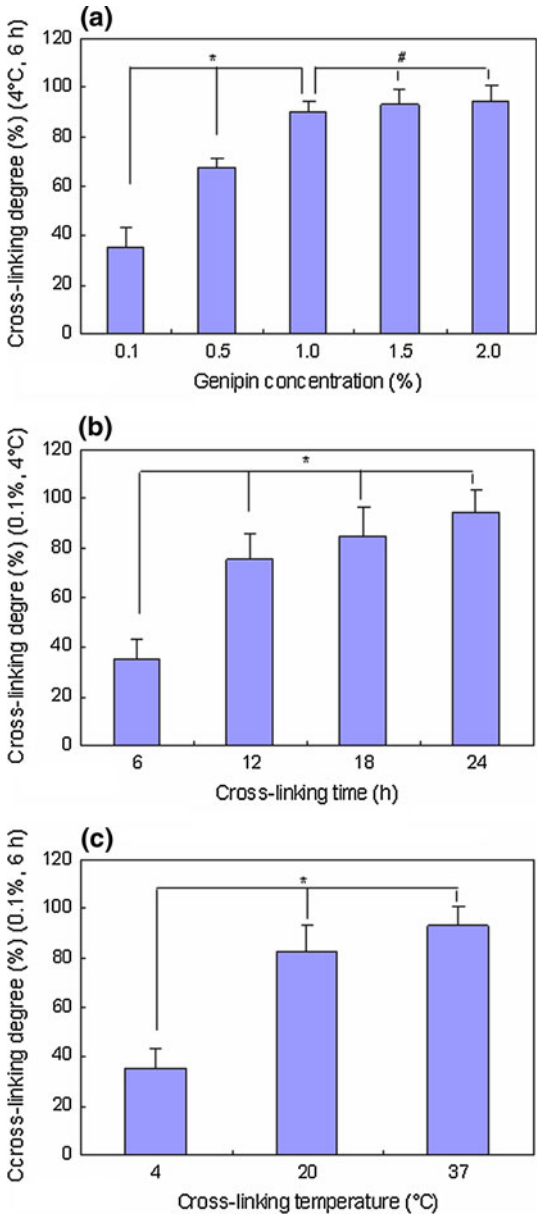

Fig. 6 Under the initial condition, the cross-linking degree increased from 0.1 to 1.0% genipin (* $P < 0.05$), and it did not change much from 1.0 to 2.0% genipin ($^{\#}$ $P > 0.05$) (**a**). However, under the initial condition, the cross-linking degree increased significantly with an increase of both cross-linking time and cross-linking temperature (* $P < 0.05$) (**b–c**). Values represent the mean (n = 5) ± SD. * $P < 0.05$ non-parametric Mann–Whitney U test and the Student's t-test

to the gradually enhanced pore size of the scaffold. The final mechanical strength then seemed to be determined by the balance between the two effects. When the cross-linking reagent was at lower concentrations (from 0.1 to 1.0%), the enhancement of cross-linking on the 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 greatly increased (on average more than 500 μm) and the inhibitive effect of cross-linking was enhanced, which finally resulted in diminished mechanical strength. Moreover, it has been

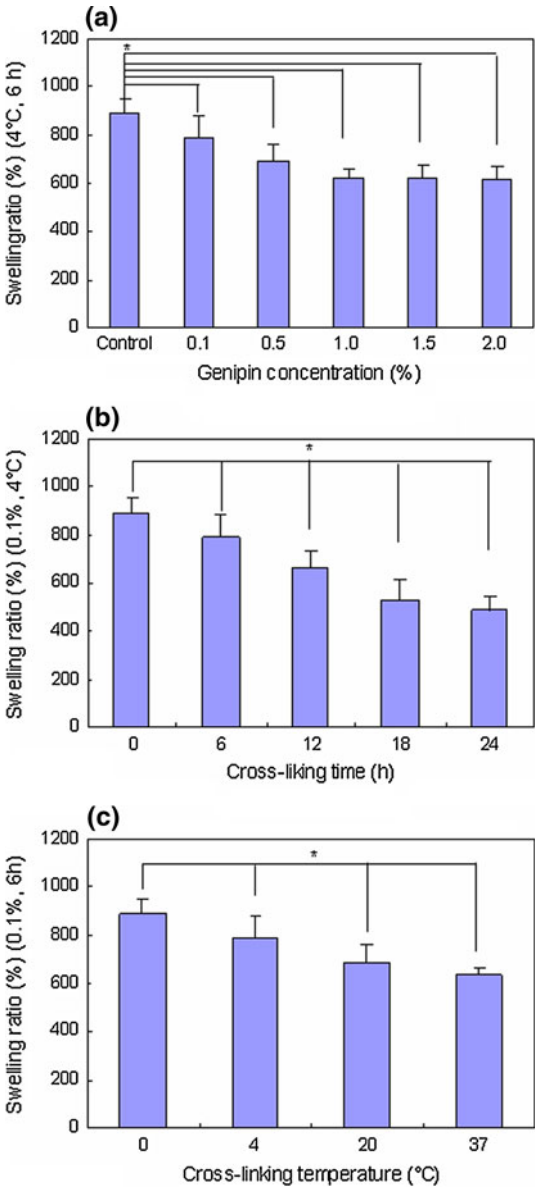

Fig. 7 The swelling ratio decreased with increasing genipin concentration, cross-linking time and temperature. The swelling ratio of each group of cross-linked scaffold was much lower than that of the uncross-linked one. Values represent the mean ($n = 5$) ± SD. * $P < 0.05$ non-parametric Mann–Whitney U test and the Student's t-test

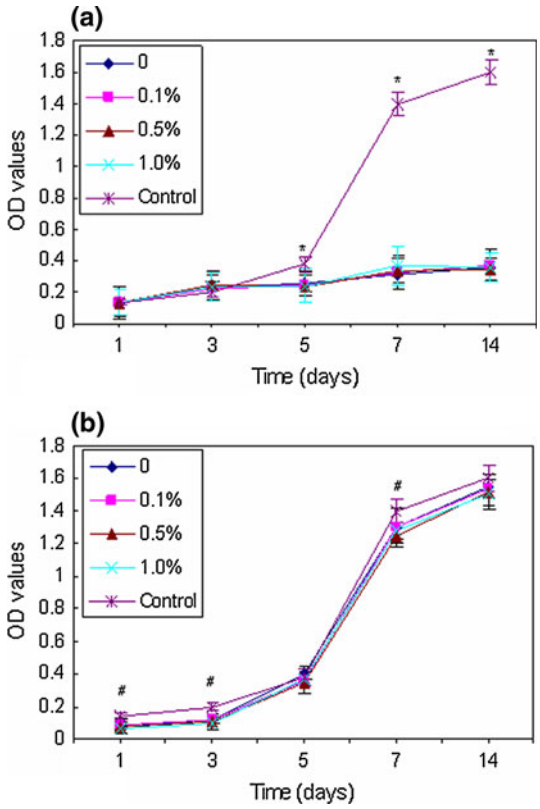

Fig. 8 The optical absorbance (OD) values of BDCs cultured with scaffolds of different genipin concentrations were significantly lower than that of the control group (* $P < 0.05$) (**a**). No differences in values were detected between the entire scaffold groups and the control group when the ADCs were cultured ($^{\#}$ $P > 0.05$) (**b**). Values represent the mean ($n = 5$) ± SD. * $P < 0.05$ non-parametric Mann–Whitney U test and the Student's t-test

reported that an excessively high concentration (more than 2%) of genipin could cause the cross-linking to occur only in the outer layers of the scaffold, and the cross-linking of the inner layers would be limited and insufficient, resulting also in a decrease of the final mechanical strength of the biomaterial [31, 32].

Similarly, direct observation revealed that the average pore size was also enlarged with an increasing cross-linking temperature. The enhancement of temperature also contributes to the reaction of cross-linking [39]. Thus, the changes of mechanical strength caused by cross-linking temperature could also be explained as follows: under lower temperatures, such as 4°C and 20°C, the negative effect of increasing pore sizes on the mechanical strength was lower than the positive effect of cross-linking, resulting in an increase in the compressive strength from 4 to 20°C; but at 37°C, the pore sizes were greatly enlarged, which caused a decline in the compressive strength. Moreover, at 37°C, the structure of the scaffold was found to be broken and many fissures had formed on the surface of the pore walls, which may also explain the decrease of compressive strength.

In contrast to the effects of genipin concentration and cross-linking temperature, the influence of cross-linking time on the mechanical strength corresponded with our presumption, which was that the mechanical strength would increase with a prolonged cross-linking time. It was reported that the cross-linking mechanism consists of two reactions, involving different sites on the genipin molecule [25, 40]. The first step is the nucleophilic attack of the genipin C3 carbon atom from a primary amine group to form an

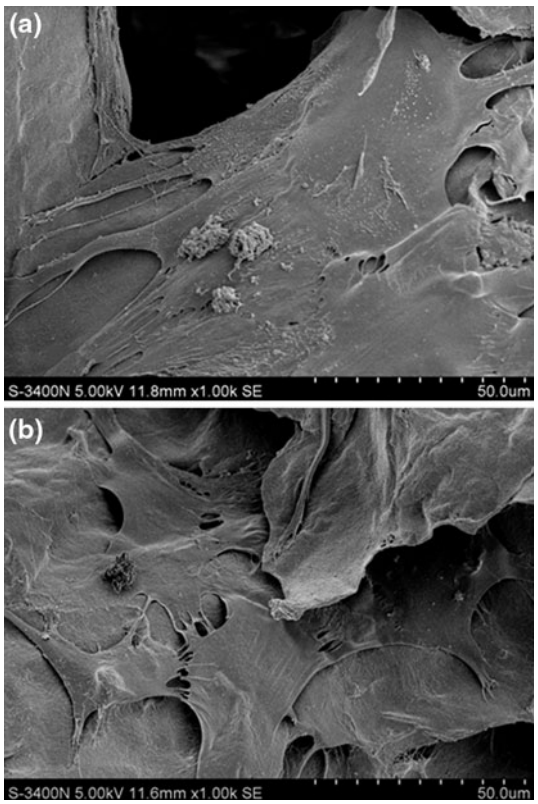

Fig. 9 Few MDCs could be found on the surface of the scaffold (**a**). By contrast, ADCs were well adhered to the scaffold surface and appeared to be morphologically normal throughout the entire experiment (**b**)

intermediate aldehyde group. The newly formed secondary amine reacts with the aldehyde group to form a heterocyclic compound. The following step is a nucleophilic substitution reaction that involves the replacement of the ester group on the G molecule by a secondary amide linkage. The reaction is complicated by the oxygen radical-induced polymerization of genipin that occurs once the heterocyclic compound has formed, giving the gel a blue color. 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 according to our direct measurement. Thus, the compressive strength showed a tendency to increase with increased cross-linking time.

The degradation rate is another important index for the evaluation of scaffolds. In this study we found that the degradation rate at early stages (from 7 to 21 days) seemed to have an inverse relationship with the cross-linking degree, i.e., the degradation ratio decreased with an increasing cross-linking degree. In this study, the cross-linking degree was detected by the NHN assay, which determines the percentage of free amino groups remaining in the material after cross-linking. It has been reported that genipin can undergo a ring-opening reaction to form an intermediate aldehyde group due to the nucleophillic attack by the amino groups in chitosan [41]. Thus, the determination of the percentage of free amino groups remaining in the material after cross-linking could reflect the degree of the cross-linking reaction. Our data demonstrated that chitosan was cross-linked by genipin, and the degree of cross-linking increased with an increasing genipin concentration, cross-linking temperature or cross-linking time. It was also observed that the color of the genipin cross-linked chitosan/collagen scaffold turned dark-bluish, and the bluish color could be attributed to double bonds in the genipin cross-linking molecules [32]. The gradual deepening of the color also revealed an increasing degree of cross-linking.

The swelling ratio is an important index used to evaluate the structural stability of the scaffold. Since chitosan contains hydroxyl and amino groups, it is readily hydrated in water, which will affect the prefabricated structure of the scaffold. Cross-linking can change the hydrophilicity of the scaffold and decrease the swelling ratio, which will contribute towards maintaining the structural stability of the scaffold [32]. Our results revealed that all three factors (concentration, temperature and time) could greatly change the swelling ratio of the chitosan/collagen scaffold. It was also found that when the concentration of genipin was increased beyond 1.0%, the swelling ratio stopped changing. These results are similar to a previous report in which the swelling of scaffolds decreased with increasing genipin concentrations up to a certain concentration, but not at higher concentrations [31]. The reason that higher concentrations of genipin do not decrease the swelling of the scaffold to a greater extent may be that higher concentrations of genipin could react and cross-link only with the outer layers of the scaffold. Subsequently, once the outer layers of the scaffold were cross-linked, the cross-linking of the inner layers was limited.

In order to investigate the biocompatibility of these scaffolds, the proliferation of two types of seed cells was investigated when they were cultured with scaffolds at different genipin concentrations. Regarding the MTT test, the results showed that these chitosan/collagen scaffolds seemed to inhibit the proliferation of BDCs. This inhibition was independent of the concentration of genipin; however, it may be a result of a component of chitosan. Chitosan has been reported to have inhibitory effects on the proliferation of some cells, including BDCs, and this side effect could be enhanced with increasing chitosan concentration [42]. It has been found that the degree of deacetylation (DD) of chitosan in chitosan–collagen scaffolds is a crucial factor in determining the biological behavior ofMC3T3-E1 cells in vitro. The chitosan–collagen scaffolds with low-DD chitosan (50%) had a statistically significantly ($P < 0.05$)

higher proliferative effect and ALP activity than those scaffolds with high-DD chitosan (80%) [43]. The DD of chitosan used in our study was more than 80%, which maybe a reason for the inhibitory effect on the proliferation of BDCs. However, a convincing explanation for this phenomenon has not yet been proposed. By contrast, the proliferation of ADCs did not seem to be inhibited in the presence of both chitosan and genipin, although lower OD values were detected at an early stage of culture due to the slower adhesive rate of ADCs at the early stage. With a prolonged culture time, ADCs showed good morphology, presenting morphological and proliferative features similar to those encountered for the blank controls (data not shown).

5 Conclusion

The properties of the genipin-cross-linked chitosan/collagen can be greatly affected by different genipin concentrations, cross-linking temperatures and cross-linking times. In order to yield optimal mechanical, structural and biological properties, a genipin concentration of 1.0% and a cross-linking temperature of 20°C are recommended. And when both genipin concentration and cross-linking temperature are kept constant longer the cross-linking time is effective for the mechanical property.

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References

1. Mehlhorn AT, Niemeyer P, Kaiser S, Finkenzeller G, Stark GB, Südkamp NP, Schmal H. Differential expression pattern of extracellular matrix molecules during chondrogenesis of mesenchymal stem cells from bone marrow and adipose tissue. Tissue Eng. 2006;12:2853–62.
2. Gross AE. Cartilage resurfacing: filling defects. J Arthroplasty. 2003;18:14–7.
3. Wu YN, Yang Z, Hui JH, Ouyang HW, Lee EH. Cartilaginous ECM component-modification of the micro-bead culture system for chondrogenic differentiation of mesenchymal stem cells. Biomaterials. 2007;28:4056–67.
4. Akizuki S, Yasukawa Y, Takizawa T. Does arthroscopic abrasion arthroplasty promote cartilage regeneration in osteoarthritic knees with eburnation? A prospective study of high tibial osteotomy with abrasion arthroplasty versus high tibial osteotomy alone. Arthroscopy. 1997;13:9–17.
5. Ghivizzani SC, Oligino TJ, Robbins PD, Evans CH. Cartilage injury and repair. Phys Med Rehabil Clin N Am. 2000;11:289–307.
6. Nehrer S, Spector M, Minas T. Histologic analysis of tissue after failed cartilage repair procedures. Clin Orthop Relat Res 1999;365:149–62.
7. Guilak F, Awad HA, Fermor B, Leddy HA, Gimble JM. Adipose-derived adult stem cells for cartilage tissue engineering. Biorheology. 2004;41:389–99.
8. Lee CR, Grodzinsky AJ, Hsu HP, Martin SD, Spector M. Effects of harvest and selected cartilage repair procedures on the physical and biochemical properties of articular cartilage in the canine knee. J Orthop Res. 2000;18:790–9.
9. Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials. 2004;25:3211–22.
10. Yamane S, Iwasaki N, Majima T, Funakoshi T, Masuko T, Harada K, Minami A, Monde K, Nishimura S. Feasibility of chitosan-based hyaluronic acid hybrid biomaterial for a novel scaffold in cartilage tissue engineering. Biomaterials. 2005;26:611–9.
11. Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. Biomaterials. 2000;21:2529–43.
12. LeBaron RG, Athanasiou KA. Ex vivo synthesis of articular cartilage. Biomaterials. 2000;21:2575–87.
13. Sechriest VF, Miao YJ, Niyibizi C, Westerhausen-Larson A, Matthew HW, Evans CH, Fu SH, Suh JK. GAG-augmented polysaccharide hydrogel: a novel biocompatible and biodegradable material to support chondrogenesis. J Biomed Mater Res. 2000;49:534–41.
14. Suzuki D, Takahashi M, Abe M, Sarukawa J, Tamura H, Tokura S, Kurahashi Y, Nagano A. Comparison of various mixtures of beta-chitin and chitosan as a scaffold for three-dimensional culture of rabbit chondrocytes. J Mater Sci Mater Med. 2008;19:1307–15.
15. Freier T, Koh HS, Kazazian K, Shoichet MS. Controlling cell adhesion and degradation of chitosan films by N-acetylation. Biomaterials. 2005;26:5872–8.
16. Madihally SV, Matthew HW. Porous chitosan scaffolds for tissue engineering. Biomaterials. 1999;20:1133–42.
17. Correlo VM, Boesel LF, Pinho E, Costa-Pinto AR, Alves da Silva ML, Bhattacharya M, Mano JF, Neves NM, Reis RL. Melt-based compression-molded scaffolds from chitosan-polyester blends and composites: Morphology and mechanical properties. J Biomed Mater Res A. 2009;91:489–504.
18. Mahmoudifar N, Doran PM. Tissue engineering of human cartilage and osteochondral composites using recirculation bioreactors. Biomaterials. 2005;26:7012–24.
19. Martin I, Miot S, Barbero A, Jakob M, Wendt D. Osteochondral tissue engineering. J Biomech. 2007;40:750–65.
20. Koide T, Daito M. Effects of various collagen crosslinking techniques on mechanical properties of collagen film. Dent Mater J. 1997;16:1–9.
21. Weadock K, Olson RM, Silver FH. Evaluation of collagen crosslinking techniques. Biomater Med Devices Artif Organs. 1983–1984;11:293–318.
22. Vizárová K, Bakos D, Rehákova M, Petríkova M, Panáková E, Koller J. Modification of layered atelocollagen: enzymatic degradation and cytotoxicity evaluation. Biomaterials. 1995;16:1217–21.
23. Speer DP, Chvapil M, Eskelson CD, Ulreich J. Biological effects of residual glutaraldehyde in glutaraldehyde-tanned collagen biomaterials. J Biomed Mater Res. 1980;14:753–64.
24. Nishi C, Nakajima N, Ikada Y. In vitro evaluation of cytotoxxicity of diepoxy compounds used for biomaterial modification. J Biomed Mater Res. 1995;29:829–34.
25. Chiono V, Pulieri E, Vozzi G, Ciardelli G, Ahluwalia A, Giusti P. Genipin-crosslinked chitosan/gelatin blends for biomedical applications. J Mater Sci Mater Med. 2008;19:889–98.
26. Sung HW, Chang Y, Liang IL, Chang WH, Chen YC. Fixation of biological tissues with a naturally occurring crosslinking agent: fixation rate and effects of pH, temperature, and initial fixative concentration. J Biomed Mater Res. 2000;52:77–87.
27. Cauich-Rodriguez JV, Deb S, Smith R. Effect of cross-linking agents on the dynamic mechanical properties of hydrogel blends

of poly (acrylic acid)-poly (vinyl alcohol-vinyl acetate. Biomaterials. 1996;17:2259–64.
28. Sung HW, Huang RN, Huang LLH, Tsai CC, Chiu CT. Feasibility study of a natural crosslinking reagent for biological tissue fixation. J Biomed Mater Res. 1998;42:560–7.
29. Zhang R, Ma PX. Poly (alpha-hydroxyl acids)/hydroxyapatite porous composites for bone-tissue engineering. I. Preparation and morphology. J Biomed Mater Res. 1999;44:446–55.
30. Nettles DL, Elder SH, Gilbert JA. Potential use of chitosan as a cell scaffold material for cartilage tissue engineering. Tissue Eng. 2002;8:1009–16.
31. Mi FL, Tan YC, Liang HC, Huang RN, Sung HW. In vitro evaluation of a chitosan membrane cross-linked with genipin. J Biomater Sci Polym Ed. 2001;12:835–50.
32. Yuan Y, Chesnutt BM, Utturkar G. The effect of cross-linking of chitosan microspheres with genipin on protein release. Carbohydr Polym. 2007;68:561–7.
33. Kim H, Lee JH, Suh H. Interaction of mesenchymal stem cells and osteoblasts for in vitro osteogenesis. Yonsei Med J. 2003;44:187–97.
34. Ahn HH, Lee JH, Kim KS, Lee JY, Kim MS, Khang G, Lee IW, Lee HB. Polyethyleneimine-mediated gene delivery into human adipose derived stem cells. Biomaterials. 2008;29:2415–22.
35. Wu H, Wan Y, Cao X, Wu Q. Proliferation of chondrocytes on porous poly (DL-lactide)/chitosan scaffolds. Acta Biomater. 2008;4:76–87.
36. Kon M, de Visser AC. A poly (HEMA) sponge for restoration of articular cartilage defects. Plast Reconstr Surg. 1981;67:288–94.
37. Tigli RS, Gumusderelioglu M. Evaluation of alginate-chitosan semi IPNs as cartilage scaffolds. J Mater Sci Mater Med. 2009;20:699–709.
38. Lund MN, Luxford C, Skibsted LH, Davies MJ. Oxidation of myosin by haem proteins generates myosin radicals and protein cross-links. Biochem J. 2008;410:565–74.
39. Fu MX, Wells-Knecht KJ, Blackledge JA, Lyons TJ, Thorpe SR, Baynes JW. Glycation, glycoxidation, and cross-linking of collagen by glucose. Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. Diabetes. 1994;43:676–83.
40. Butler MF. Mechanism and kinetics of phase separation in a gelatin/maltodextrin mixture studied by small-angle light scattering. Biomacromolecules. 2002;3:676–83.
41. Mi FL, Tan YC, Liang HF, Sung HW. In vivo biocompatibility and degradability of a novel injectable-chitosan-based implant. Biomaterials. 2002;23:181–91.
42. Tan W, Krishnaraj R, Desai TA. Evaluation of nanostructured composite collagen-chitosan matrices for tissue engineering. Tissue Eng. 2001;7:203–10.
43. Suphasiriroj W, Yotnuengnit P, Surarit R, Pichyangkura R. The fundamental parameters of chitosan in polymer scaffolds affecting osteoblasts (MC3T3-E1). J Mater Sci Mater Med. 2009;309–20.