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Preparation of collagen–chondroitin sulfate–hyaluronic acid hybrid hydrogel scaffolds and cell compatibility in vitro

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ABSTRACT

Ideal scaffolds for tissue engineering should mimic natural extracellular matrix (ECM) as much as possible. The aim of this study was to develop a biomimetic hydrogel scaffold with good operability and stability by collagen self-assembly. To improve physical and chemical performances, the hydrogels were crosslinked with different concentrations of genipin using gel-crosslinking method. Morphology, crosslinking index, and content of glycosaminoglycans of the hydrogel scaffolds were characterized. The potential of the hydrogels as cartilage tissue engineering scaffolds was evaluated by compression test, swelling measurement, in vitro degradation assay, and biocompatibility study in vitro. Results showed that hydrogel scaffold crosslinked with 0.75 mM genipin exhibited balanced optimal properties, and excellent cellular compatibility. Since gelation was temperature sensitive and crosslink occurred under mild conditions, the hydrogel scaffold may be well suited as a carrier for chondrocytes transplantation or as cartilage tissue engineering scaffolds.

1. Introduction

Biomaterials play a pivotal role as scaffolds to provide three-dimensional templates and synthetic extracellular matrix (ECM) environments for tissue regeneration. ECM serves as a reservoir of signaling molecules, both adhesion molecules and growth factors, to instruct cell decision processes. Therefore, to serve as the temporary ECM for regenerative cells, it may be beneficial for the scaffold to mimic the key characteristics of the natural ECM (Ma, 2008; Patterson, Martino, & Hubbell, 2010). Among the materials used for tissue engineering applications, hydrogels are of interest for encapsulation of cells to ensure adequate cell–cell interactions. The high water content of the scaffold allows for efficient diffusion of nutrition and oxygen into, and waste and carbon dioxide out of the hydrogel (Degoricija et al., 2008). Especially, hydrogels are promising scaffold materials for cartilage tissue engineering because they are water-saturated turgid networks that mimic the three-dimensional environment of cells in native cartilaginous tissue, and were shown to be beneficial in supporting the chondrogenic phenotype with their ability to maintain cells in spherical shape (Chao, Grayson, & Vunjak-Novakovic, 2007; Drury & Mooney, 2003). Besides that, hydrogels used in these applications should be degradable, processed under relatively mild conditions, have mechanical and structural properties similar to macromolecular-based components in the ECM (Drury & Mooney, 2003; Lee & Mooney, 2001). However, when used in cartilage tissue engineering, the hydrogels must be able to be gelled without damaging the cells, and have sufficient mechanical integrity and strength to withstand manipulations associated with implantation and in vivo existence.

Some recent attention has focused on intelligent hydrogels that undergo gel–sol phase transitions under mild (physiological) conditions. Many physical and chemical stimuli have been investigated to induce various responses of the intelligent hydrogel systems. The physical stimuli include temperature, light, pressure, sound and magnetic fields, while the biochemical stimuli include pH, ions and specific molecular recognition events (Qiu & Park, 2001). Temperature-sensitive hydrogels, such as poly (*N*-isopropylacrylamide) and chitosan (Chenite et al., 2000; Cho et al., 2004), are widely investigated because of their lower critical solution temperature in the range of 25–32 °C, closing to the body temperature. Photopolymerizing hydrogel systems also provide an effective method to encapsulate cells (Elisseeff et al., 2000). However, unless chromophores are covalently linked to the polymer backbone, they can be leached out during swelling–deswelling cycles. Alginate hydrogel, a well-known example of a polymer that crosslinked by ionic interactions, has been successfully shown to support chondrocytic phenotype by maintaining cell shape through encapsulation (Hauselmann et al., 1994). But it undergoes slow and uncontrolled dissolution through ion exchange of calcium, which results in loss

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of mechanical stiffness over time (LeRoux, Guilak, & Setton, 1999).

Type I collagen (Col), the primary structural protein of the native ECM, is the most widely used natural polymer for making scaffolds. Furthermore, it has desirable functional properties, making it favorable for cellular growth (Liao, Chan, & Ramakrishna, 2008; Patterson et al., 2010). Studies have showed that collagen hydrogels had the ability of creating a cellular microenvironment mimicking chondrogenic environment to induce chondrogenesis of MSCs in vivo, without the addition of growth factor TGF-β1 (Zheng et al., 2009). The basic structure of all collagen is composed of three polypeptide chains, which wrap around one another to form a three-stranded rope structure. The strands can self aggregate to form stable fibers in the physiological condition to provide mechanical support (Lee, Singla, & Lee, 2001). In addition, the biological and mechanical properties of collagen hydrogel could be enhanced by blending it with other polymers (i.e., poly (glycolic acid), poly (lactic-co-glycolic acid), chitosan) (Chen, Ushida, & Tateishi, 2001; Tan, Krishnaraj, & Desai, 2001), and by introducing various chemical crosslinkers (i.e., glutaraldehyde, formaldehyde, carbodiimide) (Lee, Grodzinsky, & Spector, 2001; Park, Park, Kim, Song, & Suh, 2002). Glycosaminoglycans (GAGs), mainly for chondroitin sulfate (CS) and hyaluronic acid (HA), are ubiquitous components of the extracellular matrix (ECM) of cartilage and participate in a wide variety of biological processes. CS is the most abundant GAG, varies from 20 to 80% of total GAGs (Almarza & Athanasiou, 2004). Inclusion of chondroitin sulfate in scaffold may promote the secretion of proteoglycan and type II collagen, and influence the bioactivity of the seeded chondrocytes (Sechriest et al., 2000; van Susante et al., 2001). Hyaluronic acid performs important biological functions, such as regulating cell adhesion and motility, and mediating cell proliferation and differentiation, which is presented in high amounts in the ECM during embryonic cartilage development, and also facilitates the integration of engineered cartilage (Allemann et al., 2001; Dowthwaite, Edwards, & Pitsillides, 1998; Ishida, Tanaka, Morimoto, Takigawa, & Eto, 1997; Solchaga, Goldberg, & Caplan, 2001). Consequently, the combination of collagen–chondroitin sulfate–hyaluronic acid may partly mimic the cartilage extracellular matrix and then yield out biomimetic environment for chondrocytes when the cells were encapsulated.

In this study, a novel collagen–chondroitin sulfate–hyaluronic acid hybrid hydrogel scaffold that partly mimicked the composition and structure of cartilage extracellular matrix was synthesized through collagen self-assembling. Chondroitin sulfate and hyaluronic acid were firstly crosslinked by ADH to reduce their solubility in water, and then were blended with neutral collagen solution to form composite hydrogel. As the composite hydrogel is of poor intensity and biodegradability, a chemical crosslinking agent with good biocompatibility was adopted to improve the performance. The effects of crosslinking were investigated by the characterization of the active components content, compression strength, water uptake ability, and enzymatic degradation rate, and the potential of the crosslinked hybrid hydrogel scaffold used in cartilage tissue engineering was evaluated by cellular compatibility test.

2. Materials and methods

2.1. Materials

Type I collagen (Col) was extracted from calf skin and suspended in a 0.5 M acetic acid solution at a concentration of 7 mg/ml. Hyaluronic acid (150 kDa, Shanghai, China) and chondroitin sulfate came from Shanghai Qisheng Biological Preparation Co., Ltd. 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) and adipic dihydrazide (ADH) were purchased from Sigma Chemical Co.(St. Louis, MO, USA). Genipin was purchased from Challenge Bioproducts Co., Ltd., Taiwan.

2.2. Preparation of hydrogels

CS and HA were mixed in a 15:1 ratio and dissolved in distilled water at a concentration of 80 mg/ml. ADH was added in solid form to give a final concentration of 110 mg/ml. Then EDC were dissolved in the mixture at a concentration of 40 mg/ml while maintaining the pH at 4.75 by the addition of 1.0 M hydrochloric acid. The reaction was stopped by raising the pH of reaction mixture to 7.0, followed by exhaustive dialysis (Pouyani & Prestwich, 1997). The ADH modified CS and HA were dissolved in D_2O, and analyzed by ^{1}HNMR spectroscopy (Bruker AV11-600MH spectrophotometer). The obtained mixture was homogenized using an IKA T10 basic, Ultra-Turrax homogenizer (IKA-werke, Germany) to form a colloidal dispersion, and then was added into neutral collagen solution (pH 7.4) with weight a ratio of 1:4 at 4 °C. After uniformly mixed, the solution was incubated at 37 °C for 30 min to form elastic collagen–chondroitin sulfate–hyaluronic acid hybrid hydrogels (CCH). For crosslinking, a gel-crosslinking method was adopted, in which the CCH hydrogels after physical gel formation were immersed directly into 0.6 mM, 0.75 mM, and 1 mM genipin solution at 37 °C for 5 h, respectively. The prepared crosslinked hydrogels were named CCH-0.6, CCH-0.75 and CCH-1, respectively. Pure collagen hydrogels (Col) without crosslinking were prepared to be the control.

2.3. Morphology observation

The morphologies of the hybrid hydrogels frozen at −20 °C and lyophilized at −50 °C were characterized by a scanning electron microscope (SEM, S-800, HITACHI, Tokyo, Japan). Before observation, the samples were coated by ultrathin layer of gold/Pt in an ion sputter. Pore size of the lyophilized hydrogels were measured from SEM images using Image Pro Plus 6.0 software (Media Cybernetics, USA).

2.4. Evaluation of crosslinking index

The samples were respectively heated to 120 °C in 2 ml hydrochloric acid (1.0 M) for 12 h, and then were neutralized by adding 1 M NaOH. 1 ml of the neutralized solution mixed with 1 ml acetic acid buffer and 1 ml ninhydrin solution was incubated at 100 °C in water bath for 15 min. Then the mixture was cooled down to room temperature, diluted with 3 ml 60% ethyl alcohol. At last, the optical absorbance of the mixture at 570 nm was determined with a microplate reader (Bio-Rad550). The extent of crosslinking was indicated by the crosslinking index defined as

$$\text{Crosslinking index}\,(\%) = \left[\frac{NH_o - NH_t}{NH_o}\right] \times 100$$

where NH_o is the amount of free amino groups in the sample before crosslinking; NH_t is the amount of free amino groups in the sample after crosslinking (Lien, Li, & Huang, 2008).

Three parallel measurements were averaged for each sample.

2.5. Quantification of immobilized glycosaminoglycans

The glycosaminoglycans content in hybrid hydrogels was determined by hexosamine analysis using *p*-dimethylaminobenzaldehyde (Pieper, Oosterhof, Dijkstra, Veerkamp, & Van Kuppevelt, 1999). CS and HA (with a mass ratio of 15:1) were used as standards. The glycosaminoglycans content was expressed as residual weight/original weight.

2.6. Compression test

Cylindrical hydrogels that were 34.6 mm in diameter and 13.5 mm in height were prepared for compression tests. Samples swelled in distilled water for 24 h were compressed to 50% of their original height at a constant speed of 5 mm/min. Compression testing was done on three different cylinders for each kind of hydrogels. The elastic modulus was calculated from the linear portion of the stress–strain curves.

2.7. Swelling measurements

The air-dried samples were immersed in distilled water at 25 °C. Then the samples were taken out at predetermined time points and wiped with filter paper to remove excess liquid. The weight of the air-dried samples before and after immersion was weighted. The water uptake of sponges was calculated according to the formula:

$$\text{Water uptake}\,(\%) = \left[\frac{W_t - W_0}{W_0}\right] \times 100$$

where W_0 is the weight of the dried sample and W_t is the weight of the swollen sample.

2.8. In vitro degradation

In vitro degradation of hydrogels was performed using trypsin with an activity of 200 units/mg. Samples of about 3 mg dry weight were incubated in 3 ml of trypsin solution at 37 °C. Then the samples were taken out at predetermined time points and dipped into 30 ml of distilled water three times and lyophilized. The extent of degradation was calculated as the percentage of weight difference of the dry sample before and after degradation. Samples were tested in triplicate.

2.9. Cell proliferation test and histological staining

Cell proliferation of CCH-0.75 and Col was assessed. Generally, chondrocytes were isolated from articular cartilage of newborn rabbits. The cells were seeded in plastic culture dishes and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C, using α-MEM with 10% FBS. CCH-0.75 and Col gels were air-dried to form membranes, and placed in 24-well dishes and exposed to ultraviolet light for 24 h before use. The 3rd passage (P3) chondrocytes were collected and suspended in α-MEM with 10% FBS, with a cell density of 2×10^4 cells/ml. 1 ml the cell suspension was added to each well with CCH membrane and control well, and cultured for 12, 24 h, 2, 4, and 6 days. Then, the proliferated cells were quantified by MTT assay. In detail, 200 μL MTT solution (5 mg/ml in 0.9% NaCl, filter sterilized) was added to each culture well. After incubation for 4 h, the MTT reaction medium was removed, and 1 ml dimethylsulfoxide was added. Optical densities were determined by ELISA plate reader (Spectra Max 340, Molecular Device Inc., CA) at a wavelength of 490 nm.

The CCH-0.75 membrane and Col membrane loaded chondrocytes were harvested and fixed in 75% ethanol solution for 30 min at day 6, and then were stained with safranin O and toluidine blue according to routine histology protocol to characterize GAGs secretion. The stained membranes were observed and recorded by inverted phase contrast microscope (OLYMPUS CK40).

2.10. Statistical analysis

All data were presented as means ± standard deviation. Significance levels were determined by the Student's *t*-test. All statistical calculations were performed using SPSS 15.0 statistical software (SPSS Inc., Chicago, USA).

3. Results and discussions

3.1. Reaction mechanism

Generally, collagen triple helices could aggregate to form fibers and fiber bundles by lateral and longitudinal association. Primary microfibrils aggregate through inter- and intra-crosslinking at the optimum pH and temperature to form larger collagen fibrils (Ripamonti et al., 2004). Based on this phenomenon, macromolecule could be entangled by collagen fibers to form composite. Since natural CS and HA could diffuse slowly and dissolve completely in water, we use a convenient method for the chemical modification of CS and HA under mild conditions to preserve the molecular size distribution. Covalent attachment of adipic adihydrazide (ADH) to the carboxylic acid groups results in the availability of pendant hydrazine amino functionalities. HA-ADH and CS-ADH were synthesized respectively and the reaction mechanism was as previously described (Kirker, Luo, Nielson, Shelby, & Prestwich, 2002; Luo, Kirker, & Prestwich, 2000; Pouyani, Harbison, & Prestwich, 1994). ADH may simultaneously react with HA and CS at certain proportion to form the adipic dihydrazide derivatives, which may further react with genipin, and then crosslink with collagen to form a polymer network.

The synthesis of HA-ADH and CS-ADH was confirmed by ^{1}H-NMR spectra; 58.6% of D-glucuronic acid residues were modified by ADH. This was calculated from the ratio of the integrated value of the peak for the methyl proton of ADH residues at 1.62 ppm to that of the methyl proton of N-acetyl-D glucosamine residues at 2.00 ppm (Ito et al., 2007).

To date, the methods used for scaffold crosslinking can be classified into three types, mixing-crosslinking, scaffold-crosslinking and gel-crosslinking (Lien et al., 2008). The gel-crosslinking method was adopted in this study. Compared with the other two methods, gel-crosslinking does less harm to cells embedded in hydrogel and is more convenient for operation. Moreover, the crosslinking agent adopted in this study is biocompatible. The proliferative capacity of cells exposed to genipin was approximately 5000 times greater than that exposed to glutaraldehyde (Sung, Huang, Chang, Huang, & Hsu, 1999; Sung, Huang, Huang, & Tsai, 1999; Sung, Liang, Chen, Huang, & Liang, 2001). Genipin is able to react with the amino groups of the synthesized adipic dihydrazide derivatives and collagen to form intermolecular crosslink inside the hybrid hydrogel system. Though the reaction of genipin crosslinking occurs mainly on the surface of hybrid hydrogel scaffold, the physicochemical properties of scaffold vary with the dosage of crosslinking agent.

3.2. Morphology observation

Fig. 1 shows the photo of CCH hydrogels crosslinked by different genipin concentrations and revealed that the color of hybrid hydrogels became dark blue with the increase of genipin concentration from 0 to 0.75 mM. It is because that genipin produces blue pigments on reaction with amino acids (Touyama, Inoue, et al., 1994; Touyama, Takeda, et al., 1994). The pigment itself acts as a molecular fingerprint that may be optically *in situ* to evaluate the degree of crosslinking. Though there is no evidence that blue pigments have negative influence on cell growth, it may directly affect the definition of data obtained from optical density. When the concentration of genipin increased from 0.75 mM to 1 mM, the color of hybrid hydrogels changed significantly. It suggested that the concentration of genipin should be no more than 1 mM to avoid the obvious change of color.

SEM images were obtained to characterize the microstructure and morphologies of lyophilized hydrogels (Fig. 2). The hybrid hydrogels exhibited homogeneous distribution and interconnected porous structure. Results of pore size measurements

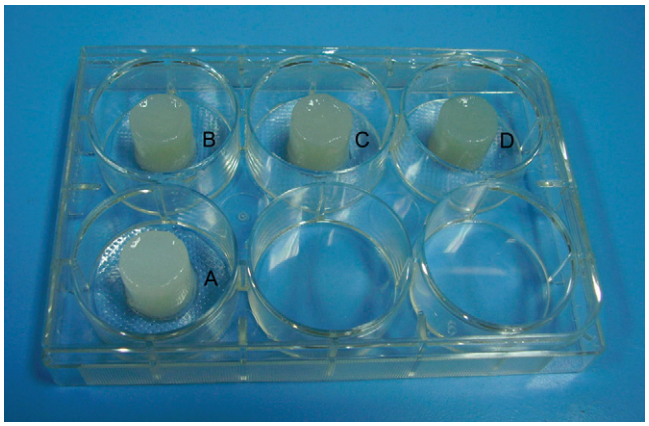

Fig. 1. Photograph of the hybrid hydrogels: (A) CCH, (B) CCH-0.6, (C) CCH-0.75, and (D) CCH-1.

showed that the pore size of CCH, CCH-0.6, CCH-0.75, and CCH-1 were 535.4 ± 126.3 nm, 355.5 ± 91.2 nm, 239.8 ± 50.9 nm and 194 ± 37.2 nm, respectively. No significant phase separation was observed. Generally, physical hydrogel are not homogeneous, since clusters of molecular entanglements, or hydrophobically- or ionically-associated domains, can create inhomogeneities (Hoffman, 2002). Due to the hydrophilicity of CS and HA, both the bound and free water in hydrogel increased. As the hydrophobic groups interact with water molecules, the fibrillogenesis impelled by hydrophobic forces may be inhibited to a certain extent, which leads to the homogeneous fibril structure. The pore size of the lyophilized hydrogels decreased significantly with the increase of genipin concentration from 0 to 0.75 mM, but had no significant decrease while increasing genipin concentration from 0.75 mM to 1 mM. Chemical crosslinked polymers usually have high crosslink density. During crosslinking, phase separation can occur, and water-filled macropores contact with the formation of polymeric network. The reduced pore diameter indicated the increase of crosslinking density. The pore size of the lyophilized hydrogels seemed to reach a plateau when genipin concentration exceeded 0.75 mM, which suggested that crosslinking degree may not increase significantly when genipin concentration exceeded 0.75 mM.

Table 1
Crosslinking index and retention of glycosaminoglycans of hybrid hydrogels crosslinked with genipin solution at different concentration.

Sample	Crosslinking index (%)	Retention of glycosaminoglycans (%)
CCH	0	65.01 ± 1.27
CCH-0.6	20.14	79.94 ± 1.21
CCH-0.75	33.32	82.79 ± 1.00
CCH-1	35.92	83.21 ± 1.76

3.3. Crosslinking index and retention of glycosaminoglycans

Table 1 summarizes the extent of crosslinking and retention of immobilized glycosaminoglycans of four kinds of hybrid hydrogels. Crosslinks have to be presented in a hydrogel in order to prevent dissolution of the hydrophilic polymer chains in an aqueous environment (Hennink & Van Nostrum, 2002). The extent of crosslinking index and retention of glycosaminoglycans increased with increasing genipin concentration up to 1 mM. The crosslinking index of the hydrogel scaffold crosslinked by 0.6 mM genipin was 20.14%, and increased to 33.32% when genipin concentration was 0.75 mM, however, it did not display significant variations when genipin concentration was greater than 0.75 mM. The retention of glycosaminoglycans was 65.01% when the CCH hydrogel was not crosslinked, and increased significantly to 79.94% when genipin concentration was 0.6 mM, while it did not display significant increase when genipin concentration was greater than 0.6 mM.

The extent of crosslinking did not display significant variations at genipin concentration that is greater than 0.75 mM, which is in accordance with the SEM analysis above. The result in Table 1 also suggested genipin is not able to link all the amino groups, even at a higher concentration. Both CS and HA have good water solubility, and are able to undergo diffusion and degradation quickly (Shu, Liu, Luo, Roberts, & Prestwich, 2002; Sintov, Di-Capua, & Rubinstein, 1995). It's worth to note that the content of glycosaminoglycans in hybrid hydrogels without crosslinking was 65%, which proved that ADH modification combining with collagen self-assemble can fix

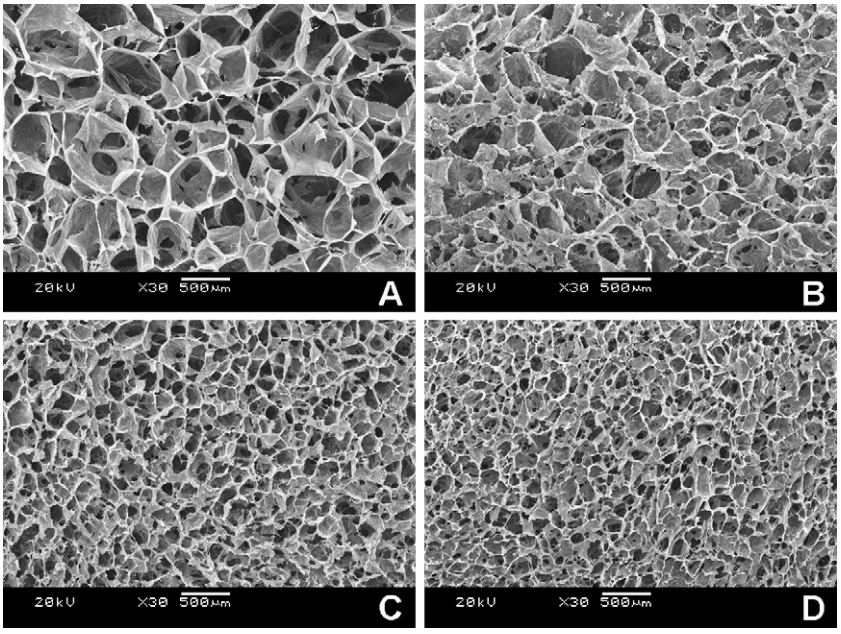

Fig. 2. SEM images of cross section of hybrid scaffold: (A) CCH, (B) CCH-0.6, (C) CCH-0.75, and (D) CCH-1.

Table 2
Compression parameters of hybrid hydrogels crosslinked with genipin solution at different concentration.

Sample	Modulus (kPa)	Compressive stress at 25% strain (kPa)
Col	44.44 ± 0.13	11.27 ± 0.33
CCH	44.93 ± 0.66	11.59 ± 0.24
CCH-0.6	48.09 ± 0.62	12.12 ± 0.31
CCH-0.75	51.53 ± 0.54	13.07 ± 0.42
CCH-1	53.67 ± 0.36	13.21 ± 0.35

most of the glycosaminoglycans. After crosslinking with genipin, the glycosaminoglycans next to the surface of hydrogel integrated into collagen fibers through covalent bonding. The results also revealed that the retention of glycosaminoglycans reached a plateau before crosslinking index with the increase of genipin concentration, which suggested that the surface layer of the hydrogel has the priority to be crosslinked compared to the inner part when genin is insufficient. Additionally, the fact that more than 80% of glycosaminoglycans was immobilized in crosslinked hydrogel scaffolds indicated that the stability of CS and HA has been improved, which is beneficial for the interaction between the biopolymers and cells.

3.4. Mechanical measurements

Table 2 summarizes the compression parameters of mechanical measurements of collagen hydrogels and hybrid hydrogels. The requirements for a suitable tissue-engineering scaffold for cartilage repair are complex and extend beyond basic biocompatibility and low toxicity. As a stress-absorbing tissue, the mechanical properties of cartilage determine its function in the body and are thus an important design criterion for a cartilage repair material. Hydrogel mechanical properties could be affected by the crosslinker type and density (Drury & Mooney, 2003). The results presented in Table 2 showed the compressive modulus of the crosslinked hydrogels with 34.6 mm in diameter increased from 44.93 to 53.67 kPa as increasing genipin concentration increased up to 1 mM. The compression results also suggested that the compressive modulus of non-crosslinked CCH hydrogels was slightly greater than that of Col hydrogels, which may be due to the compressive behavior of the negatively charged GAGs. The theory is that water molecules are attracted to the negative charges of the GAGs, and this hydration effect increases the compressive capabilities of the tissue (Almarza & Athanasiou, 2004; Chao et al., 2007). The compressive modulus was dependent on genipin concentration, and showed a slow increased from 44.93 to 53.67 kPa with the increase of genipin concentration. This may be partly because the hybrid hydrogels were mainly formed by collagen self-assembly of molecular. Although the dosage of genipin was limited, chemical crosslinking of hybrid hydrogels provided more covalent links that resulted in the increase of compressive stress. Self-assembled collagen fibrillar bundles in hydrogel served as a physically reinforcing skeleton, and the mechanical strength of pure collagen hydrogel was sufficient to be used as cell scaffold. Thus the mechanical strength of hybrid hydrogels is mainly determined by the mechanical strength of pure collagen hydrogel. To improve the mechanical properties of hybrid hydrogels, it was feasible to accelerate fibrillogenesis.

3.5. Swelling measurements

Fig. 3 shows the water uptake of collagen hydrogels and hybrid hydrogels crosslinked with genipin solution at different concentration. Park et al. (2009) proposed that chondrogenic differentiation of mesenchymal stem cells (MSCs) in oligo(poly(ethylene glycol) fumarate) (OPF) hydrogel was affected by the swelling ratio, which

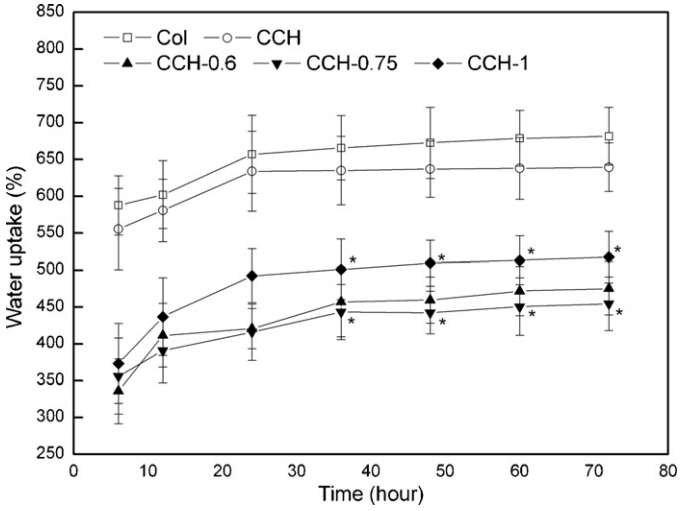

Fig. 3. Water uptake of hybrid hydrogels crosslinked with genipin solution at different concentration ($n = 3$; $^*p < 0.05$).

may be related to the diffusion of signaling molecules and nutrients in hydrogel. Many factors, such as molecular weight of macromer, concentration of macromer, cross-linker and initiator as well as cross-linking extent, can influence the swelling ratio of a crosslinked composite. The curves in Fig. 3 can be divided into two groups. In the first group, water uptake of Col and CCH were in the range 550–690%. In comparison, the water uptake of crosslinked hybrid hydrogels decreased to 330–520%. Generally, higher extent of crosslinking lead to lower water uptake, however, when the genipin concentration was 1 mM, the swelling tendency of hybrid hydrogels increased significantly. Compared to CCH-0.75 hydrogel, The swelling ratio increase of CCH-1 might be resulted from the reaction between collagen and excess genipin according to the retention results of glycosaminoglycans, and the reaction may lead to a critical structure change of the hydrogel. It could be concluded that the structure of CCH-1 with small pores might be favorable for water retaining.

3.6. In vitro degradation

Ideally, the rate of scaffold degradation should mirror the rate of new tissue formation or be adequate for the controlled release of bioactive molecules. Thus, it is important to understand and control both the mechanism and the rate by which each material is degraded (Drury & Mooney, 2003).

Fig. 4 plots weight losses in different periods. The results of in vitro degradation indicated that the degradation of hydrogels depends on the extent of crosslinking. Higher amount of genipin results in less weight loss of the hydrogel. The curves in the figure can also be divided into two groups. In the first group, the weight loss ratio of the hydrogel without crosslinking sharply increased and was approximately 80% after 36 h. In the second group with crosslinking, the weight loss ratio of the hydrogel increased gradually with immersion time and the value of weight loss is less than the first group. The weight loss of hydrogels after the same time decreases with increase of genipin concentration from 0 to 1 mM, while there was no significant difference between the degradation ratio of CCH-0.75 and CCH-1. The curve that corresponded to CCH showed a lower rate of degradation than the curve corresponded to Col, implying that clusters of molecular entanglements between the ADH derivatives and collagen could inhibit the penetration of enzymatic solution to some extent. The Col and CCH hydrogels without crosslinking were almost totally degradated at 36 h. In contrast, hydrogel crosslinked with genipin attenuated the rate of degra-

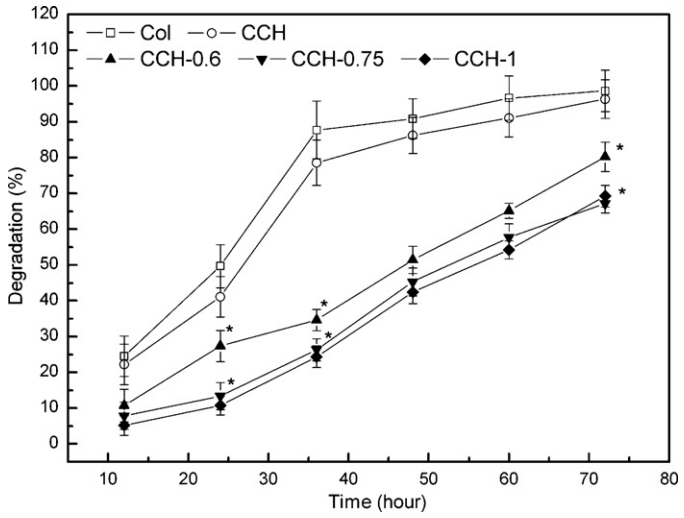

Fig. 4. In vitro degradation of hybrid hydrogels crosslinked with genipin solution at different concentration ($n = 3$; $^*p < 0.05$).

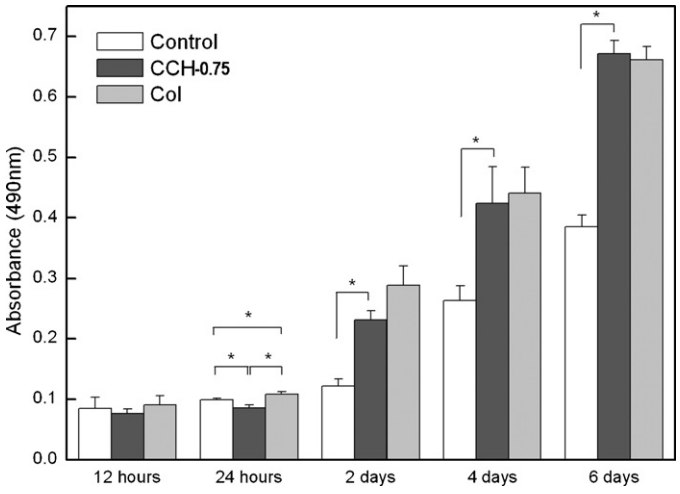

Fig. 5. Cell proliferation assessed by MTT test performed 12, 24 h, 2, 4, and 6 days after seeding ($n = 3$; $^*p < 0.05$).

dation, no more than 30% of the weight lost 36 h later. This also suggested that hydrogels formed by collagen self-assembly was not chemically stable, and they degraded and eventually disintegrated in enzymatic solution. In addition, there was no significant difference between the degradation ratio of CCH-0.75 and CCH-1, which is in accordance with the close crosslinking index between CCH-0.75 and CCH-1.

3.7. Cell proliferation test and histological staining

In consideration of the discussion of above properties, genipin concentration of 0.75 mM might be the optimal condition in this experiment.

Fig. 5 shows the cell proliferation of Col, CCH-0.75, and control group after 12, 24 h, 2, 4, and 6 days after seeding. Chondrocytes adhesion of the three groups had no significant difference for 12 h after seeding. After 24 h, the cell proliferation on Col membrane was enhanced significantly compared with control group, while the CCH-0.75 membrane seemed to inhibit cell proliferation. The different results between Col and control group after 12 h might be caused by a relatively lower chondrocytes adhesion on CCH-0.75 membrane. Both the Col and CCH-0.75 membrane showed significant effect of promoting effect on cell proliferation after 2 days, and the cell proliferation behave nearly the same after 4 days. The results also suggested that collagen–chondroitin sulfate–hyaluronic acid hybrid hydrogel scaffold could promote chondrocytes proliferation, thus possess excellent cellular compatibility.

Fig. 6 shows the histological examination of the specimens after 6 days culture. Chondrocytes changed their morphology from a differentiated round to polygonal cell shape to an elongated fibroblast-like phenotype when grown in monolayer cultures. This phenotypic alteration was accompanied by changes in collagen

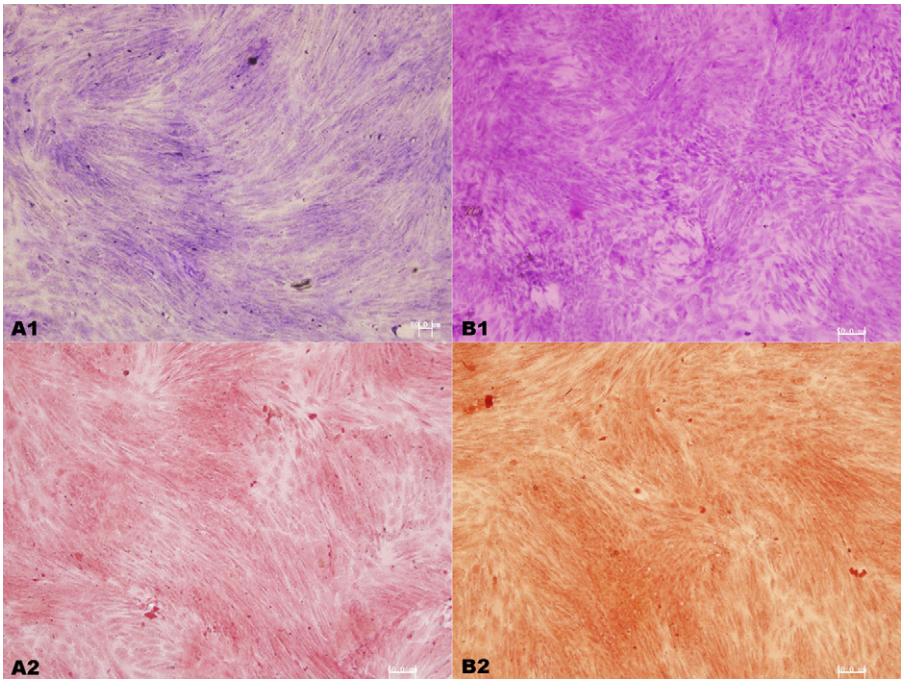

Fig. 6. Chondrocytes-membrane stained with toluidine blue (A1, B1) and safranin O (A2, B2) 6 days after seeding. (A) Col membrane and (B) CCH-0.75 membrane.

expression patterns, and most prominently monolayer cells lose their collagen II and chondromodulin expression, which is highly specific in chondrocytes (Benya & Shaffer, 1982; Schnabel et al., 2002). Histological staining results showed that, on CCH-0.75 membrane (B), the newly formed matrix was stained violet red with toluidine blue and alizarin crimson with safranin O, showing GAG secretion compared with the blank part of the membrane without chondrocytes, while chondrocytes on Col membrane (A) had no significant GAG secretion till 6 day after seeding. It revealed that chondrocytes seeded on CCH-0.75 membrane could maintain their phenotype during culture, while the chondrocytes seeded on Col membrane might undergo a de-differentiation process. Previous results have shown that HA induces a variety of stimulatory signals to regulate chondrocyte proliferation as well as matrix synthesis in cartilage microenvironment, through adhesion to CD44 on chondrocytes (Ishida et al., 1997), and CS may promote the secretion of proteoglycan and type II collagen, and thus influence the bioactivity of the seeded chondrocytes (van Susante et al., 2001). One of the major challenges in chondrocyte transplantation is chondrocyte de-differentiation during in vitro culture and fibrocartilage formation after cell implantation instead of defect healing (Le Blanc, Tammik, Sundberg, Haynesworth, & Ringden, 2003). Monolayer expanded chondrocyte cultures do not express the true chondrocyte phenotype, and their ability to regenerate damaged cartilage tissue is impaired. Upon implantation, dedifferentiated cells may form a fibrous tissue containing collagen type I that does not have the correct mechanical properties, which could lead to degradation and failure of the repair tissue (Brodkin, Garcia, & Levenston, 2004). Therefore, the CCH hybrid hydrogel scaffold may have excellent performance as scaffolds for cartilage tissue engineering, and its performance in vivo need to be investigated further.

4. Conclusions

Novel temperature sensitive hybrid hydrogel scaffolds based on collagen self-assembly were prepared and crosslinked with genipin after gelation. The structure analysis showed that crosslinking index and glycosaminoglycans increased with the concentration of genipin from 0 mM to 1 mM, resulting in increasing of modulus and decreasing of degradation ratio. The prepared hydrogel exhibited relative homogeneous phase, which might be good for cell encapsulation. In regard of the increased precipitation of pigments and the water uptake of hybrid hydrogels crosslinked with genipin at 1 mM, 0.75 mM might be the optimal concentration for hybrid hydrogel crosslinking. In vitro cell culture showed that the hybrid hydrogel scaffold crosslinked with 0.75 mM genipin exhibited excellent cellular compatibility. The prepared collagen–chondroitin sulfate–hyaluronic acid hybrid hydrogel scaffold mimicked the component and structure of extracellular matrix of cartilage and might have a promising application in cartilage tissue engineering.

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