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EDC/NHS-crosslinked type II collagen-chondroitin sulfate scaffold: characterization and in vitro evaluation

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Abstract Three-dimensional biodegradable porous type II collagen scaffolds are interesting materials for cartilage tissue engineering. This study reports the preparation of porous type II collagen-chondroitin sulfate (CS) scaffold using variable concentrations of 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxy-succinimide (NHS). The physico-chemical properties and ultrastructural morphology of the collagen scaffolds were determined. Then, isolated chondrocytes were cultured in porous type II collagen scaffolds either in the presence and/or absence of covalently attached CS up to 14 days. Cell proliferation, the total amount of proteoglycans and type II collagen retained in the scaffold and chondrocytes morphology were evaluated. The results suggest that EDC-crosslinking improves the mechanical stability of collagen-CS scaffolds with increasing EDC concentration. Cell proliferation and the total amount of proteoglycans and type II collagen retained in the scaffolds were higher in type II collagen-CS scaffolds. Histological analysis showed the formation of a denser cartilaginous layer at the scaffold periphery. Scanning electron microscopy (SEM) revealed chondrocytes distributed the porous surface of both scaffolds maintained their spherical morphology. The results of the present study also indicate that type II collagen-CS scaffolds have potential for use in tissue engineering.

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1 Introduction

Articular cartilage is well known to be hardly healed in the case of defects because of its avascular nature and the low mitotic activity of the parenchymal cells. Tissue engineering of the cartilage, in which biocompatible scaffolds are cultured with chondrocytes to prepare transplantable hyaline-like tissues, may provide a more suitable alternative [1]. The composition of the biocompatible scaffolds should maintain the chondrocytes phenotype and a pore structure that accommodates cell infiltration. Furthermore, the scaffolds need to be mechanically stable to withstand surgical handing for the purpose of implantation [2].

Many kinds of scaffolds have been developed to serve as a vehicle to deliver the precultured chondrocytes to a cartilage defect and to offer temporary mechanical support to the cells, until the cells have synthesized a new pericellular matrix. Type II collagen could be introduced as new surgical materials to deal with articular cartilage defects due to its low antigenicity, haemoatatic and cell-binding properties [3]. The disadvantage of using type II collagen as a biomaterial for tissue repair is its rapid biodegradation. To overcome these problems, chemical crosslinking methods have been used to achieve scaffolds with desired mechanical properties. Glutaraldehyde (GA) is the most widely used reagent for collagen crosslinking. However, GA is associated with cytotoxicity in vitro and in vivo [4]. With use of 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) and crosslinking, the crosslinking agent is not incorporated into the amide crosslinks, seems to yield biomaterials with good biocompatibility, higher cellular differentiation potential and increased stability [5, 6].

Type II collagen and chondroitin sulfate (CS) are the main composition in the articular cartilage and create a suitable environment for chondrocytes. Earlier studies have

demonstrated that metabolic parameters of the chondrocytes are influenced by the biochemical composition of the scaffolds. CS are negatively charged polysaccharides with biocharacteristics like hydration of the extracellular matrix and binding of effector molecules (e.g. growth factors and cytokines), thus CS might have such a stimulatory influence on the metabolic activity of seeded chondrocytes [7].

In this in vitro study, we reported the preparation of the porous type II collagen -CS scaffolds with variable concentrations of EDC. The physico-chemical and ultrastructural character of porous type II collagen scaffolds attached CS were assessed. The metabolic activity of seeded chondrocytes was also evaluated in this porous scaffold in the presence and absence of covalently attached CS.

2 Materials and methods

2.1 Type II Collagen extraction and purification

Native insoluble type II collagen was isolated from the joints and sternums of chicks (2–3 months old, local slaughter-house) as described by Miller [8].

2.2 Determination of type II Collagen purification

2.2.1 Total amino acid analysis

For amino acid analysis, the purified collagen was hydrolyzed with 6N hydrochloric acid for 24 h at 120 °C. The resulting mixture was analyzed by an Agilent1100 HPLC system following online derivatisation with *O*-phthalaldehyde (OPA) and 9-fluorenylmethoxycarbonyl (F-MOC) for proline.

2.2.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The collagen fractions obtained as described above were characterized by SDS-PAGE electrophoresis in the presence of 5% 2-mercaptoethanol on polyacrylamide separating gels (7.5%) using a miniprotein electrophoresis system (Bio-Rad). Gels were stained with protein Coomassie brilliant blue R250 [9].

2.3 The preparation of type II collagen film and crosslinking of scaffolds

Type II collagen were dissolved in 0.5 M acetic acid (HAc) solution (5 mg/mL). After centrifugation (3,000 rev/min, 15 min) to remove entrapped air-bubbles, the collagen solutions were dispensed in the 60 mm culture flasks, frozen at –20 °C for 2 days and freeze-dried for 2 days. A type II collagen film was achieved with a thickness of approximately 1.5 mm [10].

The freeze-dried collagen film, in the presence of CS, was crosslinked by using EDC and NHS. Collagen matrices of 25 mg dry weights were incubated for 0.5 h at room temperature in 10 mL of 40% (v/v) ethanol containing 50 mM 2-morpholinoethane sulfonic acid (MES) (pH 5.0). Subsequently, matrices were incubated in 10 mL 40% (v/v) ethanol containing 50 mM MES (pH 5.0) and 2% (w/v) CS for 4 h at room temperature with variable concentrations of EDC (from 1 mg/mL to 15 mg/mL). NHS was added in an EDC: NHS ratio of 4:1. After reaction for 4 h, excess EDC and CS were rinsed from the matrix using 0.1 M Na_2HPO_4 for 1 h followed by four times in deionized water for 30 min. The collagen matrices were freeze-dried [11].

2.4 Determination of physico-chemical and morphologic characteristics

2.4.1 Denaturation temperature (T_d)

The Td was determined with differential scanning calorimetry (DSC) using a Perkin-Elmer DSC-7. Four milligrams of type II Collagen scaffolds were immersed in deionized water at 4 °C for 16 h. The wet samples were wiped with filter paper to remove excess water and hermetically sealed in aluminum pans. Heating rate of 5 °C/min was applied from 20 to 90 °C and the endothermic peak of the thermogram was monitored.

2.4.2 Free amino group content

To assess the degree of collagen crosslinking, the free amino group content of the scaffolds was determined spectrophotometrically using 2, 4, 6-trinitrobenzene 1-sulfonic acid (TNBS) [11]. The free amino group content was expressed as amino groups per 1,000 residues calculated.

2.4.3 Immobilized CS content

The immobilized CS content in the scaffolds was determined by hexosamine analysis using *p*-dimethylaminobenzal dehyde. The CS content was expressed as mg CS/g scaffold [9].

2.4.4 Degradation by collagenase

Type II collagen-CS scaffolds were accurately weighed and placed in 1 mL 0.1 M Tris–HCl (pH 7.4) containing 200 U bacterial collagenase (*Clostridium histolyticum*, EC 3.4.24.3, invitrogen 17101-015). After incubation for 5 h at 37 °C, reaction was stopped by the addition of 0.2 mL 0.25 M ethylenediamine tetraacetic acid (EDTA). The samples were centrifugated (5,000*g*, 15 min, 4 °C). The precipitates were washed three-fold in distilled water (4 °C) and finally lyophilized. Scaffold degradation was determined from the weight of residual scaffold, and expressed as a percentage of the original weight [11].

2.4.5 Swelling ratio

Scaffolds of about 7 mg dry weight were swollen in deionized water for 1 h and then equilibrated overnight in phosphate saline buffer (PBS, pH 7.4) at 4 °C. After removal of the excess surface water with filter dustless paper, the collagen scaffolds were weighed immediately. Subsequently, collagen scaffolds were air-dried to constant weight. The swelling ratio was calculated as a ratio of the weight of the swollen to that of the dried sample [10].

2.4.6 Ultrastructural matrix morphology

The structures of the collagen scaffolds were observed by a QUANTA-200 scanning electron microscopy (SEM).

2.5 Isolation and culturing of chondrocytes

Chondrocytes were isolated from articular cartilage of 1-day-old rabbits. Cartilage was aseptically collected in slices with a surgical blade. The slices rinsed with cold phosphate-buffered saline (PBS, pH 7.4) two times were minced into flakes of about 2 mm × 2 mm, and digested with solutions of 0.02% collagenase (*Clostridium histolyticum*, EC 3.4.24.3, invitrogen 17101-015) in dulbecco' s modified eagle' s medium (DEME) medium for 4 h at 37 °C. Then the chondrocytes were collected by centrifugation (1,200 rev/min, 5 min) and rinsed twice with PBS. Finally, cells were suspended in DMEM containing 10% fetal bovine serum and 50 U/mL penicillin. Chondrocytes viability was determined using Trypan blue dye exclusion [11].

The scaffolds were placed in 6-well microplate wells after sterilization, and then 100 μL cell suspensions containing 5×10^6 chondrocytes was seeded into each scaffold with 5 mm in diameter and 1.5 mm depth The cell-containing scaffolds were cultured at 37 °C, 5% CO_2 and 95% humidity. Culture medium was changed every 2 d. Cultures was terminated after one, seven and 14 days.

2.6 Determination of characteristics for cell-seeded scaffolds

2.6.1 Glycosaminoglycan (GAG) and DNA content

The GAG content of cell-seeded scaffolds was determined by a modification of the 1, 9-dimethyl methylene blue method [12]. The amount of GAG was extrapolated from a standard curve prepared using spark chondroitin sulphate. The amount of DNA was measured using Hoechst 33258 dye while the calf thymus DNA was used as a standard [13]. The results were expressed as μg DNA/per scaffold.

2.6.2 Type II collagen retained in the scaffold

Type II collagen retained in the scaffold was determined by reversed-phase high performance liquid chromatography (RP-HPLC) analysis. Prior to the RP-HPLC analysis, cells-seeded scaffolds for 14 days were incubated in 100 μL 4 M Gua-HCl for 8 h (4 °C) to remove GAG. After centrifugation (10,000 rev/min, 5 min), the precipitates were washed three times in PBS (7.4) and digested in 100 μL 0.1 M HAc containing 0.5% pepsin (EC 3.4.23.1, Sigma Chemical Co.P-7000, 1 g pepsin/100 g wet weight) for 6 h. Subsequently, the mixtures were centrifugated (10,000 rev/min, 5 min) and analyzed on an Agilent1100 HPLC system with ultraviolet detector immediately. The reversed phase column was a 4.6 mm × 250 mm ZORBAX 300 SB with an injection volume of 20 μL. The mobile phase consisted of two solvents: (A) 5% acetonitrile and 0.05% trifluoroacetic (TFA) and (B) 80% acetonitrile (v/v). The separation was performed using a linear gradient of A–B (v/v). Flow rate was maintained 1 mL/min. Absorbance was monitored at 220 nm.

2.6.3 Histological analysis

The cells-seeded scaffolds on 14 days were fixed in neutral buffered formalin, and embedded in paraffin. The embedded samples were sectioned with a microtome at a thickness of 2 μm, and then stained with hematoxylin and eosin staining (HE) for histological examination and Alcian Blue stain for glycosaminoglycan examination.

2.6.4 Scanning electron microscopy

Cells-seeded scaffolds on 14 days were fixed in PBS (pH7.4, 4 °C) for at least 24 h. Then, samples were dehydrated in a graded series of ethanol and air-dried critical point, sputtered with gold, and the samples were examined using a QUANTA-200 scanning electron microscopy (SEM).

3 Results

3.1 Determination of collagen purification

Type II collagen was isolated and purified from 2 to 3 days old chick cartilage. Figure 1 shows the typical profile of SDS-PAGE electrophoresis for type II collagen of the chick cartilage and a standard Sigma-Aldrich type II collagen (c9301, collagen from chicken sternal cartilage). Both purified collagen samples had similar migration bands which displayed an α band and dimmer of α band. This result was consistent with earlier report [14–17] that type II collagen molecule was comprised of three identical α-chains, thus, depicted as $[\alpha_1(II)]_3$. The amino acid composition was also indicated that collagen from chick cartilage is in the high content of glycine, hydroxyproline and proline residues, with 313, 118 and 94 residues per 1,000 amino acids residues, respectively. It also contains small amounts of tyrosine, cysteine, histidine and methionine residues with 5, 17, 4 and 2 residues per 1,000 amino acids residues, respectively. Tryptophan residue was not analysed in all samples because hydrolysis with 6 N hydrochloric acid destroys it (Table 1).

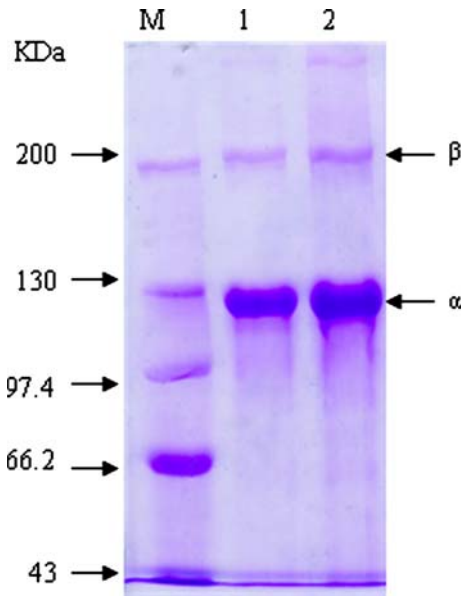

Fig. 1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profile of the Sigma-Aldrich standard type II collagen (lane 1) and the purified chick cartilage type II collagen (lane 2). Lane M, MW standards

3.2 Characterization of type II collagen scaffolds

The physico-chemical characteristics of collagen scaffolds with different concentration of EDC are presented in Table 2. The results indicate that EDC-crosslinked scaffolds increased the denaturation temperature (Td). The Td of EDC-crosslinked collagen scaffolds increased to 76.5 °C for concentration of EDC 15 mg/mL compared with 44.2 °C for 1 mg/mL. At concentration of EDC higher than 7 mg/mL, the collagen scaffolds exhibited a higher stability compared with 1 mg/mL. Scaffolds of EDC crosslinking also increased resistance to enzymatic degradation by bacterial collagenase. EDC-crosslinking collagen samples revealed only a partial degradation (8%) after 4 h of collagenase treatment compared with the under 5 mg/mL whereas 10 mg/mL EDC-crosslinking was completely resistant to collagenase treatment. The degree of crosslinking is inversely proportional to the amount of free amino groups. The number of free amino groups per 1,000 amino acid residues, which in native collagen amounts to 35, decreased to 16 for concentration of EDC 15 mg/mL. With increasing EDC concentration, a significant decrease in the swelling ratio and increase in the immobilized of CS was also detected. The immobilized of CS to collagen was maximal for 138 mg/g scaffolds at EDC concentration 10 mg/mL.

The SEM photograph of the crosslinking collagen scaffolds is shown in Fig. 2 EDC-crosslinked scaffold morphology shows porous surfaces with pore diameters

Table 1 Comparison of amino acid composition of sigma and chicken cartilage collagen

Amino acid	Cartilage collagen Residues/1,000 residues	Amino acid	Cartilage collagen Residues/1,000 residues
Aspartic acid/asparagine	47	Cysteine	17
Glutamic acid/glutamine	94	Valine	22
Serine	25	Methionine	2
Histidine	4	Phenylalanine	15
Glycine	313	Isoleucine	13
Threonine	30	Leucine	31
Alanine	102	Lysine	15
Arginine	53	Proline	94
Tyrosine	5	Hydroxyproline	118

Table 2 Physico-chemical characteristics of collagen matrices with different EDC concentration

EDC (mg/mL)	Td[a](°C)	Amine groups[b] (*n*/1,000)	CS[c] (mg/g matrix)	Remaining collagen[d] (%)	Swelling ratios[e] (%)
1	44.2 ± 0.4	35 ± 3	50.1 ± 3.2	0	5.7 ± 0.9
3	48.0 ± 0.3	24 ± 2	69.4 ± 5.4	5.3 ± 2.1	5.5 ± 0.3
5	56.2 ± 2.1	20 ± 2	97.4 ± 3.7	40.4 ± 5.3	3.8 ± 0.3
7	66.0 ± 0.2	18 ± 1	128.3 ± 1.4	92.1 ± 4.6	3.4 ± 0.2
10	70.2 ± 1.1	17 ± 3	127.5 ± 3.8	100	3.5 ± 1.1
15	76.5 ± 0.9	16 ± 2	138.5 ± 6.4	100	2.9 ± 0.2

[a] Denaturation temperature. Values are mean ± SD (n = 3)

[b] Expressed as amine group content per 1,000 amino acids. Values are mean ± SD (n = 4)

[c] CS: Chondroitin sulfate. Values are mean ± SD (n = 4)

[d] Expressed as percentage of the original weight (n = 4)

[e] Expressed as a ratio of the weigh of swollen to that of original weight.(n = 4)

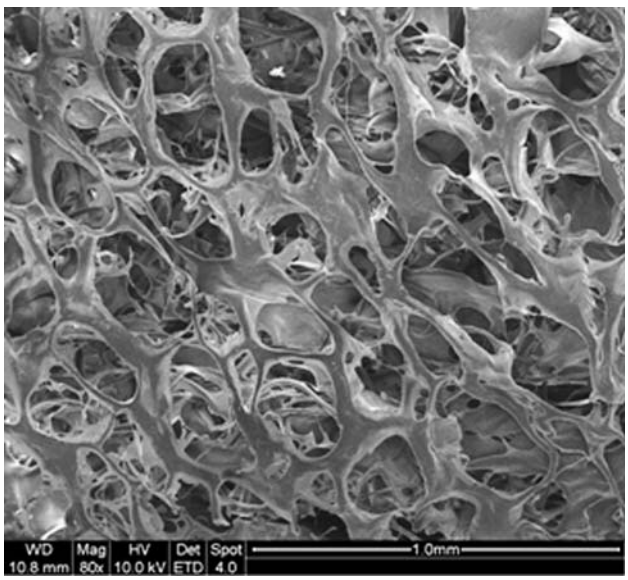

Fig. 2 Scanning electron micrograph (SEM) of EDC/NHS-cross-linked collagen scaffolds

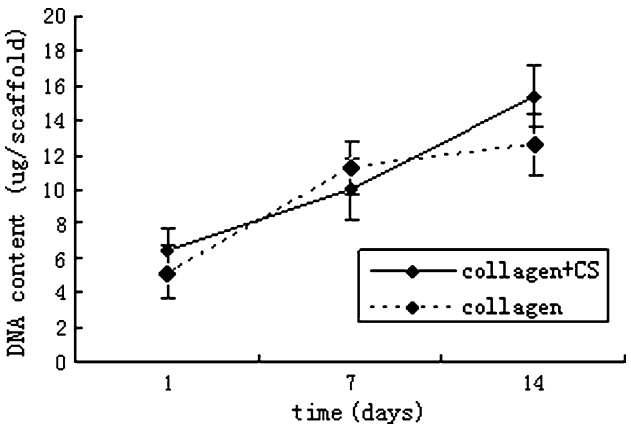

Fig. 3 The DNA content of cell-seeded scaffolds. Values are mean ± SD (n = 4). Collagen-CS: type II collagen-chondroitin sulfate scaffold crosslinked; collagen: type II collagen scaffold crosslinked

ranging from 80 to 200 μm. Inner structures contained lattice-like lamellae which formed a highly porous interconnecting network.

3.3 Characterization of cell-seeded scaffolds

3.3.1 Biochemical assays for DNA and GAG

Figure 3 shows the content of the seeded chondrocytes in both collagen scaffolds after 7 days and 14 days of culture. The total amount of DNA present on day 1 was similar for both cell-seeded scaffolds. In the absence of CS the DNA level gradually increased from 5.18 μg on day 1 to 12.4 μg per scaffold on day 14, whereas in CS-loaded scaffolds the DNA level increased from 6.5 μg on day 1 to 15.4 μg per scaffolds on day 14. After correction for the difference in the initial GAG values, it appeared that the collagen-CS scaffold contained more newly synthesized GAG than in the absence of CS after 14 days in culture (Fig. 4). The total amount of GAG increased from 8.6 μg on day 1 to 44.6 μg per scaffolds on day 14 for the CS-loaded matrices compared to from 12.7 μg to 58.8 μg in the absence of CS. Significant differences in the proliferative and biosynthetic behavior of the chondrocytes in the different scaffolds were found, consistent with Usha and Wissink [18, 19].

3.3.2 Histological examination

Histological examination of the cell-seeded scaffolds using hematoxylin-eosin staining indicates cell distribution throughout the porous surface of both collagen

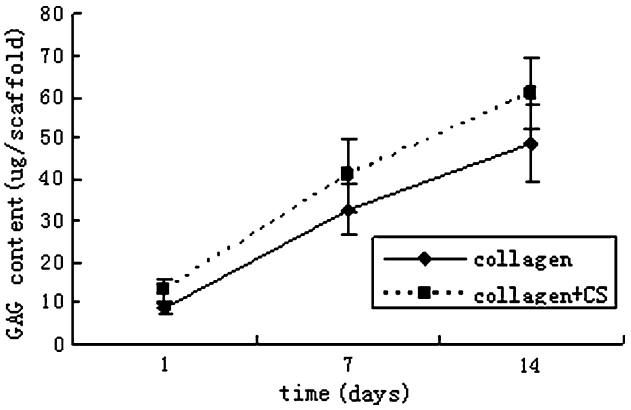

Fig. 4 The GAG content of cell-seeded scaffolds. Values are mean ± SD ($n = 4$). Collagen-CS: type II collagen-chondroitin sulfate scaffold crosslinked; collagen: type II collagen scaffold crosslinked

scaffolds as shown in Fig. 5. At 14 days, in both scaffolds, a clear increase in cell number had occurred and seeded chondrocytes had formed a dense layer of cartilaginous tissue in the superficial area of the matrices (Fig. 5). Alcian Blue stain indicated that glycosaminoglycans were abundant and homogeneously distributed around the cells (Fig. 6).

3.3.3 The RP-HPLC analysis for type II collagen

A simple RP-HPLC method for the separation of type II collagen-retained the scaffolds was applied using a ZORBAX 300 SB column. Figure 7 shows type II collagen-retained the cell-seeded scaffolds with or without CS have similar retention time at 10.5 min compared with Sigma-Aldrich type II collagen at 10.4 min for the HPLC analysis. The difference in the retentions time was insignificantly. The peak area of type II collagen-retained in the collagen-CS scaffold was higher than scaffold in the absence of CS. The result indicated that seeded chondrocytes in both collagen scaffolds synthesized new type II collagen matrix.

3.3.4 Scanning electron microscopy

Scanning electron microscopy performed at 14 days clearly revealed the seeded chondrocytes distributed throughout the porous surface of both scaffolds, and the vast majority of the seeded cells had maintained their spherical morphology for both collagen scaffolds. Initiation of new pericellular matrix production by the chondrocytes was clearly visible (Fig. 8).

4 Discussions

Tissue engineering is now showing promise as a possible method for cartilage repair, seeking a suitable scaffold has become more and more important. Type II collagen, is a biological material, is thought to provide information for cell attachment, and has good biocompatibility. The reason for choosing the type II collagen and CS in this study was

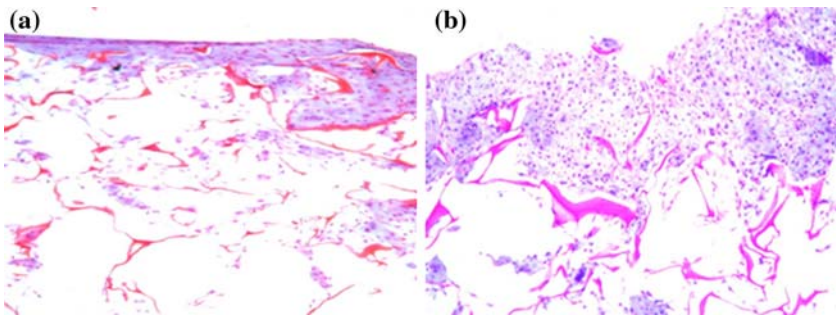

Fig. 5 Hematoxylin and eosin staining of chondrocytes of cell-seeded scaffolds. (**a**) type II collagen scaffold for 14 days; b: type II collagen-chondroitin sulfate scaffold for 14 days. Original magnification 100×

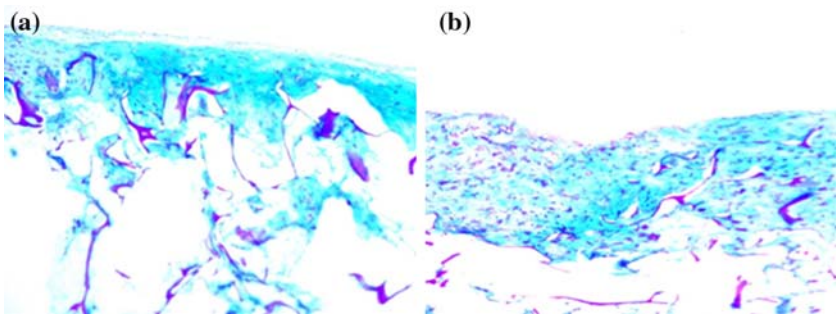

Fig. 6 Alcian Blue staining of GAG of cell-seeded scaffolds. (**a**) Type II collagen scaffold for 14 day; (**b**) type II collagen-chondroitin sulfate scaffold for 14 days. Original magnification 100×

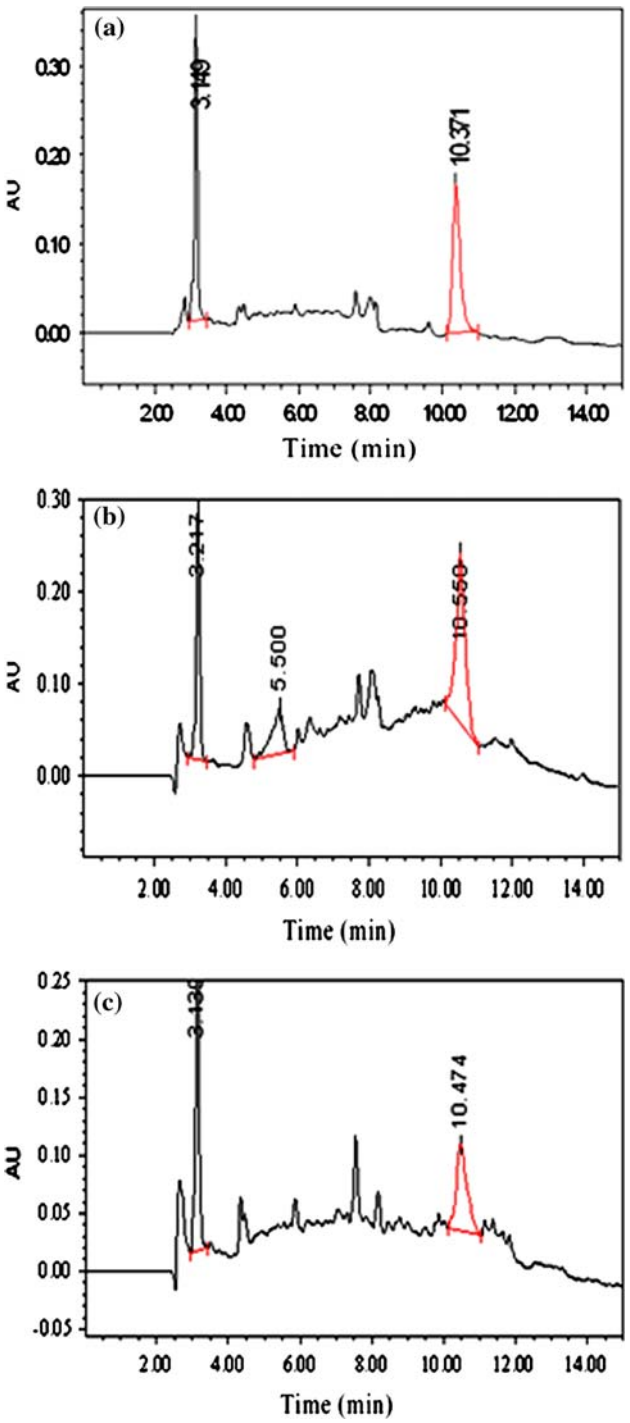

Fig. 7 The spectrum of RP-HPLC of type II collagen retained in the cell-seeded scaffolds. (**a**) Sigma-Aldrich standard type II collagen; (**b**) type II collagen-chondroitin sulfate scaffold for 14 days; (**c**) Type II collagen scaffold for 14 day

to mimic the natural cartilage matrix and to try to meet the requirement for reciprocity for cartilage tissue engineering.

For a rational design of scaffolds for tissue engineering, it is essential to study the effect of individual components, to do so, scaffolds have to be designed staring with highly purified molecules and the contribution of each component in the scaffold has to be controlled. The purification of type II collagen derived from chick cartilage has been evaluated by SDS-PAGE and total amino acid analysis. The result was consistent with most commonly found triplet in collagen chains space [20, 21] that glycine, which constitutes about one-third of all residues in collagen, would be present as every third residue in the sequence, and that high amounts of proline and hydroxyproline could be accommodated while maintaining planar peptide bonds [22–24]. The SDS-PAGE result indicates that the preparation is essentially free from contaminating proteins.

Tissue-engineered cartilage grafts should meet certain criteria to enable surgical handling and mechanical loading. Natural type II collagen as a biomaterial for tissue repair is easily biodegraded. EDC can react with carboxyl groups of the aspartic and glutamic acid residues forming an activated, but unstable form of *O*-urea. The use of NHS can improve the crosslinking yield of EDC by forming a more stable ester [25], and then improve the mechanical properties of the crosslinked collagen scaffolds. Improvement of mechanical stability for type II collagen-CS scaffolds of EDC-crosslinking was demonstrated by a decrease in free amino groups and swell ratio after crosslinking, and a corresponding increase in the shrinkage temperature and resistance to collagenase enzymatic degradation in this study. EDC-crosslinking increased mechanical stability of type II collagen-CS scaffolds may be due to a net increase in the relative molecular mass of polymers, resulting in improved degree of tropism and crystallization of the polymers. In addition, EDC-crosslinking of scaffolds also could decrease the decomposition of polymers by blocking movement of larger polymer molecular chain and blocking penetration of the water molecules thus increasing the stability of collagen scaffold [26, 27].

Ultrastructural matrix morphology of crosslinked collagen scaffolds were assessed by SEM. This difference in pore diameter was thought to be caused by the fabrication method of scaffolds, because the scaffolds were fabricated using freeze-drying. [28]. However, the highly porous structure of EDC-crosslinked scaffolds allowed cell penetration, growth, and proliferation.

In this in vitro study, EDC-crosslinking with and without CS effect on the cell-proliferation and matrix production by seeded chondrocytes in scaffolds was studied. The biochemical assays demonstrated that chondrocytes supported some degree of proliferation and biosynthetic activity in the type II collagen-CS scaffolds. The patterns of our histological examination of the cell-seeded scaffolds with haematoxylin and eosin and Alcian Blue staining also demonstrated a higher ratio of GAG and DNA content in the type II collagen-CS scaffolds,

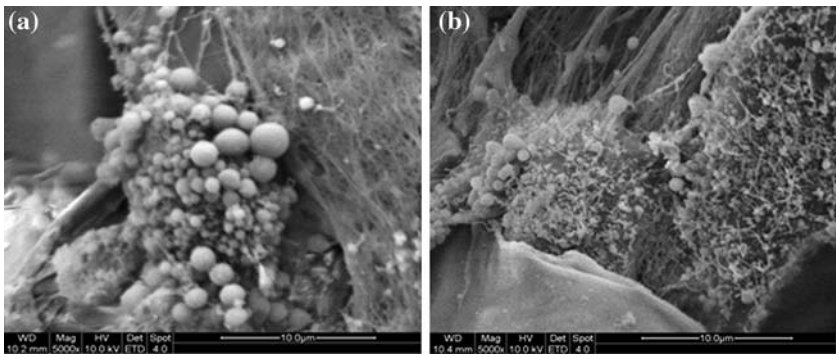

Fig. 8 Scanning electron micrographs of chondrocytes in the cell-seeded scaffolds. (**a**) type II collagen scaffold; (**b**) type II collagen-chondroitin sulfate scaffold

which correlated with the biochemical assays [29]. The increased proliferation and higher rates of synthesis of GAG indicated that chondroitin sulfate (CS) might have a stimulatory influence on the metabolic activity of seeded chondrocytes. Immobilized GAG retained large porous lamellar matrix spaces, probably due to their water-binding capacity which promotes matrix swelling. Porous matrix structures may modulate cell behavior and favor type II collagen-GAG scaffolds for host tissue deposition [30].

SEM revealed that a vast majority of the seeded cells had maintained their spherical morphology. In particular, in the direct vicinity of the chondrocytes many collagen fibrils were found, suggesting that the cells secreted collagens [31]. Type II collagen secreted by chondrocytes was also determined by RP-HPLC. Preservation of the chondrocytic phenotype was a prerequisite for the generation of a cartilage-specific environment. Three-dimensional biodegradable porous type II collagen-CS scaffolds, on which chondrocytes were cultured to prepare transplantable hyaline-like tissues, might provide greater available surface area for cell attachment and spreading than 2D surfaces. Moreover, the 3D scaffolds surface affected cell adhesion, spreading and proliferation, and controlled the spatial arrangement of cells and their transmission of biochemical and mechanical signals. Chondrocytes attached to a flat surface, they were able to spread and adopt a more fibroblast-like morphology, which was accompanied by an increase in proliferation and an altered phenotype [32]. Type II collagen, the major protein produced by chondrocytes in articular cartilage, and smaller cartilage specific collagens were down-regulated through time and division in monolayer culture, while collagen type I were increased. This phenotypic switch was performed rapidly by each cell on an individual basis, as collagen type I and II were not expressed simultaneously in dedifferentiating cells [33, 34]. A three-dimensional matrix is required, not only as a carrier for the transplantation of cells and for providing temporary mechanical support, but also for maintaining the characteristic round morphology of the chondrocytes.

In conclusion, results indicate that EDC improve the mechanical properties of the crosslinked collagen scaffolds and type II collagen-CS scaffolds may create an appropriate environment for culturing chondrocytes and for the generation of an engineered cartilage construct.

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