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In vitro and *in vivo* test of PEG/PCL-based hydrogel scaffold for cell delivery application

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Abstract

Biodegradable elastic hydrogel scaffolds based on hydrophilic poly(ethylene glycol) (PEG) and hydrophobic poly(ε-caprolactone) (PCL) were fabricated and investigated as a delivery vehicle of rabbit chondrocytes for the formation of neocartilage. The diacrylated forms of PEG and PCL were used as building blocks to prepare a series of hydrogel scaffolds with different block compositions and, thus, different physico-chemical properties. The porous hydrogel scaffolds were prepared by using the salt leaching method that is generally used for the creation of porous scaffolds, and their *in vitro* cell interactions were examined using chondrocytes. The hydrogel scaffold with a relatively high PEG content showed better cell growth for chondrocytes, while the scaffold with a relatively low PEG content showed lower chondrogenic differentiation. It was observed that different kinds of scaffolds and rabbit chondrocytes were shown to have different swelling ratios in the scaffold for effective cell growth and tissue regeneration. RT–PCR results for the resultant cartilage tissue revealed that a PEG–PCL ratio of 14 to 6 scaffold was optimal for cartilage tissue formation in terms of collagen Type II, aggrecan, SOX9, and COMP gene expression. In addition, the hydrogel scaffold with a PEG–PCL ratio of 14 to 6 showed faster formation of new cartilage than those shown by other scaffolds.

Keywords: Poly(ethylene glycol) (PEG); Poly(ε-caprolactone) (PCL); Hydrogel scaffolds; Chondrocytes; Cartilage formation

1. Introduction

Porous biodegradable polymeric scaffolds have been widely used for regeneration of cell-based artificial organs. Specially in cartilage engineering, scaffold served as the matrices of tissue formation plays a pivotal role, and has to fulfill a few basic requirements, that is, high porosity and proper pore size, required surface properties permitting cell adhesion, differentiation and proliferation, desirable mechanical integrity to maintain the predesigned tissue structure, non-cytotoxicity and osteoconductivity [1–3].

Numerous investigations, including scaffold fabrication [4], surface modification [5–7], and a bioreactor system [8], have been actively conducted for the development of the scaffolds, which can provide a desirable environment for cell growth. In particular, a great deal of research effort has been expended recently for repairing articular cartilage lesions by combining biodegradable scaffolds with chondrocytes [5–9]. In these investigations, a small population of articular chondrocytes was extracted from each patient, cultivated at a large scale, and seeded within porous biodegradable polymeric scaffolds. The resultant cell/scaffold construct was implanted back into the defect site for the regeneration of articular cartilage. In this plan for cartilage tissue engineering, the scaffold plays a pivotal role in dictating cell adhesion, proliferation, and differentiation for expressing desirable phenotypes. The scaffold must have an open, porous structure for sufficient cell seeding

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and for facilitating mass transfer of oxygen and nutrients. These scaffolds were fabricated by incorporating ammonium bicarbonate salt particles as a gas foaming salt porogen into a gel-like PLGA phase precipitated in an organic solvent [10–12]. Upon contacting this salt-PLGA mixture with a citric acid solution, macroporous scaffolds with highly interconnected pore structures could be obtained [13–15]. These scaffolds exhibited very high cell seeding density, as well as a homogeneous cellular distribution.

The aim of this study was to fabricate biodegradable elastic hydrogel scaffolds, of which properties such as biodegradability, swelling property, elasticity, and hydrophilicity can be easily modulated by simply changing the synthetic parameters, which include block composition, ratio, and other factors. The diacrylated forms of hydrophilic poly(ethylene glycol) (PEG) and biodegradable poly(ε-caprolactone) (PCL) were used as building blocks for the synthesis of a series of hydrogels [16–19]. Our previous study showed that PEG/PCL-based hydrogels could be useful for applications in drug delivery and tissue engineering due to several promising properties, such as elasticity, degradability, and other tailor-made properties [20]. This study investigated a porous hydrogel scaffold mixed with differentiation materials as a three-dimensional (3-D) culture for the macro-encapsulation of chondrocytes in conditioned media. The hydrogel scaffold can help to penetrate the inner state of the scaffold [21–23], which prevents the dedifferentiation of chondrocytes when they are implanted into the body [24–26]. Moreover, essential factors that are helpful for enhancing chondrogenic differentiation are also easily loaded into the inner state of the scaffold when they are mixed with chondrocytes. In this study, we hypothesized that the PEG/PCL-based hydrogel scaffold can offer a suitable environment for the retention of the chondrocytic phenotype, and can allow the synthesis of mechanically functional cartilage of the extracellular matrix (ECM) for cell therapy.

2. Materials and methods

2.1. Materials

Polycaprolactone diol (PCL diol, Mn=1250), benzene (anhydrous grade), acryloyl chloride, triethylamine, dimethyl sulfoxide (DMSO, anhydrous grade), and PEG diacrylate (PEG–DA, Mn=700) were purchased from Sigma-Aldrich. 2,2′-Azobisisobutyronitrile (AIBN) was obtained from JUNSEI Chemicals (Japan) and used after purification by recrystallization in methanol. Sodium chloride powder (size distribution: 180–400 μm, 99%) was purchased from Samchun Pure Chemical. The other chemicals were of reagent grade, and were used as received. Dulbecco's modified Eagle's medium (D-MEM low and high), fetal bovine serum (FBS) and pen-streptomicin were from GIBCO BRL, Life Technologies (Grand Island,NY); 3–(4,5–Dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT), Trypsin–EDTA and dexamethasone (Dex) were from Sigma Chemical Co. (St. Louis, MO, USA); recombinant transforming growth factor-β3 was from R&D Systems (Minneapolis, MN); ITS-plus Premix was from BD Biosciences (Bedford, MA); and anti-collagen Type I and Type II were from Chemicon International Inc. (Temecula, CA, USA).

2.2. Diacrylation of PCL

A predetermined amount of PCL diol (5 g, 4 mmol) was dissolved in anhydrous benzene. Triethylamine (1.01 g, 10 mmol) and acryloyl chloride (0.91 g, 10 mmol) were added to the solution, and the mixture was stirred for 3 h at 80 °C. Triethylamine hydrochloride formed as a reaction by-product in the reaction solution and was filtered off. The filtrate was precipitated in an excessive amount of cold *n*-hexane. The resulting product was collected and dried in a vacuum oven for 24 h (Yield ~87%).

2.3. Preparation of PEG–PCL hydrogel scaffolds

The predetermined amounts of PCL–DA and PEG–DA were dissolved in 5 ml of DMSO and placed into 15 ml of polypropylene conical tubes containing 7 g of sodium chloride salt particulates (size distribution: 180–400 μm, 99%). The feed ratio between PCL–DA and PEG–DA was varied from 7:3 to 3:7, but the total polymer concentration was fixed to 20 wt.%. After the addition of a small amount of AIBN, the reaction solution was placed in a convection oven maintained at 70 °C for 12 h. The resultant hydrogel was removed from the tube and cut into discs with thicknesses of 3 mm. The hydrogel samples were immersed in distilled water to dissolve the salt, and were then immersed in ethyl alcohol to remove any residual chemicals. Finally, the scaffolds were washed with distilled water several times and freeze-dried for 2–3 days.

2.4. Characterization

The reaction for diacrylation of PCL diol was confirmed by ^{1}H–NMR (JNM-AL400 spectrometer, JEOL Ltd, Akishima, Japan) and Fourier transform-infrared (Nicolet, USA) measurements. To measure the swelling ratio, the disk-typed hydrogel samples were immersed in distilled water. After reaching the equilibrium swelling state, the excessive water on the surface was removed by tapping with filter paper. The weight swelling ratio (Sr) was calculated from the equation, Sr=Ws/Wd, in which Ws and Wd are the weights of swollen and dried scaffolds, respectively. The contact angle of the hydrogels was measured (DSA100, KRÜSS). The PEG–PCL hydrogels with the same chemical compositions but without porous structure were prepared in the film type and used for contact angle measurements because the porous hydrogels could not be measured due to their tendency to absorb water instantaneously through pores on the surface. The morphologies of the scaffolds were measured by a scanning electronic microscope (S-2460N, Hitachi, Tokyo, Japan). Cross-sections of PEG–PCL scaffolds were mounted onto aluminum studs and sputter-coated with gold.

2.5. Chondrocyte isolation and cell culture

Chondrocytes were isolated from White New Zealand rabbit knee articular cartilage by collagenase digestion [27]. In brief, female rabbits weighing 250 g were sacrificed by an overdose of Nembutal. The non-fibrillated articular cartilage of the knee was removed by sterile dissection. The cartilage was finely minced, suspended in calcium-and magnesium-free phosphate-buffered

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saline (CMPBS), and washed. The fragments were sequentially digested in 0.2% collagenase (Worthington Biochemical: Lakewood, NY) in CMPBS for 3 h at 37 °C. The cells obtained from the collagenase digests were pooled and passed through a cell strainer (70 μm Nylon: Falcon: Franklin Lake, NJ) in order to remove the undigested matrix. The cells released in the supernatant were collected by centrifugation (1200 *g*, 15 min) and washed twice with CMPBS. The number and viability of the cells were determined using a hemocytometer and the trypan blue (0.25%) exclusion dye test, respectively. The collected cells were suspended in Dulbecco's modified Eagle's medium (DMEM: Gibco BRL: Grand Island, NY) containing 10% fetal bovine serum (FBS: Gibco BRL), 100 U/ penicillin G, and 100 μg/ml streptomycin (Gibco BRL), and were subsequently plated in tissue culture flasks at an initial density of 1.5×10^5 cells/cm^2. The cells were incubated at 37 °C in a 5% CO_2 atmosphere, and the culture medium was changed every third day. After 2 weeks of primary culturing, each dish of cells was passaged into three 10-cm culture dishes every seven days. The chondrocytes used in this study was passage 4. The subcultured cells (5×10^5 cells/5 mm^3) were seeded on the each type of porous scaffolds. After cell embedding, porous scaffold was moved into Millicells (12 μm) in 12-well and then chondrogenic medium (DMEM high glucose supplemented with ITS+1 Premix: 6.25 μg/ml insulin, 6.25 μg/ml transferring, 5.33 μg/ml linoleic acid, and 1.25 mg/ml bovine serum albumin (BSA), 10^{-7} dexamethasone, 50 μg/ml ascorbic acid, 100 μg/ml sodium pyruvate, 40 μg/ml L-proline and pen-streptomucin, 100 U/ml-100 μg/ml) in the absence or presence of TGF-β3 (5 and 10 ng/ml) were added. The subcultured cells (5×10^5 cells) were added into the different types of scaffolds. The size of scaffolds used in this study was 5 mm^3. This method illustrated in Fig. 1.

2.6. MTT assay

The number of viable cells in the scaffolds after cell cultures for 0, 1, 2, and 3 weeks were estimated by the MTT assay method. A stock solution of 3–(4,5–Dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT, Sigma) was made with phosphate-buffered saline (PBS, Invitrogen) solution at a concentration of 2.5 mg/ml. Cell proliferation was determined by MTT mitochondrial reduction on days 0, 7, 14, and 21. In brief, after removal of the supernatant, scaffolds were washed with PBS, transferred into new Petri dishes containing 3 ml of MTT solution (1 mg/ml in PBS), and incubated for 3 h at 37 °C. Each scaffold was transferred to an Eppendorf tube, and 1 ml of extraction solution (0.01 N HCl in isopropanol) was then added. Tubes were vigorously vortexed for 5 min to allow total color release from the scaffold and centrifuged at 12,000 rpm for 5 min. The supernatant was read at 570 nm. At least five scaffolds were analyzed for each condition and at each time point.

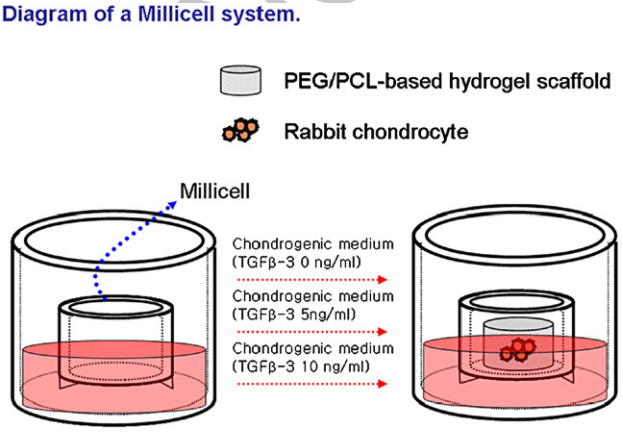

Fig. 1. Schematic representation of the different conditions used to evaluate the bioactivity of the released TGF-β3 in chondrogenic media.

Table 1
Oligonucleotide primers used for reverse transcriptionpolymerase chain reaction and expected product sizes

Targets	Sequences	Product size (bp)
Collagen type II	F : 5′-GCACCCATGGACATTGGAGGG-3′	350 bp
	R : 5′GACACGGAGTAGCACCATCG-3′	
Aggrecan	F : 5′-CCTTGGAGGTCGTGGTGAAAGG-3′	360 bp
	R : 5′-AGGTGAACTTCTCTGGCGACGT-3′	
COMP	F : 5′-CAGGACGACTTTGATGCAGA-3′	360 bp
	R : 5′-AAGCTGGAGCTGTCCTGGTA-3′	
SOX-9	F : 5′-TTCATGAAGATGACCGACGA-3′	355 bp
	R : 5′-CACACCATGAAGGCGTTCAT-3′	
MMP-13	F : 5′-GGAGAGGTTTCAGGGCAAAG-3′	224 bp
	R : 5′-TGACAAACCCTGCATCCTGT-3′	
GAPDH	F : 5′-TCACAATCTTCCAGGAGCGA-3′	293 bp
	R : 5′-CACAATGCCGAAGTGGTCGT-3′	

2.7. RNA extraction and RT–PCR

Scaffold-cultured cells (5×10^5 cells/5 mm^3) grown in chondrogenic medium in the absence or presence of TGF-β3 (5 and 10 ng/ml), were analyzed by RT–PCR in order to investigate temporal changes in collagen Type II, aggrecan, COMP and SOX-9 mRNA expression. The scaffolds were collected on days 3, 7, 14, and 21, placed in Microcon 100 filtration devices (Millipore Corporation, Bedford, MA), and centrifuged at 1500 *g* for 5 min at 4 °C in order to remove the medium. Cells were directly lysed in the culture scaffold by the addition of 0.8 ml of TRIzol reagent (Invitrogen). Total RNA (0.5 μg) was reverse transcribed in a 50 μl reaction using MMLV reverse transcriptase and random hexamers by following the protocol of the manufacturer (Perkin Elmer, Norwalk, CT, USA). One microliter of cDNA was used for RT–PCR. The PCR reactions were performed by using DNA Taq polymerase (Promega) with the following conditions: 1 cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining. The primer sequences and product sizes are described in Table 1.

2.8. Nude mouse implantation

Implantation of several types of scaffold mixed with cells was performed under sterilized conditions in a clean room. The construct scaffolds that contained chondrocytes were cultivated

in vitro for 1 day and then implanted subcutaneously into the backs of ten nude mice. These mice were sacrificed at 4 weeks after implantation, and each specimen was recovered. After sacrifice, the gross appearance of the sample was observed and tested for histological analysis.

2.9. Histology and immunohistochemistry analysis

The injection site was completely excised and processed for classical histology. In brief, samples from each time point were embedded in O.C.T. compound (TISSUE-TEKs 4583, Sakura Finetek USA, Inc) and frozen. The specimens were cut into 10 m-thick sections at −20 °C, and were stained with hematoxylin and eosin (H&E) for the nucleus and cytoplasm, respectively. The stained sections of each test sample were examined using light microscopy for cell proliferation and photographed with a digital camera. Cryosections (10 μm) of chondrogenic cultures were mounted on adhesion microscope slides (Marienfeld). Cell-seeded scaffolds were placed in 4% paraformaldehyde solution for 4 h, which fixed the constructs on the slides. Embedded sections were stained with Safranin-O, Alcian blue, and Masson's trichrome staining for histological evaluation. Sections were stained with hematoxylin and eosin to determine the cell morphology and distribution, and with Safranin-O for sulfated GAG. Total collagen content was revealed with Masson's trichrome stain. Immunohistochemistry was performed to identify Type I and Type II collagens. Nonspecific binding sites were blocked with normal horse serum diluted 1:10 in 0.3% bovine serum albumin for 30 to 60 min, and then incubated at 4 °C for 4 h with primary antibody targeting collagen type I or II (Chemicon, Temecula, CA, USA) at 1:1000 in a humid environment. After rinsing in PBS containing 2% BSA, sections were incubated in FITC goat anti-mouse antibody (1:1000, Molecular Probes, OR, USA) for 30 min.

2.10. Statistical analysis

All data were plotted and analyzed using Sigma Plot 2000 software (SPSS Inc.). A paired *t*-test was performed to compare the obtained results for the PEG–PCL scaffolds. A *p*-value <0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Preparation of PEG–PCL hydrogel scaffolds

As a cross-linkable biodegradable polymer block, PCL–DA was synthesized by the reaction of PCL diol with acryloyl chloride. The synthetic result was confirmed by FT–IR and ^{1}H–NMR measurements. The absorption bands at 1635 and 813 cm^{-1} in the FT–IR spectrum could be assigned to a C&z.dbnd;C bond due to the acrylation of PCL diol. The proton peaks from vinyl groups that appeared in the 5.79–6.43 ppm range could be confirmed from the corresponding ^{1}H–NMR spectrum. From the above results, the terminal hydroxyl groups of the PCL diol were considered to have successfully reacted to produce acrylated end-groups. The degree of acrylation, which was determined from the ^{1}H–NMR spectrum, was greater than 90%. Biodegradable PEG–PCL hydrogel scaffolds were synthesized by a radical cross-linking reaction of PEG–DA and PCL–DA in the presence of sodium chloride salt particulates. From the following salt leaching process, a highly porous structure could be generated in the hydrogel. As listed in Table 2, three kinds of hydrogel scaffold with different block compositions (PCL:PEG=14:6, 10:10, and 6:14 by weight %) were fabricated by varying the feed ratio of PEG–DA and PCL–DA to demonstrate a different hydrophilicity. The total polymer concentration was fixed to 20% w/v. The hydrogels prepared from a lower polymer concentration did not have enough mechanical strength, which caused them to be fragile during fabrication and freeze-drying.

Table 2
Chemical composition of hydrogel scaffold

Samples	PEG–DA (% w/v)	PCL–DA (% w/v)	Sr (Ws/Wd)	Contact angle (degree)
PEG–PCL #1. (n=3)	6	14	2.2	65.6±0.46
PEG–PCL #2. (n=3)	10	10	4.1	60.6±1.25
PEG–PCL #3. (n=3)	14	6	7.5	37.3±9.73

[a]Total polymer concentration was fixed to 20% w/v.

3.2. Characterization of PEG–PCL hydrogel scaffolds

As listed in Table 2, the hydrophilicities of the hydrogel scaffolds with different block compositions were compared to each other according to contact angle measurements. The measured values for hydrogel #1, #2, and #3, were 65.6, 60.6, and 37.3, respectively. It is reasonable that the hydrogel with a higher PEG content would demonstrate higher hydrophilicity and, thus, a lower contact angle value. The hydrophilicity of a hydrogel is closely related to the swelling behavior. The weight swelling ratios of the PEG–PCL hydrogel scaffolds were observed to be 2.2, 4.1, and 7.5, respectively, in the order of increasing PEG content. Because a hydrogel with higher hydrophilicity tends to swell more, a larger swelling ratio was observed with the increasing content of hydrophilic PEG. As could be expected from the swelling data, the hydrogel scaffold with higher PEG content swelled more in aqueous media, accompanied by an increase in the volumetric dimension and a slightly expanded pore structure.

3.3. MTT assay for cell proliferation

Rabbit chondrocytes were seeded into ethylene oxide (EO)-sterilized PEG–PCL scaffolds at a cell density of 5.0×10^5 cells per scaffold section in order to estimate the swelling effect on cell interactions. Due to the hydrophilic characters evidenced by their swelling properties, the cell suspensions could be directly seeded into the scaffold without any pre-wetting treatments. Usually, biodegradable scaffolds fabricated from synthetic

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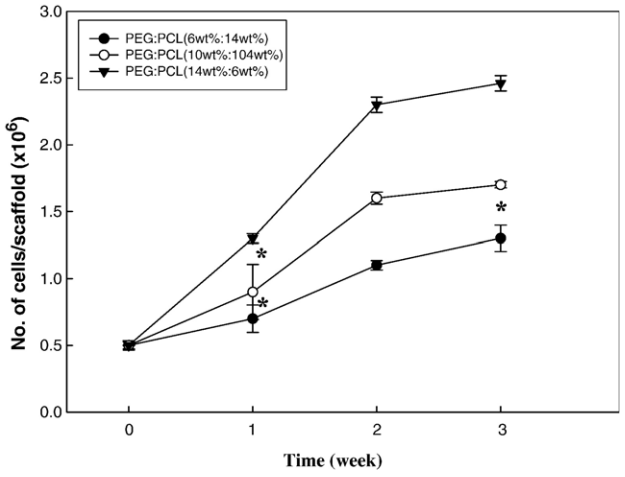

Fig. 2. Number of chondrocytes cultivated within different PEG/PCL-based hydrogel scaffolds after 21 days. The cells were cultured in the scaffolds for given periods of time (0, 1, 2, 3 weeks), and the viable cell numbers in each scaffold were estimated by the MTT assay method ($n=5$). Error bars represent standard error of the mean ($^*p<0.05$).

polymers, including PLA, PLGA, and PCL, require a pre-wetting process with alcohol for cell seeding. Because of their hydrophobicity, the scaffolds cannot be wetted in cell culture medium. Herein we illustrated a highly efficient method of cell embedding hydrogel typed PEG–PCL porous scaffold (Fig. 1).

The cells were cultured in the scaffold sections for the given periods of time (7, 14, and 21 days). The hydrogel scaffold of PEG–PCL (14:6) showed almost 100% cell seeding efficiency (after the cell-seeded specimens were maintained for 1 day at 37 °C in a CO_2 incubator for cell adhesion), which indicates that the scaffolds satisfied the requirements of cell entrapment, i.e., highly porous and interconnected pore structures that can be further developed by swelling.

As shown in Fig. 2, the embedded cells in hydrogel scaffold of PEG–PCL (14:6) were grown with significant differences in 14 days. Thereafter, the different characteristics of scaffolds showed different growth behaviors in the scaffolds; the chondrocytes showed better cell growth in the scaffold sections with a hydrophilic nature. In general, the cells need large enough pores to allow them to migrate into the pores of the scaffold and to allow for effective nutrient supply and metabolic waste removal, which are essential for effective cell growth; cells also must be small enough to establish a sufficiently high surface area for efficient binding to the scaffold and for cell–cell interactions for better cell growth, as discussed earlier. The exact mechanism of differential growth behavior in different scaffold types with varying hydrophilicity characteristics is not yet clear. However, the result shown in Fig. 2 suggests that the chondrocytes may prefer a hydrophilic nature (better conditions for efficient transport of nutrients or metabolites by swelling) for their growth.

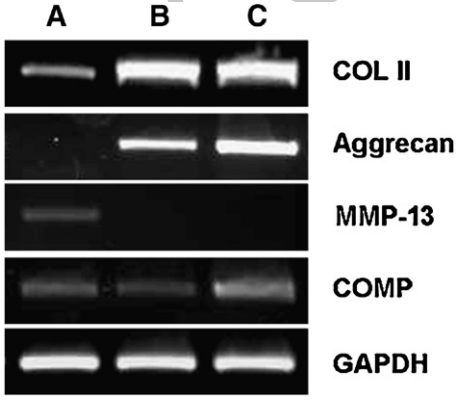

Fig. 3. RT–PCR analyses of the expression levels of chondrogenic markers in rabbit chondrocytes. Chondrocytes cultivated within different PEG/PCL-based hydrogel scaffolds after 21 days ($n=3$). (A) PEG–PCL (6 wt.%:14 wt.%); (B) PEG–PCL (10 wt.%:10 wt.%); (C) PEG–PCL (14 wt.%:6 wt.%). Type II collagen gene expression is evident by day 21 in all the scaffolds except the PEG–PCL (6 wt.%:14 wt.%) scaffold.

3.4. RT–PCR results

RT–PCR was used to investigate the expression of collagen Type II, aggrecan, SOX-9, and cartilage oligomermatrix protein (COMP) mRNAs in several types of scaffold. As can be seen in Fig. 3 C, collagen Type II was strongly expressed in differentiated cells, and its expression was enhanced in differentiated chondrocytes. Chondrogenic-specific proteins (including aggrecan and COMP) were also strongly expressed from the chondrocytes embedded in the PEG–PCL (14:6) scaffolds. It means that the chondrocytes were embedded well in the PEG–PCL (14:6) scaffolds and then the embedded cells were proliferated and differentiated in the same scaffolds.

To further analyze the effect of TGF-β3 on chondrogenic differentiation, we tested chondrocytes in the FBS model after 21 days using doses of TGF-β3 (0, 5, and 10 ng/ml). As can be seen in Fig. 4, collagen Type II was strongly expressed in chondrocytes stimulated by addition of TGF-β3, and its expression was enhanced by increasing the TGF-β3 concentration. Chondrogenic-specific proteins (including aggrecan and COMP) were strongly expressed in the presence of 10 ng/ml of TGF-β3, and expressions of these proteins decreased at low TGF-β3 concentrations (Fig. 4).

We evaluated the chondrogenic differentiation in the serum-free model after 21 days by time-dependent manner. Chondrocytes cultured in PEG–PCL (14:6) scaffolds raised SOX-9 mRNA levels after 3 days, while chondrocytes cultured in PEG–PCL (6:14 and

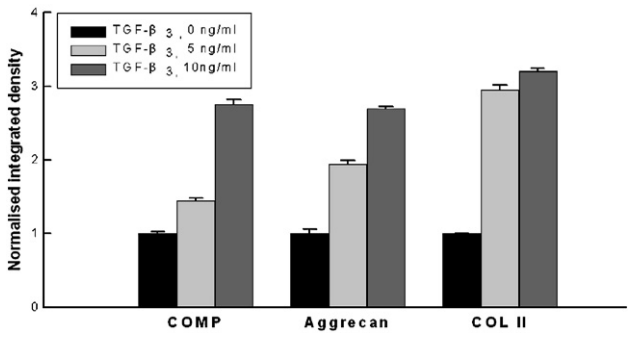

Fig. 4. RT–PCR result for the expression of collagen Type II, aggrecan, SOX9, and COMP in chondrocytes cultivated within PEG–PCL in a concentration-dependent manner ($n=3$).

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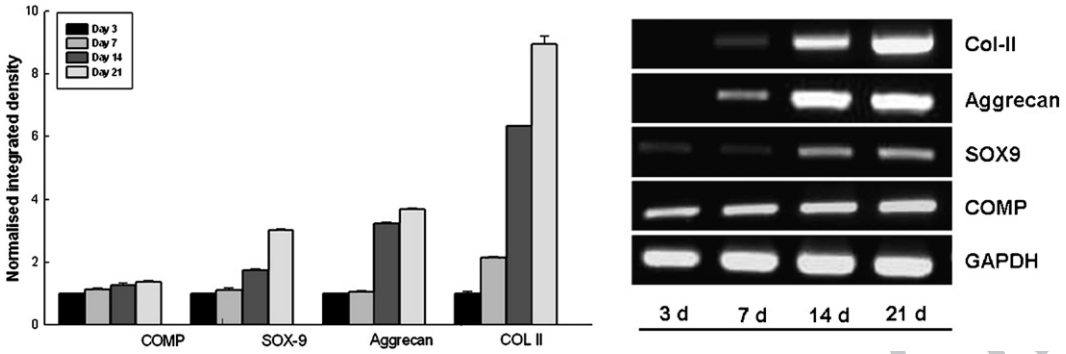

Fig. 5. RT–PCR analyses of PEG–PCL (14 wt.%:6 wt.%) showing the expression of genes associated with chondrogenic differentiation. Chondrocytes cultivated within PEG/PCL-based hydrogel scaffold after 21 days. Increasing aggrecan and COL II expression are shown, and SOX-9 and COMP expression can also be seen. Experiments were performed in triplicate, and the bands represent typical results. Expression levels of all of these critical genes seeded in a hydrogel-like scaffold were increased in a time-dependent manner ($n=3$).

10:10) did not increase the mRNA levels (Fig. 5). By day 21, levels of SOX-9 mRNA in PEG–PCL (14:6)-cultured cells were higher than those of controls, but expression was not shown in PEG–PCL (6:14)-cultured cells (Fig. 5 A). Expression of Type II collagen mRNA cultured in PEG–PCL (14:6) increased over the 14 days, while expression in cultures of chondrocytes embedded in PEG–PCL (6:14 and 10:10) was increased over those of controls. Cells cultured in the PEG–PCL (14:6) scaffold showed only slight changes in SOX9 expression compared to controls (Fig. 5 B). When the chondrocytes were cultured in PEG–PCL (14:6) for 21 days, aggrecan mRNA was found to be at higher levels. However, when the cells were cultured in PEG–PCL (6:14),

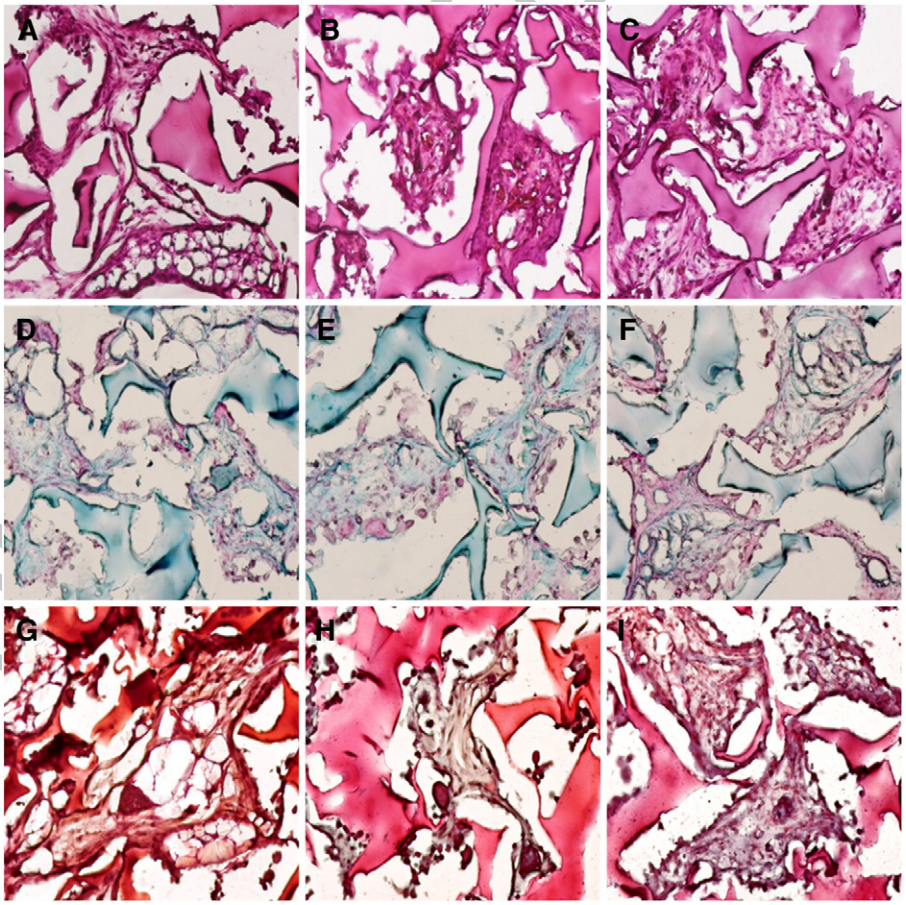

Fig. 6. Histological analysis of rabbit chondrocyte cultures maintained in a chondrogenic medium supplemented with TGF-β3 for 28 days. Sections were stained with hematoxylin (A–C) to show cell morphology and distribution. Sections were also stained with Alcian Blue (D–F) and Safranin-O (G–I) in rabbit chondrocytes cultured within PEG/PCL-based hydrogel scaffolds after 28 days ($n=3$). (A, D, G) PEG–PCL (6 wt.%:14 wt.%); (B, E, H) PEG–PCL (10 wt.%:10 wt.%); (C, F, I) PEG–PCL (14 wt.%:6 wt.%).

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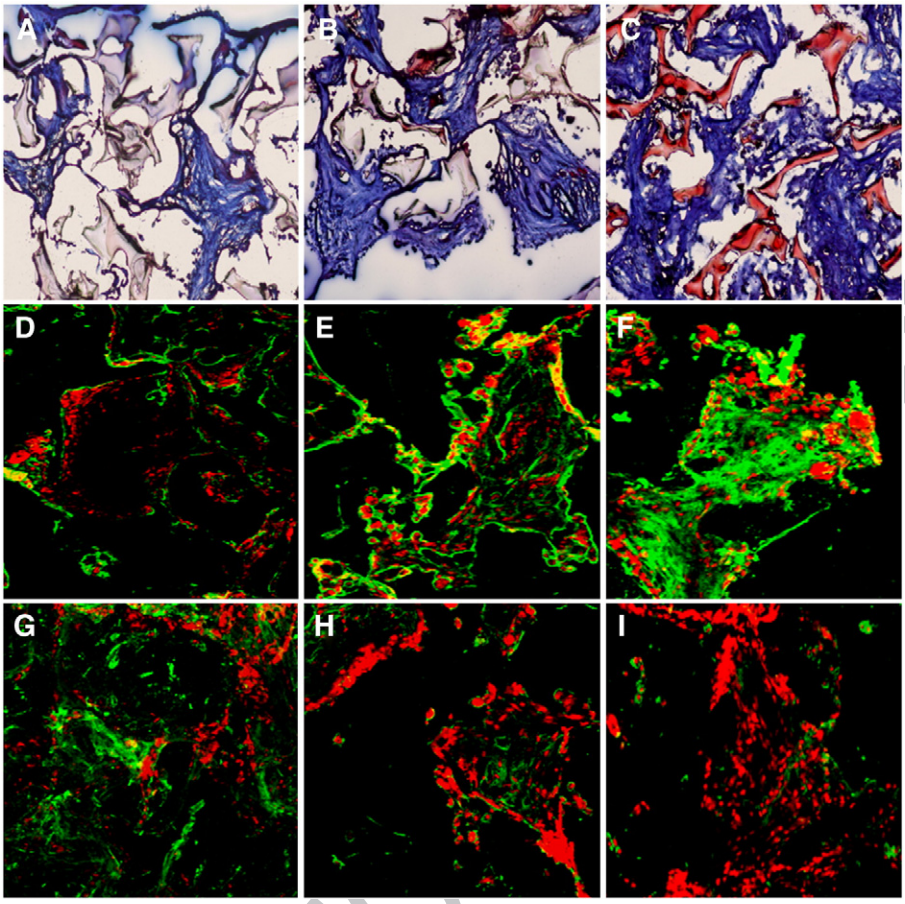

Fig. 7. Histological analysis of rabbit chondrocytes cultivated within PEG/PCL-based hydrogel scaffold after 28 days. Sections were stained with Masson's trichrome (A–C) for collagen content (blue stains collagen, red stains cytoplasm and residual scaffold constructs), and were immunohistochemically stained for type II collagen (D–F, red indicates positive staining) and type I (G–I) ($n=3$). (A, D, G) PEG–PCL (6 wt.%:14 wt.%); (B, E, H) PEG–PCL (10 wt.%:10 wt.%); (C, F, I) PEG–PCL (14 wt.%:6 wt.%).

mRNA expression was not observed at strong levels, even after 21 days of culturing (Fig. 5 C).

Quantitative results showed that mRNA expression of chondrocytes in the PEG–PCL (14:6) scaffold was increased in a time-dependent manner. By week 3, expression of the SOX-9 gene was close to three times higher than that at 3 days of culturing (Fig. 5). By week 3, Type II collagen mRNA expression was increased by 9-fold compared to that at 3 days of culturing. After 3 weeks, PEG–PCL scaffold-seeded chondrocytes showed a four-fold increase in aggrecan mRNA expression. Interestingly, by week 3, less than a one-fold change was observed in COMP mRNA expression. These results suggested that the PEG–PCL scaffold-seeded chondrocytes enhanced the gene expression of chondrogenic differentiation in a time-dependent manner.

3.5. Histological analysis

The formation of noecartilage within several types of PEG–PCL scaffold was observed at 4 weeks after implantation in nude mice. Histological characteristics of transplanted chondrocytes from PEG–PCL scaffolds were evaluated (Fig. 6). In cartilage regeneration, a distinct cartilage-specific morphological appearance and structural characteristics such as lacunae are normally observed. In order to certify the specific morphological changes, the implanted constructs were examined by H & E, Safranin-O and Alcian Blue staining. As seen in Fig. 6 (A–C), chondrocytes embedded in the PEG–PCL (14:6) scaffold were homogenously distributed throughout the scaffolds and, additionally, MSCs maintained a round shape and formed cell aggregates during culture periods.

These methods indicated that the chondrocytes encapsulated in a hydrogel typed scaffolds mixed with cells for differentiation accumulated an abundant extracellular matrix that was rich in proteoglycans and polysaccharides (Fig. 6 D–I). In contrast, the cells encapsulated in the scaffold produced extracellular matrix only in the immediate vicinity of each cell. After 4 weeks of culturing, the main difference between hydrogel typed or not in terms of cell proliferation and differentiation was the significant difference in neocartilage formation. The accumulation of proteoglycans and polysaccharides in the hydrogel scaffold prepared with PEG–PCL (14:6) for differentiation was increased significantly and spread throughout the whole construct.

Total collagen content, which was produced by freshly isolated chondrocytes, was found to be predominantly expressed by the

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chondrocytes cultivated in PEG–PCL scaffolds by Masson trichrome staining (Fig. 7 A–C). This histological result is consistent with the RT–PCR findings, and supports the finding that scaffold-made chondrocytes in the PEG–PCL (14:6) scaffold retain their phenotypes to a greater extent. Although cell-to-cell interactions among chondrocytes are known to be important in preventing dedifferentiation, a poor growth rate in scaffolds is one of the major problems with *in vitro* cultivation of chondrocytes, as it often results in dedifferentiation.

In order to provide direct evidence of a specific marker of chondrogenic differentiation, double staining and immunofluorescence reactions were performed on the specimens from the transplanted cells using the antibodies that react with Type II collagen, as well as by fluorescent cytochemistry for chromosomes with PI. The immunohistological characteristics and collagen Type II phenotype expression of the cartilage tissues from the different types of scaffolds were evaluated by immunostaining with the collagen Type II antibody and staining with PI (Fig. 7). The formation of the cartilage tissue within the scaffolds was observed 4 weeks after injection of the hydrogel into nude mice. Collagen Type II, which was produced by freshly isolated chondrocytes, was mainly expressed by the chondrocytes cultivated in the hydrogel scaffold prepared with PEG–PCL (14:6). As shown in Fig. 7, an overlap in fluorescence reactions was observed using antibodies to Type II collagen and PI. Fluorescence-staining cytochemistry of the nuclei with the blue fluorescent dye PI showed no overlap with the anti-collagen fluorescence pattern. The present study shows that hydrogel-like PEG–PCL scaffolds can be a simple means by which to promote the formation of cartilage tissue.

4. Conclusion

We fabricated a PEG–PCL hydrogel scaffold that could be useful for cell embedding and growth. Based on the swelling (water-absorbing) properties, cells could be directly and uniformly seeded into the scaffold sections without any further pre-wetting treatments. From the *in vitro* and *in vivo* cell culture with rabbit chondrocytes, it was observed that the cells and tissue had different swelling ratios in the scaffolds for effective cell growth and tissue regeneration. Due to their versatile properties, including hydrophilicity, swelling, biodegradability, and mechanical strength, the PEG/PCL-based hydrogel scaffolds can be a good tool for use in systematic studies of the interactions between cells or tissues and scaffolds.

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