# Biomimetic Porous Scaffolds Made from Poly(L-lactide)-g-chondroitin Sulfate Blend with Poly(L-lactide) for Cartilage Tissue Engineering

Chih-Ta Lee, Ching-Ping Huang, and Yu-Der Lee*

*Department of Chemical Engineering, National Tsing Hua University 101, Section 2, Kuang Fu Road, Hsin Chu, Taiwan, 300, ROC.*

*Received May 9, 2006; Revised Manuscript Received May 11, 2006*

A novel biodegradable graft copolymer chondroitin sulfate-grafted poly(L-lactide) (CS−PLLA) was synthesized. The graft copolymer was blended with PLLA to form biomimetic porous scaffolds. Natural CS was introduced into the polyester matrix to promote the proliferation of cells. Three-dimensional spongelike scaffolds were fabricated by a combination of salt leaching and solvent casting methods. The morphology of the scaffolds was observed with scanning electron microscopy with an average pore size between 50 and 250 $\mu$m, and its porosity was high (>85%). Compression analysis indicated that the mechanical properties of the scaffold were adequate to support the proliferation of cells. The hydrophilicity increased with an increase in the copolymer content in the blend, as determined by measuring the contact angle. Hematoxylin and eosin, Masson, and Safranin-O staining showed that cells formed a chondro tissue gradually. Histological results revealed that abundant cartilaginous matrixes surrounded spherical chondrocytes in the center of the explants. Chondrocytes cultured in this extracellular-matrix-like scaffold maintained a round morphology phenotype, characterized by a significant quantity of extracellular matrixes of sulfated glycosaminoglycans and collagens. Additionally, phenotypic gene expression (reverse transcriptase−polymerase chain reaction) indicated that chondrocytes expressed transcripts that encoded type II collagen and aggrecan and generated sulfated glycosaminoglycans.

## Introduction

Damaged articular cartilage has very little capacity for spontaneous healing, because of the absence of both vascularization and innervation in the cartilage to support repair and remodeling. The autologous transplantation of articular chondrocytes using tissue engineering has been studied extensively to treat articular cartilage defects. A cell-carrier substance that closely mimics the natural environment of the cartilage-specific extracellular matrix (ECM) must be developed to enable the cartilage tissue to regenerate. Recently, artificial biodegradable polymers have been utilized widely to manufacture three-dimensional (3-D) scaffolds for cell cultures. The temporary scaffolds must exhibit suitable biocompatibility, have mechanical properties that are very similar to those of the target tissue, and have an interconnected structure. The scaffolds must be highly porous for seeding cells; regenerating ECMs; and promoting the admission of delivered nutrients, the removal of waste, and tissue in-growth[1]. Moreover, the scaffold should be designed by mimicking the native ECM as much as possible.

Aliphatic polyesters, such as poly(L-lactide) (PLLA),[2] poly(lactide co-glycolide),[3] and poly($\epsilon$-caprolactone),[4] are employed extensively to construct temporary scaffolds for use in tissue engineering, because they are biodegradable and have good mechanical characteristics and a favorable degradation rate that matches the rate of healing of damaged tissue. However, these polyesters are hydrophobic and have no cell recognition sites, so the mass transport in scaffolds is poor and the cells cannot easily adhere to the scaffolds. Many strategies have been adopted, including a change of the surface charge,[5] increases in the surface roughness[6] and hydrophilicity,[7] or the immobilization of a biocompatible layer on the surface,[8] to overcome these disadvantages. Another example involves the use of natural polymers, such as collagen,[9] alginate,[10] and chitosan,[11] among others, as scaffolds. Although these natural polymers provide cell adhesion due to having specific active sites, they associate with poor mechanical properties and degrade easily. Therefore, scaffolds with the merits of both natural polymers and polyesters may be desirable and will be advantageous if they contain characteristics similar to those of the target tissue's ECM.

The growth, differentiation, replication, and metabolic activity of a cell in a culture depend on the substrate attachment site.[12] Extensive studies have demonstrated that cell−ECM interactions promote cell adhesion, migration, growth, and differentiation directly.[13] Biodegradable polysaccharide-based polymers have recently been suggested to have great potential for use in various applications in the biomedical field, including in drug delivery,[14,15] as compatiblizers,[16] and as tissue engineering scaffolds.[17,18] These polysaccharide-based materials are generally deemed to be noncytotoxic and able to induce a satisfactory response from the tissue into which they are implanted.[19]

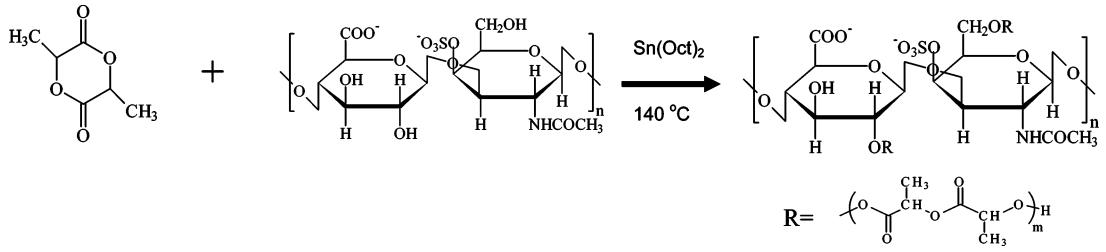
Chondroitin sulfate (CS) is a glycosaminoglycan (GAG) that plays an important role in regulating the expression of the chondrocyte phenotype. It comprises alternating units of $\beta$-1,3-linked glucuronic acid and *N*-acetyl-galactosamine (GalNAc) with sulfation at either the 4 or the 6 position of the GalNAc residues. Chondroitin sulfate is also involved in intracellular signaling, cell recognition, and the connection of ECM components to cell-surface glycoproteins.[20]

In this study, PLLA was grafted onto CS by chemical bonding, and the graft copolymer ($CS_n$-PLLA) was blended with PLLA to fabricate biomimetic porous scaffolds for cartilage

* Corresponding author. Tel.: +886-3-571-3204. Fax: +886-3-571-5408. E-mail: ydlee@che.nthu.edu.tw.

10.1021/bm060451x CCC: $33.50 
Published on Web 06/13/2006

**Scheme 1.** Reaction Scheme for the Synthesis of Graft Copolymer




tissue engineering. Mouse articular chondrocytes were seeded within the blend scaffolds, and their adhesion, the secretion of the ECM, the quantity of the synthesized collagen and GAGs, and the chondrocytic phenotypic characteristics were examined and compared to those on pure PLLA scaffolds. The morphological changes and deposition of the cartilaginous ECM were examined by histological staining. The ability of the blend scaffold to maintain a chondrocytic phenotype was investigated by reverse transcriptase−polymerase chain reaction (RT-PCR) analysis.

## Experimental Section

**Materials.** L-Lactide was purchased from Lancaster and recrystallized twice from ethyl acetate; it was then stored in a vacuum before use. CS [average MW = 12 000 g/mol, GPC, *N*,*N*-dimethylformamide (DMF)] was obtained from Calbiochem and dried under reduced pressure at 90 °C under $P_2O_5$ for one night before use. Stannous octoate [$Sn(Oct)_2$] was purchased from Sigma and used without further purification. Dimethyl sulfoxide (DMSO; Aldrich Chemical Co., anhydrous), tetrahydrofuran, ethyl acetate, hexane, chloroform, and dichloromethane were used as received.

**The Synthesis of the $CS_n$-PLLA Grafted Copolymer.** The grafted copolymers of chondroitin sulfate-g-L-lactide ($CS_n$-PLLA) were prepared according to the method described by Donabedian and McCarthy[21] (Scheme 1). Chondroitin sulfate was modified by L-lactide via ring-opening polymerization, and $Sn(Oct)_2$ was used as the catalyst. Chemical grafting of the PLLA chains onto CS was expected to follow the initiation of the ring-opening polymerization by the hydroxyl functions of the CS. Briefly, a 25 mL tri-neck flask was charged with preset amounts of L-lactide and chondroitin sulfate, and these were dissolved in a minimal amount (about 2 mL) of anhydrous DMSO. The flask was placed in an oil bath at 70 °C in a vacuum to degas for around 40 min, and then, it was heated in another oil bath at 140 °C for about 15 min, and 0.01 wt % of the $Sn(Oct)_2$ catalyst was injected. The reaction was kept running for another 3 h. After polymerization, the product was cooled to room temperature. Various nonsolvents were used to recover the polymers, depending upon the extent of modification of CS. Copolymers with a large amount of lactide modification were insoluble in water, and the products were extracted with an ethyl acetate/water (6:4) mixture. The products were further purified to remove any unreacted L-lactide monomer and PLLA homopolymer that might have formed. The unreacted L-lactide monomer was extracted twice with hexane, and the PLLA homopolymer was removed by washing the product in toluene. Finally, the copolymer was precipitated in hexane and was dried in a vacuum (1 mm Hg) at 40 °C for 24 h.

**Preparations of $CS_n$-PLLA Blend Films and Scaffolds.** The polymers used in this study are based on blends of $CS_{11.5}$-PLLA (the CS content in the copolymer was estimated to be 11.5% by $^1$H NMR) and PLLA (MW ∼ 50 000). The compositions of the scaffolds are listed in Table 1. A mixture of PLLA and $CS_{11.5}$-PLLA was dissolved in dichloromethane (10 wt %), stirred evenly, and then cast on a Teflon plate; it was kept at room temperature to evaporate most of the solvent and then vacuum-dried for another 24 h. A PLLA film was also prepared similarly. The thickness of the film was 0.2 ± 0.03 mm.

**Table 1.** Composition and Characterizations of the Scaffold

| sample code | PLLA/$CS_{11.5}$-PLLA (wt/wt) | compression modulus[a] (MPa) | contact angle[b] (deg) |
|---|---|---|---|
| 1 | 100/0 | 3.35 ± 0.433 | 111.9 ± 1.16 |
| 2 | 60/40 | 2.73 ± 0.341 | 82.8 ± 3.88# |
| 3 | 40/60 | 2.34 ± 0.383* | 69.2 ± 0.52# |
| 4 | 20/80 | 1.44 ± 0.255* | 68.3 ± 0.77# |

[a] The compression mechanical tests were set to 0.4 mm/min, and the load was applied until the scaffold was crushed completely. The scaffold size was at a thickness of 3 mm and a diameter of 7 mm. Each sample was tested in triplicate. A * indicates that the moduli decreased significantly after being blended with the copolymer ($P < 0.05$) compared with those of the PLLA homopolymer. [b] Each value was tested five times at different sites in a film. A # indicates that the contact angle (hydrophilicity) decreased significantly after being blended with the copolymer ($P < 0.05$) compared with that of the PLLA homopolymer.

3-D scaffolds were prepared by combining salt leaching and solvent casting methods. PLLA and the copolymer were dissolved in dichloromethane (15 wt %) to form a polymer solution. It was added to NaCl particles (100∼250 μm) at a mixing ratio of 1:5 (w/w). Then, the solution was cast in a Teflon mold. After the solvent was evaporated, the composite was immersed in deionized water for 24 h to remove the salt, and the water was changed several times to ensure that all of the salt was removed. Subsequently, the water remaining in the scaffold was removed by freeze-drying, and porous scaffolds were obtained, with a thickness of 3 mm and a diameter of 7 mm and a porosity of over 85%.

**Characterizations of the Polymeric Blend Films and Scaffolds.** The hydrophilicity of the blend film was tested by measuring the water contact angles (FACE CA-D Contact Angle Meter, Kyowa Kaimen-kagaku Co., Japan), via a sessile drop method. Each sample was tested five times at different sites. The surface and cross-section morphologies of the scaffold were observed by scanning electron microscopy (SEM; JSM-5600, JEOL), and the mean pore sizes were calculated from SEM images by software. The mechanical properties of the scaffold were determined by measuring their compression modulus in a wet state with a thickness of 3 mm and a diameter of 7 mm. An Instron 5500 mechanical tester with a 10 kN load cell was used for the compression mechanical test. The crosshead speed was set at 0.4 mm/min, and the load was applied until the scaffold was crushed completely.

**Cell Isolation.** The articular cartilages were removed from the knees and the hip joints of young (7 days old) Wistar rats, which were obtained from the National Taiwan University Hospital laboratory animal center. After the endothelium adventitia had been removed, the connective tissues were cut into small pieces and incubated under agitation in a sterile conical flask containing an enzymatic dissociation buffer in an orbital shaker (60 rpm) for 24 h at 37 °C.[22] Chondrocytes were released from cartilage slices by collagenase II (0.2% w/v) digestion. Following the complete dissolution of the matrix, the resultant cell suspension was filtered through a 40 μm Nitex filter and centrifuged at 2000 rpm for 5 min. The isolated cells were then cultured in a Dulbecco's Modified Eagle Medium (DMEM; Sigma) supplement with 10% fetal calf serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. They were incubated in an incubator at 37 °C under 5% $CO_2$, and the medium was changed every 3 days. The Institutional Animal Care and Use Committee at the National Tsing Hua University in Taiwan

reviewed the application for the animal study and agreed it was compatible with the standards for the care and use of laboratory animals (approval number: 09521).

**Cell Culture on Blend Films and Scaffolds.** As the cultured cells had grown to confluence, they were digested with 2 mL of 0.25% trypsin (Sigma) for 5 min; then, 3 mL of culture medium was added to stop the digestion, and the culture medium was aspirated to disperse the cells. Later, the cells were counted and used.

Polymeric blend films were cut into small disks (15 mm in diameter) and were put into a 24-well culture plate and pressed by a glass ring. It was sterilized with 70% ethanol for 1 h, and then washed with excess phosphate buffered saline (PBS) to remove alcohol. A total of $1 \times 10^4$ mouse cartilage cells were seeded on each well, and 2 mL of culture medium was added. The cell-seeded disk was maintained in the incubator at 37 °C under 5% $CO_2$ for different time intervals (2, 4, and 6 days), and then, the culture medium was removed. In the meantime, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) was dissolved in DMEM at 5 mg/mL and filtered (0.22 $\mu$m filter) for sterilization. A total of 20 $\mu$L of this stock solution was added to each well, to a final concentration of 0.5 mg/mL of MTT, and the plate was incubated for a further 4 h. Unreacted dye was removed by aspiration, and the purple formazan product was dissolved in 200 $\mu$L/well of DMSO; the solution was then quantitated spectrophotometrically using an enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 570 nm, blanked with DMSO. Mild shaking for 10 min was performed to ensure that the MTT formazan was dissolved completely before measurement.

**Cell Morphology Observation.** The morphologies of the cells cultured on scaffolds were observed by SEM (JSM-5600, JEOL). PLLA and the $CS_n$-PLLA blend scaffolds were cultured for 2 days at 37 °C under 5% $CO_2$. For SEM examination, the cells were fixed with 4% glutaraldehyde in PBS for 2 h at 4 °C. After they had been thoroughly washed with PBS, the samples were immersed in a 1% $OsO_4$ solution for another 30 min and then dehydrated sequentially in 50%, 70%, 95%, and 100% ethanol for 10 min × 2. Finally, the samples were critical-dried, coated with Au, and examined with SEM.

**Histology of Explanted Scaffolds.** Scaffolds seeded with cells were fixed in 5% formaldehyde for histological examination. Implants were harvested after 1, 2, and 4 weeks of culturing. Samples were dehydrated using a graded series of ethanol (50, 70, 95, and 100%) and embedded in paraffin. They were then cut into 4-$\mu$m-thick sections. The histological characteristics were evaluated by light microscopy. Chondrocyte viability and matrix production were assessed on hematoxylin and eosin (H&E) stains for the nucleus, Masson stains for total collagen, and Safranin-O stains for GAGs.

## Biochemical Evaluation

**Glycosaminoglycans (GAGs) Assay.** The quantification of all sulfated glycosaminoglycans in a chondrocyte culture is necessary for the complete assessment of the metabolic profile of the system. The color reagent was prepared by dissolving 16 mg of 1,9-dimethylmethylene blue (DMB) in 1 L of water containing 2.37 g of NaCl, 3.04 g of glycine, and 95 mL of 0.1 M HCl, to obtain a solution of pH 3.

Each sample was added to a 1 mL digestion solution at 60 °C for 24 h. The digestion of interfering proteins present in the cartilage was performed with papain. Then, 40 $\mu$L of each sample in the digestion solution was placed in a 96-well cultural plate, and 200 $\mu$L of a color reagent was added. The solution was quantitated spectrophotometrically using an ELISA reader at a wavelength of 525 nm, blanked with a color reagent. The assay was calibrated by use of the standards containing up to 5 $\mu$g of Bovine trachea chondroitin sulfate (Sigma) in the same solvent as that of the samples.[23] The material without cells was also quantitated before measurement. The amount of GAGs in each tissue construction was assayed five times.

**Collagen Assay.** The total collagen was determined by measuring the amount of hydroxyproline present in each construction. Aliquots of proteinase k digest to be evaluated for hydroxyproline were hydrolyzed in 6 N HCl at 110 °C for 18 h. The HCl was then evaporated, and the hydrolysate was assayed for hydroxyproline using a spectrophotometer at a 550 nm wavelength.[24]

**RNA Isolation and Reverse Transcriptase−Polymerase Chain Reaction (RT-PCR) Analysis.** After 2 weeks of culture, samples of each group were frozen in liquid nitrogen prior to RNA extraction. The total RNA was extracted by the TRIspin method described previously.[25] Briefly, frozen samples were powdered in liquid nitrogen cooled with Brown Dismembrater vessels (B. Braun Biotech., Allentown, PA). A trizole reagent (Life Technologies, Gaitherburg, MD) was added to the powdered tissue at 1 mL/100 mg of sample weight, and it was warmed to room temperature. The samples, to which chloroform was added, were centrifuged at 12 000*g* for 15 min at 4 °C. Thus, the total RNA was obtained and washed with 70% ethanol. Finally, the total RNA was isolated using the RNeasy total RNA kit (Qiqgen, Chatsworth, CA) according to the manufacturer's protocol.

A RT-PCR analysis of the transcripts was conducted on mouse articular cartilage cultured for 2 weeks either in a 2-D cultural plate or in a 3-D scaffold. The total RNA yield was fluorometrically quantified with the SYBR Green reagent (Molecular Probes, Eugene, OR) according to the manufacturer's recommendations. According to previous reports, a RT-PCR analysis was performed using mouse-specific primer sets for type I collagen, type II collagen, aggrecan, and a housekeeping gene, glyceraldehydes-3-phosphate dehydrogense (GAPDH). First, 1 $\mu$g of the total RNA was reverse-transcribed into cDNA using the StratScript RNase $H^-$ kit (Stratagene, La Jolla, CA). Second, aliquots (1.5 $\mu$L of 50 $\mu$L total value) of the resulting cDNA were amplified in a total volume of 50 $\mu$L containing PCR buffer, a 0.2 $\mu$M dNTP mixture, 1.5 mM $MgCl_2$, 0.5 $\mu$M of each primer, and one unit of the Taq DNA polymerase (Life Technologies Inc., Gaithersburg, MD). The absence of DNA contamination in RNA preparation was tested including RNA samples that had not been reverse-transcribed, as reported previously.[26] PCR was done with an initial denaturation of 1 min at 95 °C, followed by a total of 25 cycles, each consisting of 1 min at 60 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR products were separated by the electrophoresis of 20 $\mu$L of each reaction mixture in a 2% agarose gel at 100 V/cm in 1× Tris-acetate-EDTA buffer. Following electrophoresis, the gels were stained with ethidium bromide, destained in distilled water, and photographed with a charge-coupled device camera. A comparison to the standard 1 kb DNA ladder (Life Technologies Inc., Gaithersburg, MD) size of the PCR products could be ensured and separated by electrophoresis.

**Statistical Analysis.** A statistical analysis was performed using an independent sample *t*-test to justify whether the differences of culture conditions on cell proliferation and GAGs synthesis were significant. Data are expressed using the mean ± standard deviations (SD) for representative examples ($n = 3\sim5$), and $p$ values less than 0.05 were considered significant.

## Results and Discussion

**Synthesis of $CS_n$-PLLA Graft Copolymer.** Chondroitin sulfate, present in numerous ECMs, is a natural biodegradable hydrophilic polymer. The graft copolymer was prepared according to Scheme 1. Chondroitin sulfate was grafted with L-lactide by ring-opening polymerization. Figure 1a shows the representative $^1H$ NMR spectrum of the graft copolymer ($CS_{11.5}$-PLLA). The proton signals belonging to the −CH or $-CH_2$ of the disaccharide units in chondroitin sulfate are in the range of 3−5 ppm, and the peak at 1.9 ppm corresponds to the methyl group (Figure 1b). The peak at 1.5 ppm with a doublet corresponds to the methyl group of the PLLA moiety [−CH-(C**H**$_3$)O−CO−]; another peak at $\delta = 5.2$ ppm with a quartet is attributed to the internal methine proton of the PLLA [−C**H**-($CH_3$)O−CO−], and the peak at $\delta = 4.1$ ppm with a quartet

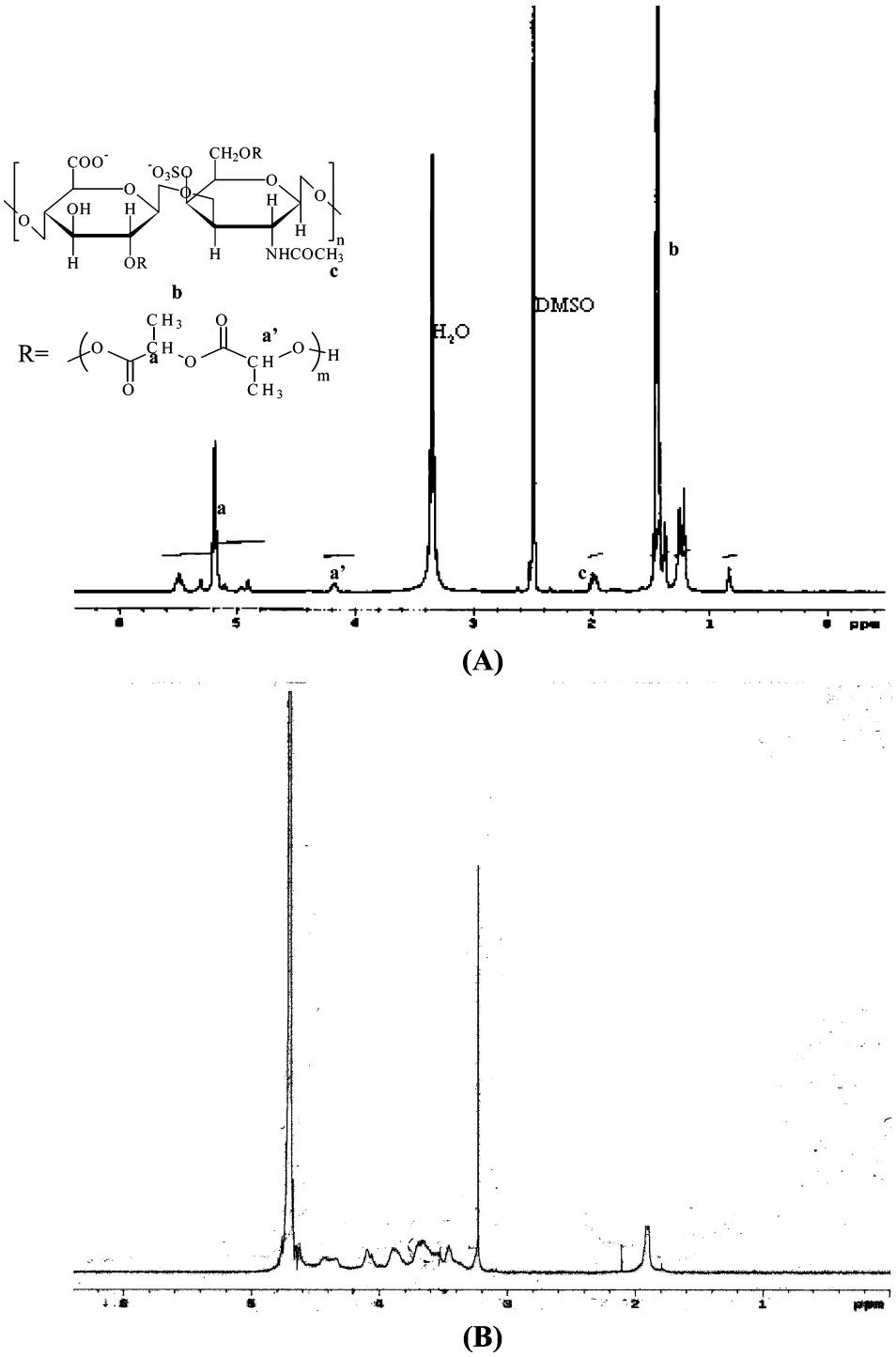



**Figure 1.** $^1$H NMR spectra of (A) $CS_{11.5}$-PLLA copolymer measured in $d_6$-DMSO and (B) chondroitin sulfate measured in $D_2O$.

belongs to the terminal methine proton of PLLA [−C**H**($CH_3$)-OH]. The methine and methylene proton signals of the chondroitin sulfate at $\delta = 3$ and 5 ppm are extremely broad and weak because the chondroitin sulfate content in the copolymer is low and the mobility of the chondroitin sulfate in organic solvents is poor. The chondroitin sulfate content in the graft copolymer can also be expressed by the degree of substitution (DS) and the degree of polymerization (DP), as described previously.[27] The DS was determined by $^1$H NMR spectroscopy, on the basis of the area ratio of the signals from the methine protons of the PLLA segments at 5.2 ppm (H) and the methyl protons of the chondroitin sulfate at 1.9 ppm (3H) (Figure 1a). The DS is the average number of hydroxyl groups on the disaccharides of CS that had reacted with L-lactide. The DP of L-lactide was also obtained by $^1$H NMR spectroscopy, on the basis of the area ratio of the terminal methine proton signal of PLLA at 4.1 ppm to the internal methine proton signal of the PLLA segments at 5.2 ppm; this area ratio is the average length of each PLLA branch. On the basis of the DS (1.12) and DP (21.45), the chondroitin sulfate content was estimated to be 11.5% by $^1$H NMR with a yield of approximately 73% and was used as the blend constituent. The copolymer thus grafted was soluble in acetone, dicromethane, DMF, and other organic solvents, but not in water. Therefore, it was of interest to study the possibility of using the $CS_{11.5}$-PLLA copolymer as a compatiblizer to improve the biological effects of PLLA.

**Preparation of Scaffolds.** The scaffolds were fabricated by both salt leaching and solvent casting methods. Table 1 presents

the composition of each scaffold. Sodium chloride particles (with sizes between 100 and 250 $\mu$m) were mixed thoroughly with the polymer solution, and then, the mixture was poured into a Teflon mold. Finally, spongelike porous scaffolds were formed. The compressive moduli of the scaffolds (without cells) are listed in Table 1. The modulus of the scaffold decreased considerably as the grafted copolymer content in the matrix increased because the mechanical strength of the graft copolymer was poor. Thus, PLLA/$CS_{11.5}$-PLLA blends may provide a range of mechanical strengths for use as a cartilage scaffold. After the copolymer is effectively blended with PLLA, the $CS_{11.5}$-PLLA copolymer can blend with PLLA with stability and distribute evenly in the blending material without an apparent phase-separation phenomenon.

**Hydrophilicity Measurements.** The hydrophilicity of the scaffold is an important index for cell adhesion. Table 1 lists the contact angles measured between the water and the sample surface. Clearly, the contact angle of the water on the samples decreased substantially from 110 °C (pure PLLA) to 68 °C [PLLA/$CS_{11.5}$-PLLA = 20:80 (w/w)], indicating an improvement in the hydrophilicity of the scaffold as the $CS_{11.5}$-PLLA copolymer was added. CS is a hydrophilic polysaccharide, and incorporating the $CS_{11.5}$-PLLA copolymer into the hydrophobic polymer is a feasible approach for improving the hydrophilicity of the scaffolds. A report reviewed that the cell adhesion was greatest on the surface with intermediate wettability (with a contact angle of around 70 °C).[28] Thus, in further cell culturing experiments, a scaffold with a blend composition of 80% $CS_{11.5}$-PLLA [PLLA/$CS_{11.5}$-PLLA = 20:80 (w/w)] was chosen.

**Scaffold Morphology.** The porous structure of the scaffold has been demonstrated to affect significantly the binding and migration of cells in vitro and to influence the rate and depth of cellular in-growth in vitro and in vivo.[29] The surface and cross-section morphologies of a typical scaffold were observed by SEM as shown in Figure 2. The spongelike scaffold had intraconnected pores with diameters of 50−250 $\mu$m. The mean porosity exceeded 85% as measured by mercury intrusion porosimetry. Polymeric scaffolds provide a 3-D microenvironment that enables chondrocytes to anchor, permits the exchange of gas and nutrients, and promotes the synthesis of the ECM.[30,31] Hence, a scaffold with high porosity provides more surface area for cell−polymer interactions, sufficient space for extracellular matrix regeneration, and minimal diffusion constraints during in vitro cell culture.[32]

**Cytotoxicity and Viability of Cells.** In this study, an MTT assay was used to measure the cytotoxicity and relative cell viability. The proliferation of mouse cartilage cells on PLLA and PLLA/$CS_{11.5}$-PLLA blend films after culturing for 2, 4, and 6 days was examined. Figure 3 shows the viabilities of chondrocytes seeded on blend films and on a PLLA (control) film. The cartilage cells' viability was demonstrated by the increase in absorbance with incubation time. The introduction of the $CS_{11.5}$-PLLA copolymer into PLLA markedly enhanced the cell growth. CS is a specific ECM that may stimulate the metabolic activity of seeded chondrocytes.[33] Another possible cause may be that the hydrophilic chondroitin sulfate facilitates the penetration of the medium into the matrix, such that the cells attach easily to the surface and grow well. In contrast, the MTT assay showed that the absorbance was low for the control (PLLA). Therefore, introducing the $CS_{11.5}$-PLLA copolymer into the matrix could enhance the viability of the cells on the scaffold because of its good cell affinity.

**Mechanical Tests after the Cell Culture on the Scaffold.** The scaffold made of 80 wt % $CS_{11.5}$-PLLA and 20 wt % PLLA, which was identified above to have favorable hydrophilicity, was studied for its effectiveness in promoting the adhesion of cells. Essential to this study, the scaffolds used in tissue engineering must have sufficient mechanical strength to support tissue regeneration at the site of implantation and to maintain sufficient integrity during both in vitro and in vivo cell growth.[34] Figure 4 plots the compressive moduli of the porous scaffolds versus different cell culturing intervals. The compression modulus of the scaffold (PLLA/$CS_{11.5}$-PLLA = 20:80) without cell seeding is 1.441 MPa (Table 1). This low mechanical

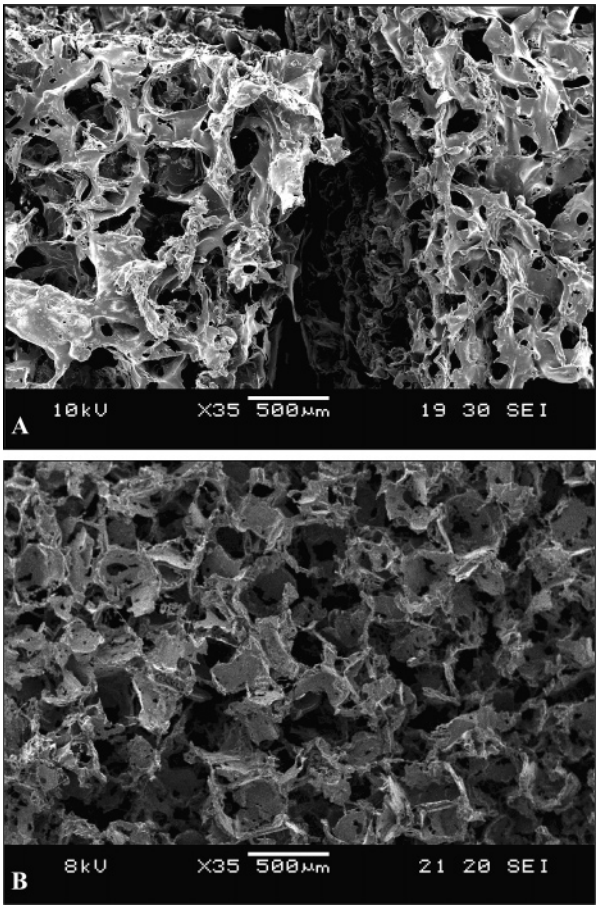



**Figure 2.** SEM micrograph of a scaffold surface (A) and cross section (B); the pore size was 50−250 $\mu$m, and the porosity was above 85%. (The composition of this scaffold is PLLA/$CS_{11.5}$-PLLA = 20:80.)

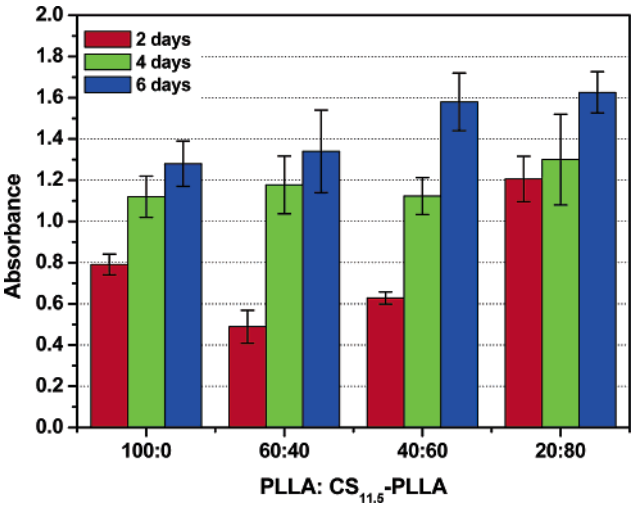



**Figure 3.** MTT assay for viable cartilage cells remaining on PLLA and PLLA/$CS_{11.5}$-PLLA blend films after different cultural intervals.

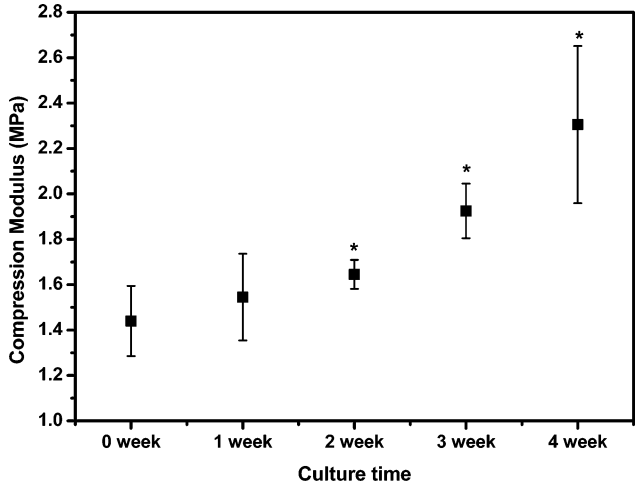



**Figure 4.** Compression modulus of the scaffolds after different cell culture time intervals (PLLA/$CS_{11.5}$-PLLA = 20:80). The scaffold size was at a thickness of 3 mm and a diameter of 7 mm. Each sample was tested in triplicate. A * indicates that the moduli increased significantly during the cell culture interval ($P < 0.05$) compared with those of the time-zero control.

property is probably related to the high porosity of the scaffold. The modulus increases slowly (1.545, 1.645, 1.925, and 2.305 MPa) with the culturing period of seeded cells. The increase in the mechanical characteristics of the porous scaffold was attributed to the growth of the cells and the secretion of the ECM (including collagen and GAGs etc.) within the scaffold. The modulus after 4 weeks of culturing (2.3 MPa) is close to that compression modulus of mouse cartilage ($2.7 \pm 0.7$ MPa). Cartilage consists of two distinct phases—a fluid phase composed of water and electrolytes and a solid phase composed of collagen fibrils (primarily, type II collagen), proteoglycans, and other glycoproteins.[35] Accordingly, the mechanical property of the artificial scaffold increases gradually as the chondrocyte secretes more ECM, which makes the cell-seeded scaffold more resemble the target cartilage tissue.

**Cell Morphology and Histochemistry Assay.** Cell infiltration and proliferation are critical for a scaffold to support and guide the regeneration of tissue. SEM observations of the cell-seeded scaffold (Figure 5), in which the cartilage cells were cultured for 2 days, revealed that the cells well-adhered to the surface of the scaffold. It indicates that the scaffold is compatible with the proliferation of cells on the scaffold, meaning good cell adhesion. In the control PLLA scaffold, the chondrocytes cannot spread and remain round (Figure 5B), which might be due to the hydrophobic nature of PLLA. Figure 6 shows the results of a histological examination of the explanted scaffold using H&E staining. The seeded chondrocytes were well-distributed in every portion of the scaffold, although the cell density was not uniform because of the difference in microstructure of the scaffold. A histological examination of these specimens using H&E stains revealed that the percentage of chondrocytes in their natural round morphology increased with an increase in the incubation period. The chondrocytes remain viable and secrete ECM components to form homogeneously compact cartilage tissues, while the cells close to the surface of the constructs have a flat and elongated morphology and are densely aggregated (Figure 6C). Masson trichrome staining (Figure 7) reveals the presence of collagen in the vicinity of the round cells. A progressive increase in Masson staining intensity from 1 through 4 weeks was observed, indicating that the secreted collagen increased. Safranin-O positive staining (Figure 8) shows that GAGs are abundant and distribute

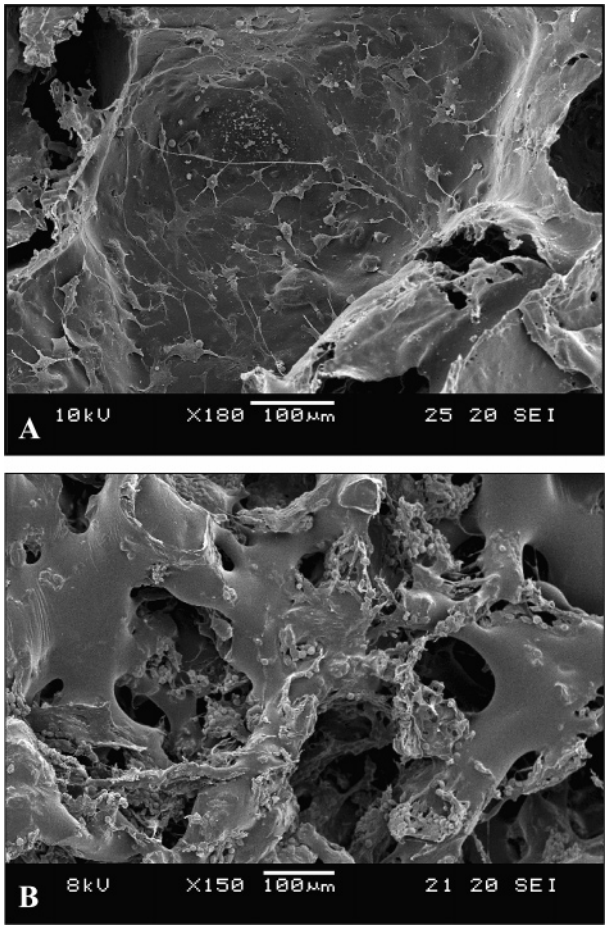



**Figure 5.** SEM photographs of chondrocytes attached to the surface of the PLLA/$CS_{11.5}$-PLLA (20:80 wt/wt) blend scaffold (A) and on the PLLA scaffold (B) after culturing for 2 days.

homogeneously around the cells, and the amount of secreted GAG also increased with an increase in the incubation time. The presence of intense Masson and Safranin-O staining provides evidence of the secretion of a cartilaginous matrix by chondrocytic cells, indicating that the chondrocytes in the scaffold are functionally active. The histologies of these constructs display a mature tissue, comprising proteoglycan and collagen with uniformly dispersed cells of chondrocytic morphology in lacunae. Moreover, the explants could be divided into two parts: central and peripheral, according to the morphology of the cells. In the central part, most of the chondrocytes have a round morphology, are embedded in lacunae, and are surrounded by an abundant cartilaginous ECM. In the peripheral part, the cells are of a spindle shape, suggesting the formation of fibro tissue. Cell in-growth is also observed in the blend scaffold, and the cells maintain their spherical chondrocyte phenotype. A histological examination of the explants by H&E, Masson, and Safranin-O staining demonstrate that a dense cartilaginous ECM surrounds the spherical chondrocytes, suggesting that the blend scaffold supports the formation of neocartilage and generates an extensive amount of ECM. Additionally, lacunas were formed in the matrix that surrounded the chondrocytes after 2 weeks of culture time, while their morphologies were similar to that of natural cartilage.

**Synthesis of Sulfate Glycosaminoglycan (GAGs) and Collagen.** GAGs and collagens are necessary for culturing cartilage in tissue engineering. The DMB assay relies on the spectrophotometric detection of metachromatic changes in DMB as the cationic dye binds to the sulfate and carboxyl groups

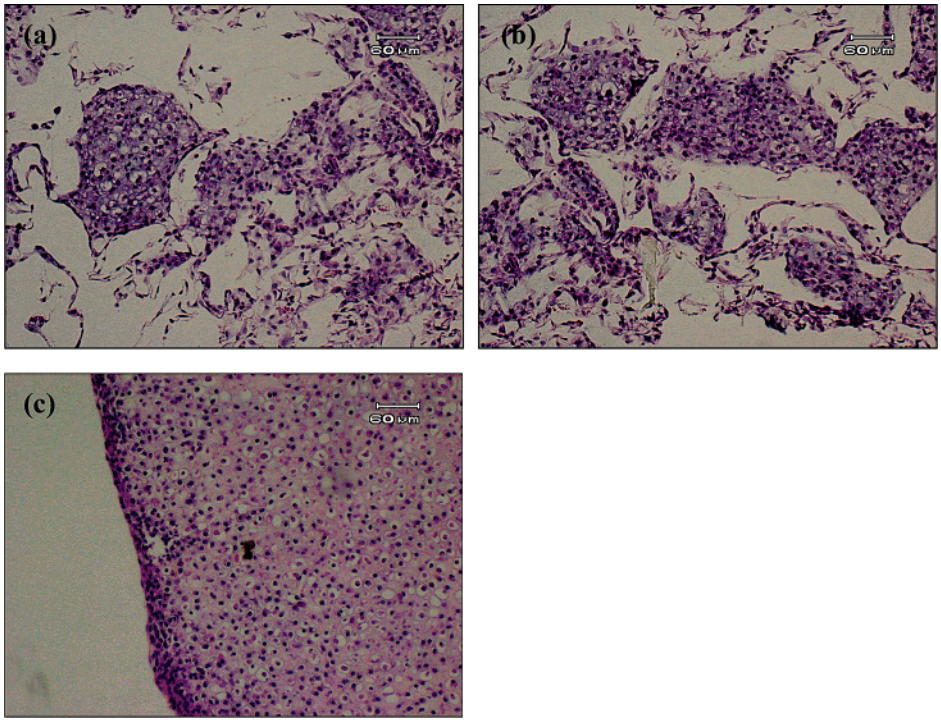



**Figure 6.** Histochemical examination of the cartilage tissue formed at various culture intervals on the blend scaffold (PLLA/$CS_{11.5}$-PLLA = 20:80): (a) 1 week, (b) 2 weeks, and (c) 4 weeks.

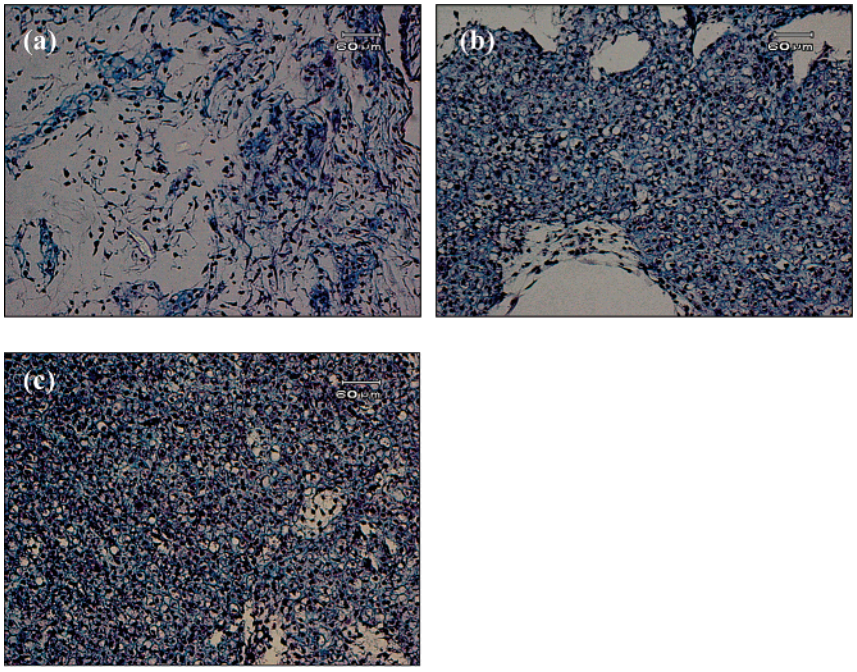



**Figure 7.** Masson trichrome staining of the scaffolds cultured in vitro after 1, 2, and 4 weeks: (a) 1 week, (b) 2 weeks, and (c) 4 weeks.

present in GAG chains.[36] The metachromatic of DMB was monitored at 525 nm and exhibited a significant monotonic increase with an increase of the concentration of chondroitin sulfate. It was linear up to 100 ppm ($R^2 = 0.992$). Therefore, the DMB assay can be used to estimate the amount of GAGs in the artificial scaffold.[37,38] The synthesis of GAGs is an important function of chondrocytes and plays a significant role in regulating the chondrocyte phenotype. Figure 9 shows that the GAG content does not change very much for the first and second weeks of culturing in scaffolds of various compositions. However, it increases significantly for longer culturing times (3−6 weeks). Meanwhile, more GAGs were synthesized by chondrocytes on blend scaffolds ($P < 0.05$) than on the control PLLA scaffold (including GAGs accumulated on materials and released into the medium); perhaps, a CS-based copolymer contained in the blend scaffolds could stimulate the cells to secrete more GAGs. Another observation was that the GAG contents were substantially lower than those in native tissue, partially because the incubation times were short and the starting cell density in the chondrocytes/scaffold constructs was low.[39]

Collagen is another important and the most abundant constituent of the natural ECM. Here, collagen was quantified by evaluating the amount of uronic acid in the cellular fraction. Figure 10 shows the amount of collagen in the cell extracts. During a given culturing period, the cells grown on blend scaffolds produced more collagen than those grown on the

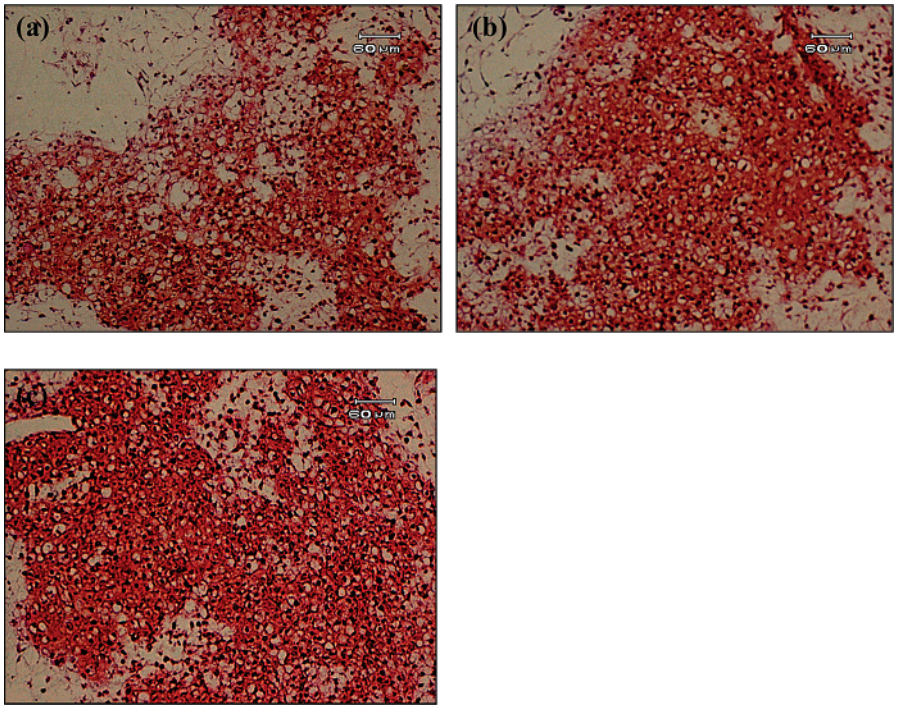



**Figure 8.** Safranin-O staining of the scaffolds cultured in vitro after 1, 2, and 4 weeks: (a) 1 week, (b) 2 weeks, and (c) 4 weeks.

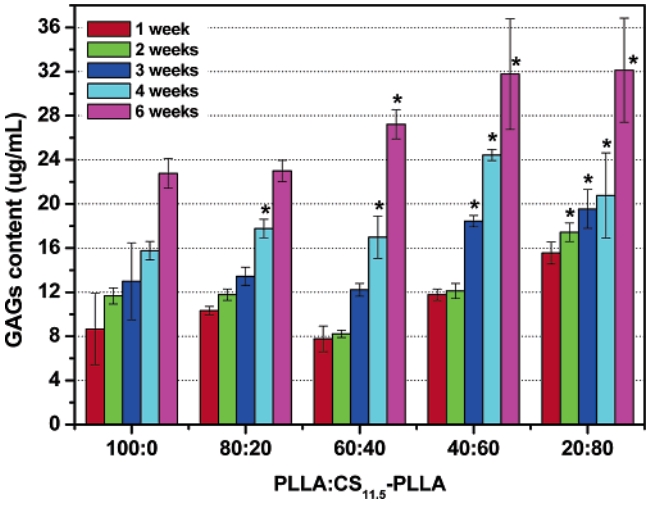



**Figure 9.** GAGs content for different scaffolds measured at various culture intervals. Data are expressed as a mean value ± SD ($n = 5$). A * indicates that the GAGs content increased significantly ($P < 0.05$) compared with that of the PLLA control.

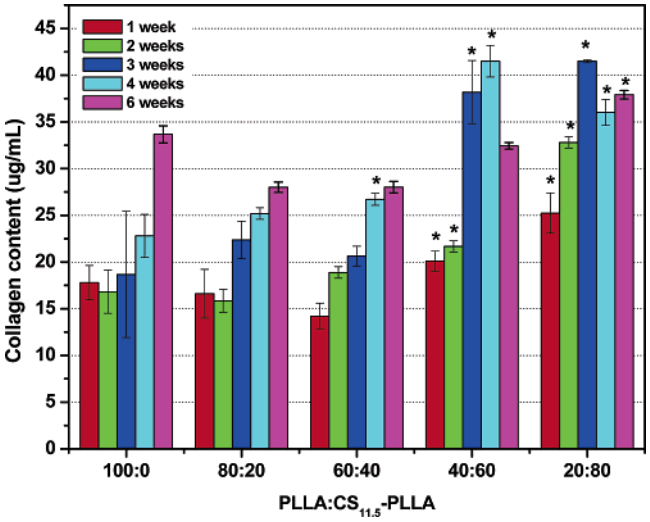



**Figure 10.** Collagen content analysis for different scaffolds measured at various culture intervals. Data are expressed as a mean value ± SD ($n = 5$). A * indicates that the collagen content increased significantly ($P < 0.05$) compared with that of the PLLA control.

PLLA scaffold (control), especially at longer culturing times. However, the collagen content decreased slightly in PLLA/$CS_{11.5}$-PLLA = 40:60 at 6 weeks compared with that at 4 weeks and in PLLA/$CS_{11.5}$-PLLA = 20:80 at 4 and 6 weeks compared with that 3 weeks and might be due to the withered cells secreting some collagenase in the center of the scaffold and some collagen being released into the culture medium. The effectiveness of any artificial scaffold depends strongly on the biological and physicochemical properties of ligands immobilized on the scaffold surface.[40,41] Molecular recognition between bound ligands and cell receptors is known to induce a series of signal transduction cascades that are transmitted from the ECM to the cell. It is evident that the CS-based copolymer provides such properties.

**Extracellular Matrix Products.** A major shortcoming in the cell-based therapy of cartilage is the dedifferentiation of chondrocytes, which occurs during in vitro expansion. The gene expressions of type I collagen, type II collagen, and the aggrecan of chondrocytes after culturing for 2 weeks were examined by RT-PCR (Figure 11). After 2 weeks of cultivation, the mRNA for GAPDH was well-expressed in all of the samples. GAPDH, the housekeeping gene, served as the control in the analysis of the expression profiles of chondrogenic genes. The GAPDH band density did not vary significantly among the four groups. When cultured in a monolayer (Figure 11, part 3), chondrocytes lost their original round morphologies and adopted a more spindle fibroblast-like appearance, accompanying the expressed low level of type II collagen transcripts, and failed to express aggrecan compared to freshly isolated mouse articular cartilage. Meanwhile, a fibroblast-specific ECM (type I collagen) was detected, revealing that mouse chondrocytes lost their phenotypes (dedifferentiated). Therefore, they exhibit the development of a fibroblastic morphology, a switch in the production of

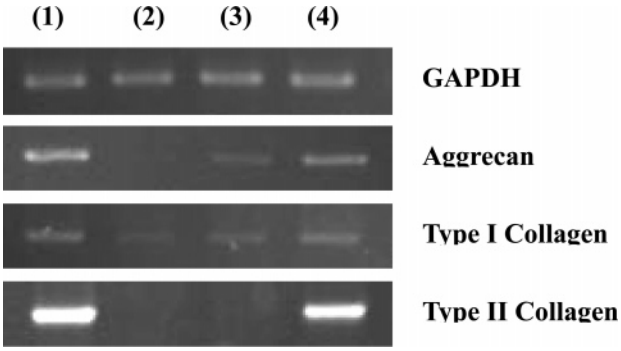



**Figure 11.** Expression of cartilage-specific genes analyzed by RT-PCR. Total RNA was analyzed for type I collagen, type II collagen, and aggrecan, with the housekeeping gene GAPDH as the control. (1) PLLA/$CS_{11.5}$-PLLA = 20:80 (P0); (2) PLLA/$CS_{11.5}$-PLLA = 20:80 (P4); (3) 2-D culture (P0); (4) PLLA (P0). PX means the passage number of the cell.

cartilage-specific type II collagen to the production of type I collagen, and a switch in the production of aggrecan to the production of proteoglycan with low molecular weights. When the chondrocytes were cultured in the blend scaffold (Figure 11, part 1), the expressions of mRNA for type II collagen and aggrecan were upregulated and that of mRNA for type I collagen was downregulated. Interestingly, when chondrocytes were cultured in a 3-D scaffold, the expressions of type II collagen and aggrecan were maintained at a level similar to those observed in primary mouse articular chondrocytes. Furthermore, the results of gene expression are in agreement with histological observations, also indicating the differentiation of the cells within the scaffold. Moreover, no expression of the aggrecan and a weak expression of type II collagen were identified in the scaffold with four passages cells (Figure 11, part 2), which suggested that the cells could not redifferentiate the dedifferentiated cells after four passages.

## Conclusions

Because tissue engineering is now showing promise as a possible method for cartilage repair, cell-based strategies for cartilage tissue engineering are considered to have great potential in preserving physiological hyaline-like cartilage tissue. In this study, a new degradable graft copolymer $CS_n$-PLLA was synthesized. The introduction of the $CS_n$-PLLA graft copolymer into the hydrophobic polymer (PLLA) to form a biomimetic scaffold significantly promoted its hydrophilicity. Scaffolds with pore sizes between 50 and 250 $\mu$m and of high porosity were prepared from $CS_{11.5}$-PLLA/PLLA blends. Histological examination showed that the chondrocyte maintained its phenotype and secreted the ECM normally in the blend scaffold in vitro. In the central parts of the scaffold, round chondrocytes were formed, embedded in the lacunae, and surrounded by an abundant ECM. The histological observations matched the results of gene expression. The characteristics of the cartilage tissue produced by this in vitro system were similar to those of nativelike tissue. It is concluded that the CS-based copolymer in the biodegradable matrixes (PLLA) enhanced chondrocytes to synthesize more cartilaginous ECM, maintained the characteristic cell morphology and thus cell phenotype, and may serve as a potential candidate for cartilage tissue engineering.

**Acknowledgment.** The authors would like to thank the Ministry of Economic Affairs of the Republic of China, Taiwan, for financially supporting this research under Contract No. 93-EC-17-A-17-S1-0009 of the Technology Development Program.

## References and Notes

(1) Peter, M. C.; Mooney, D. J. Synthetic extracellular matrices for cell transplantation. *Mater. Sci. Forum* **1997**, *250*, 43−52.
(2) Kinoshita, Y.; Kobayashi, M.; Hidaka, T.; Ikada, Y. Reconstruction of mandibular continuity defects in drug using poly(l-lactide) mesh and autogenic particulate cancellous bone and marrow: Preliminary report. *J. Oral Maxillofacial Surg.* **1997**, *55*, 718−723.
(3) Pattison, M. A.; Wurster, S.; Webster, T. J.; Haberstroh, K. M. Three-dimensional nano-structured PLGA scaffolds for bladder tissue engineering. *Biomaterials* **2005**, *26*, 2491−2500.
(4) Khor, H. L.; Ng, K. W.; Schantz, J. T.; Phan, T. T.; Lim, T. C.; Teoh, S. H. Poly(ϵ-caprolactone) films as a potential substrate for tissue engineering an epidermal equivalent. *Mater. Sci. Eng., C* **2002**, *20*, 71−75.
(5) Zhu, Y.; Sun, Y. The influence of polyelectrolyte charges of polyurethane membrane surface on the growth of human endothelial cells. *Colloids Surf., B* **2004**, *36*, 49−55.
(6) Chung, T. W.; Liu, D. Z.; Wang, S. Y.; Wang, S. S. Enhancement of the growth of human endothelial cells by surface roughness at nanometer scale. *Biomaterials* **2003**, *24*, 4655−4661.
(7) Wang, Y. W.; Wu, Q.; Chen, G. Q. Reduced mouse fibroblast cell growth by increased hydrophilicity of microbial polyhydroxyalkanoates via hyaluronan coating. *Biomaterials* **2003**, *24*, 4621−4629.
(8) Ma, Z.; Gao, C.; Ji, J.; Shen, J. Protein immobilization on the surface of poly-L-lactic acid films for improvement of cellular interactions. *Eur. Polym. J.* **2002**, *38*, 2279−2284.
(9) Lu, Q.; Ganesan, K.; Simionescu, D. T.; Vyavahare, N. R. Novel porous aortic elastin and collagen scaffolds for tissue engineering. *Biomaterials* **2004**, *25*, 5227−5237.
(10) Rowley, J. A.; Madlambayan, G.; Mooney, D. J. Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* **1999**, *20*, 45−53.
(11) Madihally, S. V.; Matthew, H. W. T. Porous chitosan scaffolds for tissue engineering. *Biomaterials* **1999**, *20*, 1133−1142.
(12) Yamane, S.; Iwasaki, N.; Majima, T.; Funakoshi, T.; Masuko, T.; Harada, K.; Minami, A.; Monde, K.; Nishimura, S. Feasibility of chitosan-based hyaluronic acid hybrid biomaterial for a novel scaffold in cartilage tissue engineering. *Biomaterials* **2005**, *26*, 611−619.
(13) Chang, C. H.; Liu, H. C.; Lin, C. C.; Chou, C. H.; Lin, F. H. Gelatin-chondroitin-hyaluronan tri-copolymer scaffold for cartilage tissue engineering. *Biomaterials* **2003**, *24*, 4853−4858.
(14) Sinha, V. R.; Kumria, R. Polysaccharides in colon-specific drug delivery. *Int. J. Pharm.* **2001**, *224*, 19−38.
(15) Chen, J.; Jo, S.; Park, K. Polysaccharide hydrogels for protein drug delivery. *Carbohydr. Polym.* **1995**, *28*, 69−76.
(16) Cai, Q.; Wan, Y.; Bei, J.; Wang, S. Synthesis and characterization of biodegradable polylactide-grafted dextran and its application as compatilizer. *Biomaterials* **2003**, *24*, 3555−3562.
(17) Gomes, M. E.; Ribero, A. S.; Malafaya, P. B.; Reis, R. L.; Cunha, A. M. A new approach based on injection moulding to produce biodegradable starch-base polymeric scaffolds: Morphology, mechanical and degradation behavior. *Biomaterials* **2001**, *22*, 883−889.
(18) Illum, L. Nasal drug delivery-possibility, problems and solutions. *J. Controlled Release* **2003**, *87*, 187−198.
(19) Mendes, S. C.; Reis, R. L.; Bovell, Y. P.; Cunha, A. M.; Blitterswijk, C. A. V.; Bruijn, J. D. Biocompatibility testing of novel starch-based materials with potential application in orthopaedic surgey: A preliminary study. *Biomaterials* **2001**, *22*, 2057−2064.
(20) Ayad, S.; Boot-Handford, R. P.; Humphries, M. J.; Kadler, K. E.; Shuttleworth, C. A. *The Extracellular Matrix-Facts Book*; Academic Press: San Diego, CA, 1994.
(21) Donabedian, D. H.; McCarthy, S. P. Acrylation of Pullulan by ring opening of Lactones. *Macromolecules* **1998**, *31*, 1032−1039.
(22) Liang, H. J.; Tsai, C. L.; Chen, P. Q.; Lu, F. J. Oxidative injury induced by synthetic humic acid polymer and monomer in cultured rabbit articular chondrocytes. *Life Sci.* **1999**, *65*, 1163−1173.
(23) Farndale, R. W.; Buttle, D. J.; Barrett, A. J. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim. Biophys. Acta* **1986**, *883*, 173−177.
(24) Deng, Y.; Lin, X. S.; Zheng, Z.; Deng, J. G.; Chen, J. C.; Ma, H.; Chen, G. Q. Poly(hydroxybutyrate-co-hydroxyhexanoate) promoted production of extracellular matrix of articular cartilage chondrocytes in vitro. *Biomaterials* **2003**, *24*, 4273−4281.

(25) Reno, C.; Marchuk, L.; Sciore, P.; Frank, C. B.; Hart, D. A. Rapid isolation of total RNA from small samples of hypocellular, dense connective tissues. *Biotechniques* **1997**, *22*, 1082−1086.
(26) Heid, C. A.; Stevens, J.; Livak, K. J.; Williams, P. M. Real time quantitative PCR. *Genome Res.* **1996**, *6*, 986−994.
(27) Ohya, Y.; Maruhashi, S.; Ouchi, T. Graft polymerization of L-lactiod on pullulan through the trimethylsilyl protection method and degradation of the graft copolymers. *Macromolecules* **1998**, *31*, 4662−4665.
(28) Groth, T.; Altankov, G. Cell-surface interactions and the tissue compatibility of biomaterials. In *New Biomedical Materials*; Haris, P. I., Chapman, D., Eds.; IOS Press: Washington, DC, 1998; pp 12−23.
(29) van Tienen, T. G.; Heijkants, R. G. J. C.; Buma, P.; de Groot, J. H.; Pennings, A. J.; Veth, R. P. H. Tissue ingrowth and degradation of two biodegradable porous polymers with different porosities and pore sizes. *Biomaterials* **2002**, *23*, 1731−1738.
(30) Yang, L.; Korom, S.; Weti, M.; Hoerstrup, S. P.; Zund, G.; Jung, F. J.; Neuenschwander, P.; Weder, W. Tissue engineered cartilage generated from human trachea using DegraPol scaffold. *Eur. J. Cardio-thoracic Surg.* **2003**, *24*, 201−207.
(31) Kuberka, M.; von Heimburg, D.; Schoof, H.; Heschel, I.; Rau, G. Magnification of the pore size in biodegradable collagen sponages. *Int. J. Artif. Organs* **2002**, *25*, 67−73.
(32) Oh, S. H.; Kang, S. G.; Kim, E. S.; Cho, S. H.; Lee, J. H. Fabrication and characterization of hydrophilic poly(lactide-co-glycolic acid)/poly(vinyl alcohol) blend cell scaffolds by melt-molding particle-leaching method. *Biomaterials* **2003**, *24*, 4011−4021.
(33) van Susante, J. L. C.; Pieper, J.; Buma, P.; van Kuppevelt, T. H.; van Beuningen, H.; van der Kraan, P. M.; Veerkamp, J. H.; van der Berg, W. B.; Veth, R. P. H. Linkage of chondroitin sulfate to type I collagen scaffolds stimulates the bioactivity of seeded chondrocytes in vitro. *Biomaterials* **2001**, *22*, 2359−2369.
(34) Thomson, R. C.; Yaszemski, M. J.; Powers, J. M.; Mikos, A. G. Fabrication of biodegradable polymer scaffolds to engineering trabecula bone. *J. Biomater Sci., Polym. Ed.* **1995**, *7*, 23−38.
(35) Lee, C. R.; Grodzinsky, A. J.; Hsu, H. P.; Martin, S. D.; Spector, M. Effects of harvest and selected cartilage repair procedures on the physical and biochemical properties of articular cartilage in the canine knee. *J. Orthop. Res.* **2000**, *18*, 790−799.
(36) Enobakhare, B. O.; Bader, D. L.; Lee, D. A. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. *Anal. Biochem.* **1997**, *243*, 1189−191.
(37) Groves, P. J.; Shellis, R. P.; Dieppe, P. A. Use of polyvinyl alcohol to stabilize binding of sulfated glycosaminoglycans to dimethylmethylene blue. *Anal. Biochem.* **1997**, *245*, 2247−248.
(38) Kawasaki, K.; Ochi, M.; Uchio, Y.; Adach, N.; Matsusaki, M. Hyaluronic acid enhances proliferation and chondroitin sulfate synthesis in cultured chondrocyte embedded in collagen gels. *J. Cell. Physiol.* **1999**, *179*, 142−148.
(39) Betre, H.; Setton, L. A.; Meyer, D. E.; Chilkoti, A. Characterization of a genetically engineered elastin-like polypeptide for cartilaginous tissue repair. *Biomacromolecules* **2002**, *3*, 910−916.
(40) Risbud, M.; Ringe, J.; Bhonde, R.; Sittinger, M. In vitro expression of cartilage-specific markers by chondrocytes on a biocompatible hydrogel: Implications for engineering cartilage tissue. *Cell Transplant.* **2001**, *10*, 755−763.
(41) Buckwalter, J. A.; Mankin, H. J. Articular cartilage: Tissue design and chondrocyte-matrix interactions. *Instr. Course Lect.* **1998**, *47*, 477−486.

BM060451X