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Crosslinked chitosan: Its physical properties and the effects of matrix stiffness on chondrocyte cell morphology and proliferation

Anuradha Subramanian, Hsin-Yi Lin

Abstract: Chitosan [<(1-4)-2 amino-2-deoxy-d-glucose], the natural polyaminosaccharide derived from *N*-deacetylation of chitin [<(1-4)-2 acetamide-2-deoxy-d-glucose], has been shown to possess attractive biological and cell interactive properties. Recently chitosan and chitosan analogs have also been shown to support the growth and continued function of chondrocytes. In the present study, chitosan substrates are crosslinked with a functional diepoxide (1,4 butanediol diglycidyl ether) to alter its mechanical property, and the viability and proliferation of the canine articular chondrocytes seeded on the crosslinked surface are further assayed. Of interest is the impact of substrate stiffness on the growth and proliferation of articular canine chondrocytes. Cross linked scaffolds were also subjected to degradation by chitosanase to examine the impact of cross linking on enzyme-assisted degradation. The hydrophilicity and compression modulus of the crosslinked surfaces were measured via contact-angle measurements and compression tests, respectively. Scanning electron microscopy (SEM) and fluorescent staining were used to observe the proliferation and morphology of chondrocyte cells on noncrosslinked and crosslinked surfaces. The crosslinked chitosan was found to be nontoxic to chondrocytes and more hydrophilic. Its compression modulus and stiffness increased, which may improve the scaffold resistance to wear and *in vivo* shrinkage once implanted. The increased stiffness also seemed to serve as an additional mechanical stimulus to promote chondrocyte growth and proliferation. The cell morphology on crosslinked scaffolds seen by SEM and fluorescent stain was the typical chondrocytic rounded shape. The method

proposed provides a nontoxic way to increase the mechanical strength of the chitosan scaffolds.

Key words: cartilage tissue engineering; chitosan scaffold; chondrocytes; crosslinking; biomaterial mechanical testing

INTRODUCTION

The articular cartilage is the dense white tissue covering the articulating surfaces of bones. The regenerative capability of the articular cartilage is very limited when injured or damaged with aging. One approach to repair defected cartilage is to generate functional tissue by seeding functional chondrocyte cells in a biocompatible scaffold and then implant the cell–material complex to repair chondral defects.1 The use of polymeric scaffolds to generate tissue-engineered cartilage has been extensively studied.2–8 Given the importance of GAG in stimulating chondrogenesis, use of GAG or GAG analogs as components of a cartilage tissue scaffold appears to be a logical approach. Chitosan has been reported to share some structural features with glycosaminoglycan, which occurs naturally in articular cartilage, and has been reported to support chondrogenesis and biosynthesis of markers associated with chondrocyte metabolism.7–10 By using a combination of freeze drying and leaching techniques, chitin/chitosan and its derivatives (carboxymethyl chitin and bicarboxymethyl chitosan) have been blended with hydroxyapatite to yield composites for use in bone-tissue engineering.11 Chitosan sponges were made by freeze-drying tripolyphosphate crosslinked chitosan and loaded with platelet-derived growth factor. These scaffolds were evaluated for their ability to regenerate periondontal

bone. Freeze-drying techniques have been used to prepare porous membranes and 3D scaffolds of chitosan for use in tissue engineering applications.12 In a previous study, nonporous chitosan membranes were modified with chondroitin sulfate (CSA) and CSA-chitosan membranes were evaluated for their ability to support chondrogenesis.7,11 In a separate study, a scaffold in the form of an interpenetrating polymeric network was made from a mixture of collagen and chitosan.13 The biocompatibility of the chitosan-based scaffolds have been evaluated in mice,14 where porous chitosan scaffolds (unseeded) were implanted in mice, and animals were sacrificed after 1, 2, 4, 8, or 12 weeks. Macroscopic inspection of the implantation site revealed no pathological inflammatory responses, gram staining and limulus assays revealed no evidence of infection or endotoxin, and lymphocyte proliferation assays and antibody responses indicated a low incidence of chitosan-specific reactions.14 This study serves to demonstrate that chitosan-based scaffolds had a high degree of *in vivo* biocompatibility in the animal model studied.

The regulation of chondrocyte function (e.g., biosynthesis of Type II collagen, PG synthesis and down regulation of nitrous oxide production) by growth factors (TGF<), cytokines, and biomechanical forces (dynamic compression) has been demonstrated. 15–21 An area that has not been studied as well, but is critical to the understanding of cartilage function and maintenance during normal and disease states, is the regulation of chondrocyte function by an intrinsic mechanical environment or substrate mechanics. For example, osteoarthritis is brought about by the degradation of the collagen fibrils in the ECM, leading to a cartilage matrix with decreased tensile modulus, which in turn perhaps leads to decreased biosynthetic activity of chondrocytes. Chondrocytes, when seeded on substrates, are known to exert contractile forces,22,23 which usually results in the shrinkage of the substrate, referred to as the chondrocyte-mediated contraction (CMC). The changes in size and microstructure of the implants/ substrates due to cell contraction may

reduce the pore size and further impede the migration of cells into the scaffolds and free exchange of the nutrients and the metabolites.24 The potential of articular chondrocytes to exert a contractile force upon scaffolds and change the size and pore structure of the scaffolds has been reported both *in vivo* and *in vitro*.22–25 Collagen-based matrices, when seeded with chondrocytes, were reported to undergo up to 35% shrinkage.22,23 Noncrosslinked collagen sponges, when implanted in full-thickness osteochondral defects in canine models, were observed to undergo cell-driven contraction of the matrix and thus prevent complete fitting of scaffolds in the chondral defects.25,26 Preventive methods against mechanical changes of polymer scaffolds in the articular cartilage implant site have been studied.27,28 Crosslinking treatments have been used to enhance the mechanical properties of the polymeric scaffolds.22,23,29 –31 The effects of four crosslinking methods on the compressive stiffness of collagen-glycosaminoglycan (CG) matrices and the interaction between adult canine articular chondrocytes and the stiffened matrix have been evaluated. 22 Dehydrothermally treated and ultra violet irradiated matrices were found to be the most compliant, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and glutaraldehyde (GTA) –treated matrices were the stiffest. Over the 4-week culture period, the GTA and EDAC matrices (the stiff ones) resulted in the highest rate of cell proliferation. RGD-derivatized alginate hydrogels were crosslinked with Ca2< and Ba2< ions and were reported to provide 10-fold higher chondrocyte attachment when compared to uncrosslinked alginate hydrogels.31 More recently,29 porous collagen scaffolds were crosslinked with EDAC in the presence of lysine; however, no obvious difference in resistance to contraction was observed over matrices crosslinked with EDAC. Furthermore, changes in stiffness upon crosslinking and the impact of the same of the morphology of seeded fibroblasts were not reported. The aim in this study was to chemically crosslink the ONH2 groups on the chitosan backbone and to modify the stiffness of

chitosan-based scaffolds and substrates. Furthermore, increased stiffness is expected as a result of crosslinking to impact chondrocyte behavior, and to test the hypothesis that the rigidity of the matrix can itself serve as a mechanical signal and direct seeded chondrocytes to possess higher biosynthetic activity of crosslinked matrices.

MATERIALS AND METHODS

Reagents

All chemicals were of analytical grade or better. Chitosan with a degree of deacetylation of 81.7 was purchased from Vansom (Redmond, WA) and was used without further purification. The diepoxide was purchased from Sigma Chemical Company (St. Louis, MO). Articular chondrocytes of canine origin (shoulder joints of dogs) were obtained from Dr. Mark Beatty, College of Dentistry, University of Nebraska Medical School (Lincoln, NE). Cellculture media and other reagents were obtained from Invitrogen. Mechanical tests were performed on an MTS Instrument available at the College of Dentistry (University of Nebraska Medical School, Lincoln, NE). SEMs were obtained on a JEOL-6100 microscope at the Center of Biotechnology (UNL, Lincoln, NE). All reagents were used without further purification.

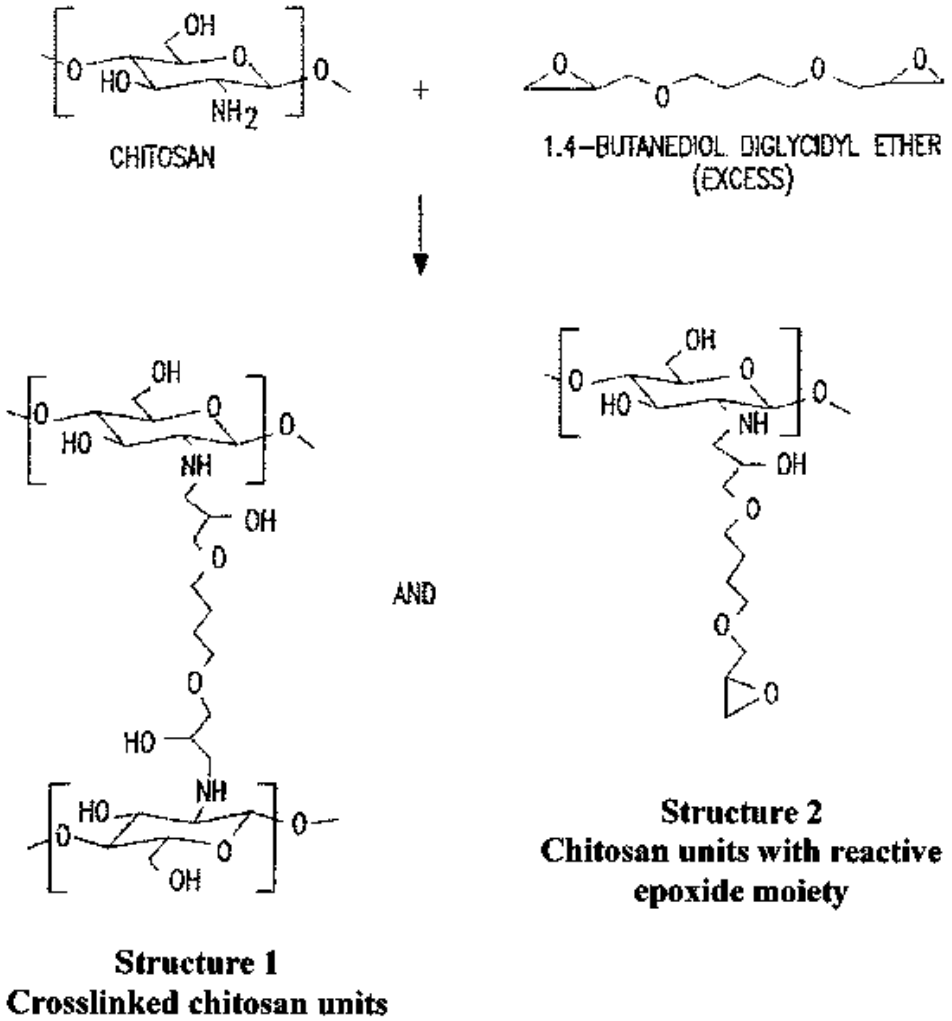

Figure 1. Schematic for the strategy for cross-linking with bifunctional epoxides. Chitosan-based films and scaffolds were first neutralized with 0.05*N* NaOH, washed with sterile PBS, and dehydrated with isopropanol. The solid line indicates the chitosan backbone. Surfaces were incubated with a known stoichiometric excess of functional diepoxide.[29] To obtain maximum crosslinking (Structure 1), the reaction was carried out at 50°C for 24 h. The surfaces were washed in IP, PBS, and then used. The surfaces were washed in sterile PBS and stored until further use.

Preparation of films and scaffolds

A 2% w/v solution of chitosan (81.7% deacetylated, MW < 276 kDa, Lot No. 01-CISQ-1702, Vanson HaloSource, WA) in 1% acetic acid was freshly prepared prior to scaffold preparation. Glass slides (Fisher) were coated with 1-mL chitosan solution and air dried at room temperature. The chitosan coated slides were further treated in an 80°C oven over-

night to prevent the detachment of the chitosan film. Dried films were then neutralized with 1*N* NaOH and sequentially rinsed with deionized water, air dried and stored until further use. For the preparation of scaffolds via freeze-dry and lyophilization (FDL), a 2-mL solution of 2% chitosan solution was prepared as mentioned earlier, and pipetted into each well of a 24-well tissue-culture polystyrene plate (TCP, Falcon). The samples were then frozen at <20°C and the frozen samples were lyophilized for 24–36 h. The scaffolds were then neutralized with 1*M* NaOH and rinsed as detailed earlier. In order to preserve the shape (pores) of the scaffolds, the scaffolds were gradually dehydrated with a series of ethanol solutions (20–100%) before air drying the scaffolds. The scaffold sample used in the cell seeding experiments was 0.8 –1.0 cm in diameter and 0.3– 0.5 cm in thickness.

Crosslinking films and scaffold

A diepoxide-based bifunctional linker32 (1,4 butanediol diglycidyl ether) was used to crosslink the chitosan chains via the reactive amino group on the chitosan backbone. The schematic of the crosslinking method is shown in Figure 1. By varying the molar stoichiometric excess of the diepoxide, films were prepared and scaffolds were freeze-dried with varying degrees of crosslinking. A crosslinking agent (1,4-butanediol diglycidyl ether, Sigma, MO) was added to isopropanol to make solutions in which the mole ratio of the diepoxide to the NH2 groups on the chitosan was 1/2 to 1 (0.5<), 1 to 1 (1<), and 5 to 1 (5<). Controls (0<) were samples submerged in plain isopropanol without adding the epoxide. Samples were placed in a 45°C shaker incubator at 60 rpm for 16 h. After 16 h, samples were rinsed with isopropanol to remove excess ether and air dried. Prior to cell-

seeding experiments, scaffolds and films were sequentially washed with sterile deionized water followed by sterile PBS.

Cell culture

All cells used in this study were passage 2 or 3 canine chondrocytes, harvested from the articular cartilage obtained from the shoulder joint of adult dogs. The cell-culture media used was RPMI <10% fetal bovine serum supplemented with antibiotics and antimycotics (all cell culture reagents are from Invitrogen). Experiments with cells were maintained in a cell-culture incubator (Isotemp, Kendro, NC) at 37°C, 95% humidity and 5% CO2.

Surface hydrophilicity

A contact angle system (model OCA 15<, Dataphysics Co.) was used to test the hydrophilicity of the crosslinked films. Water was used as the liquid phase. Five microliters (<L) of water was injected on the film surface at a rate of 3 <L/s. A Laplace-Young fitting was used to calculate the contact angle in degrees. For each epoxide to (ONH2) molar ratio, three slides were used and six data points from each slide with error <1.0 were taken for average and standard-deviation calculation.

Compression test

Scaffolds were rehydrated in deionized water 1 day before the test. The test was performed on an Instron machine (model 1123, Instron, MA). The crosshead speed was 2 mm/ min and a 50-kgf load cell was used with Instron. Six specimens of each epox-

ide/ONH2 mole ratio were tested at room temperature. The load (kgf) -displacement (millimeter) data were recorded by the computer software provided by Instron and converted to stress-strain curves to obtain elastic modulus (kPa). No maximum compression strength was obtained because the hydrogel did not break when subject to compression. Films were tested according to the procedure detailed elsewhere.33

Degradation with chitosanase

The dry weight (*W*0) of the scaffolds was measured before the test. Chitosanase (EMD Biosciences) was dissolved in acetate solution (sodium acetate buffer: 200 m*M*, pH 4.5 plus 0.02% sodium azide) to a final concentration of 0.1 U/mL. Scaffolds were submerged in chitosanase solution (1:100 w/v) and incubated at 37°C with constant shaking over indicated periods of time (1, 2, 4, and 24 h). Samples were removed from the enzyme solution after each time period and the scaffolds were rinsed with DI water. The samples were dried and weighed (*W*f), and the percentage of degradation was calculated [(*W*0 < *W*f)/*W*0 < 100%].

Chondrocyte cell adhesion and viability test with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromide] assay

Glass slides coated with chitosan films were cut into 1 < 1-cm2 squares and placed in the wells of 12-well TCP plates with the film facing upward. Samples were disinfected with

70% ethanol. Canine chondrocytes were then seeded on the films at 3 < 104/film. Cells seeded on TCP served as controls. After 3 days, the cell-culture medium was removed and a 1 mg/mL solution of MTT was added to the cells. After 4 h, the MTT solution was removed and isopropanol was added and held for 20 min. The color of formazan salt was measured by an ELISA plate reader (ELx800, Biotek) at 595 nm (A595). Cell viability was calculated as a percentage of the TCP control [(*A*sample/*A*control) < 100%].

Cell morphology and proliferation (SEM and fluorescent stain)

To observe the cells growing on two-dimensional substrate, chondrocyte cells were seeded at 1 < 104/cm2 on films that were *not* attached to slides (free-swelling) and cultured for 1 week before visualization by microscopy. At the end of the culture period, the medium was removed from each well, 300 <L of trypsin (Gibco) solution (0.25% trypsin in sterile PBS) was added to each well, and the plates were held for 30 min for cells to detach. The solution in the well was pipetted up and down to get cells into suspension, and the cell counting was done with the use of a hemocytometer.33 Upon cell seeding, the seeded scaffolds were transferred to a fresh TCP plate, and prior to trypsinization the scaffolds were carefully removed to a new TCP. This step was done to avoid an increase in cell numbers arising from cells bound to the TCP plate. To quantify the total double-stranded DNA, the scaffolds were cut into small pieces, digested in 1 mL of a 6 <g/mL of papain solution for 6 h at 60°C, and a PicoGreen™ DNA assay kit (Molecular Probes) was used according to manufacturer's specifications. To observe cells on a three-dimensional matrix, scaffolds were placed in a 24-well TCP submerged in 2 mL of cell suspension with cell density of 1 < 105/mL. After 24 h, scaffolds were moved to another TCP with fresh media. Cells were cultured for a week before SEM and for 2 weeks before

fluorescent stain. For SEM microscopy, cells were cross linked with 2.5% formaldehyde (Sigma) in phosphatebuffered saline (PBS), rinsed with deionized water, and gradually dehydrated with series of ethanol solutions. Hexamethyl disilazane (Fisher, PA) was used to remove 100% ethanol. Samples were sputter coated with Au-Pd before being examined under SEM (Hitachi, s3000N, Japan). For fluorescent staining, cells were fixed in 3.7% formaldehyde in PBS, penetrated with 0.1% Triton X-100 (Sigma, MO) in PBS, and stained with rhodamine–phalloidini (Molecular Probe) in 0.1% bovine serum albumin. A confocal laser scanning microscope (BioRadi MRC1024ES, Hercules, CA) was used to examine the cells.

Statistical analysis

The numerical data from each experiment were the average from at least triplicate samples. The same experiments were repeated three times to ensure the repeatability of the methods used. A Student *t* test was used for statistical analysis and the statistical differences were established as $p < 0.05$.

RESULTS AND DISCUSSION

Although the impact of external stimuli (i.e., dynamic compression or hydrostatic pressure), and the presence of growth factors and cytokines on chondrocyte attachment, proliferation, and biosynthetic activity has been extensively studied, the impact of substrate stiffness on chondrocyte attachment and proliferation has only been studied recently.34 – 36 Chondrocyte-mediated contraction of matrices can be expected to impact the pore

size and porosity, which, in turn, would impact the response to mechanical stimuli as well as chondrocyte biosynthetic and metabolic activity.24 In a previous study,22 crosslinking was shown to directly reduce the chondrocyte- mediated contraction of collagen-based matrices, wherein the stiffest matrix contracted by 30% and the complaint matrices contracted by 60%. Interestingly, matrices with a higher-level contraction led to higher levels of Type II collagen biosynthesis when compared to stiffer matrices, possibly due to high-density cultures in contracted matrices. Thus in this study the effects of crosslinking and matrix density on chondrocyte differentiation could not be delineated. Recently alginate matrices were first derivatized with RGD peptide and then cross- linked with Ca2< and Ba2< ions to increase the stiffness of the base alginate matrix.31 The rate and the extent of attachment were found to be dependent on the stiffness, and stiffer substrates were shown to impact chondrocyte morphology. However, in this study, the relative impact of the biochemical stimuli (RGD peptide) versus the intrinsic mechanical stimuli (i.e., matrix stiffness) could not be separated. Furthermore, the high levels of Ca2< ions used to crosslink alginate may have modulated intracellular calcium levels, which has been reported to affect integrin expression and attachment in several cell types.37,38 Although Ba2< has been reported to interfere with integrin function39 and block cell adhesion,40 the use of Ba2< to crosslink alginate surfaces has resulted in increased cell adhesion.31 In the present study, a change in the intrinsic mechanical environment was made by altering the morphological state of the matrix (planar film and scaffolds prepared via freeze drying and lyophilization), and the stiffness of the matrix was changed by crosslinking of the chitosan substrate. The intention here is to study the impact of intrinsic stimuli provided by morphologies or altered matrix stiffness independent of the stimuli provided by changes in matrix densities, or the presence of a bioactive peptide. It is known that the proposed reaction scheme (shown in Fig. 1) will result in both Structures 1 and 2, and that

the ratio of the same can be controlled by an optimization of the reaction conditions, namely, the temperature and percent diepoxide in the reaction mixture. Briefly, the concentration of the terminal epoxide moiety (Structure 2) was estimated as function of reaction temperature and percent diepoxide, by sodium thiosulfate titration (data to be published). The present reaction optimization experiments have suggested that a high degree of crosslinking (Structure 1) can be obtained by carrying out the reactions at 45 to 60°C, with less than 10% of free terminal epoxide groups (Structure 2). Infrared (FTIR) spectroscopy was used to determine the modification of the chitosan surface qualitatively. IR data were taken on desiccated chitosan films both before and after epoxide modification. The signature of the chitosan molecule can be significantly recognized from the intensity of the amine peak at wavelengths of 3300–3500 cm<1 and the strong NOH stretch at 1560–1650 cm<1, as seen from the IR spectrum shown in Figure 2(a). The IR spectrum of the diepoxide modified chitosan (1-molar excess of diepoxide) is shown in Figure 2(b). After the modification of chitosan with 1, 4 butanediol diglycidyl ether, the intensity of the characteristic amino peak at 3300–3500 cm<1 was found to decrease, and a strong stretch at 1590 cm<1 was observed, signifying the modification of the primary amine group on chitosan with the diepoxide. There is also an increase in the peak at 500 cm<1 which is due to COH2 stretch. The peak 1060 cm<1 is that from the COOOH resulting from the opening of the oxirane moiety by the primary amine group. These observations support the conclusion that chitosan was crosslinked at the terminal amino groups via the diepoxide linker. Similar FTIR spectra were obtained for films at other crosslinking ratios. Furthermore, it was observed that the crosslinked scaffolds/films were stable over the culture period (data not included).

Surface hydrophilicity (contact angle)

The results, shown in Figure 3, showed that crosslinking the chitosan film with 1,4 butanediol diglycidylether decreased the contact angle and increased the hydrophilicity of the surface. Figure 3 shows a significant drop ($p < 0.01$) in contact angle from 0< to 0.5<, but not between 0.5< and 1<. The contact angle decreased again ($p < 0.01$) when the ether/amino ratio was raised to 5<. Although the contact angles of crosslinked films were lower than the noncrosslinked films, the hydrophilicity of the crosslinked films studies were similar.

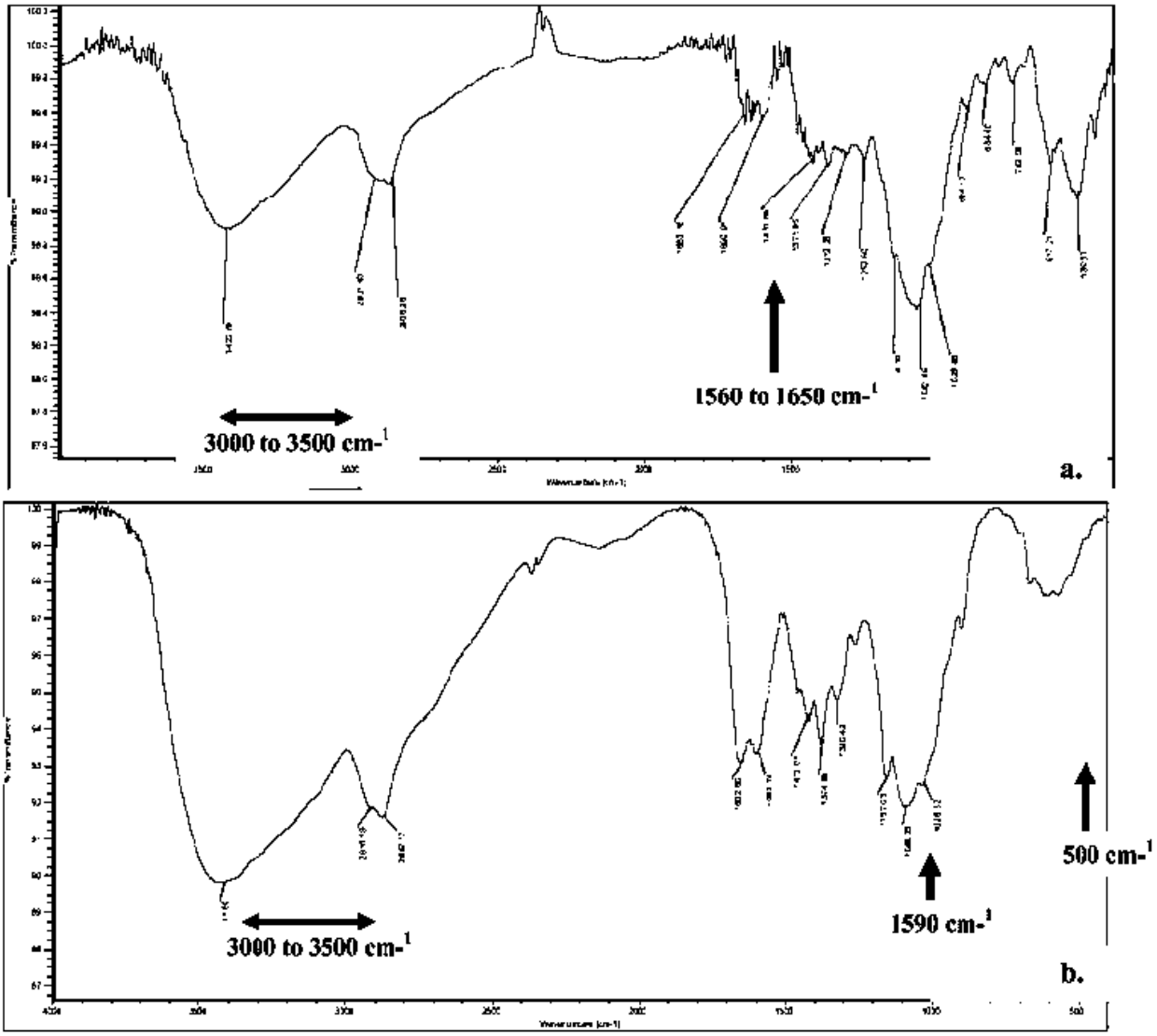

Figure 2. IR spectra of unmodified chitosan and crosslinked chitosan. Percent transmittance (%T) is denoted on the Y axis and wavelengths (in cm^{-1}) are denoted on the X axis. Relevant wavelengths and regions are indicated with arrows. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Mechanical properties (compression test)

It is expected that crosslinked films and scaffolds will possess higher stiffness and elastic modulus. The results show that chemically crosslinked films had a 1.5-times higher modulus than uncrosslinked films. The elastic moduli of the crosslinked scaffolds can be seen in Figure 4. The crosslinking process significantly increased the elastic modulus of the chitosan substrate ($p < 0.01$), indicating an increase in stiffness after crosslinking. The crosslinked scaffolds (0.5< to 5<) have about 2–5 times higher elastic modulus (7.4 –19.9 kPa) compared to uncrosslinked scaffolds (3.8 kPa). Chondrocytes, when seeded on flexible substrates (like collagen), have been shown to exert contractile forces.

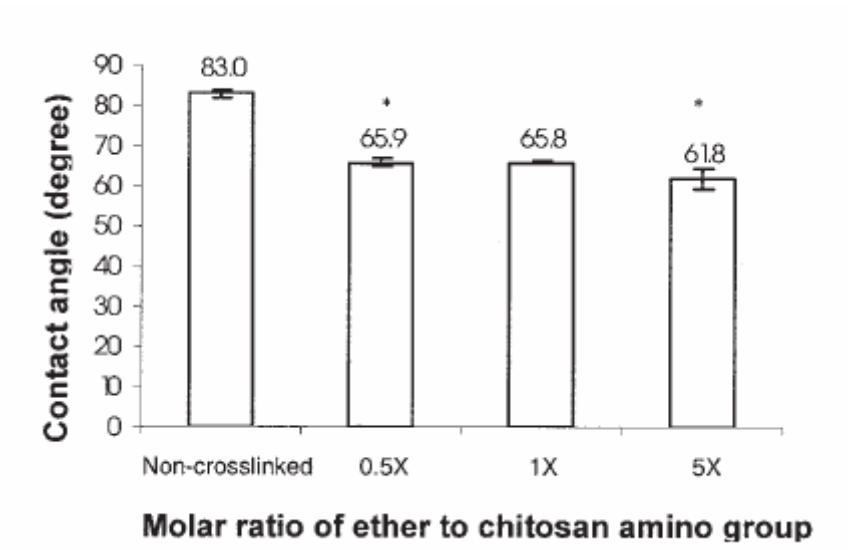

Figure 3. *Contact angle of uncrosslinked and crosslinked chitosan films. Chitosan films were washed and an OCA-20 was used to measure the contact angles. A minimum of three drops were collected for each liquid used at different locations on the sample surface and mean error was reported.*

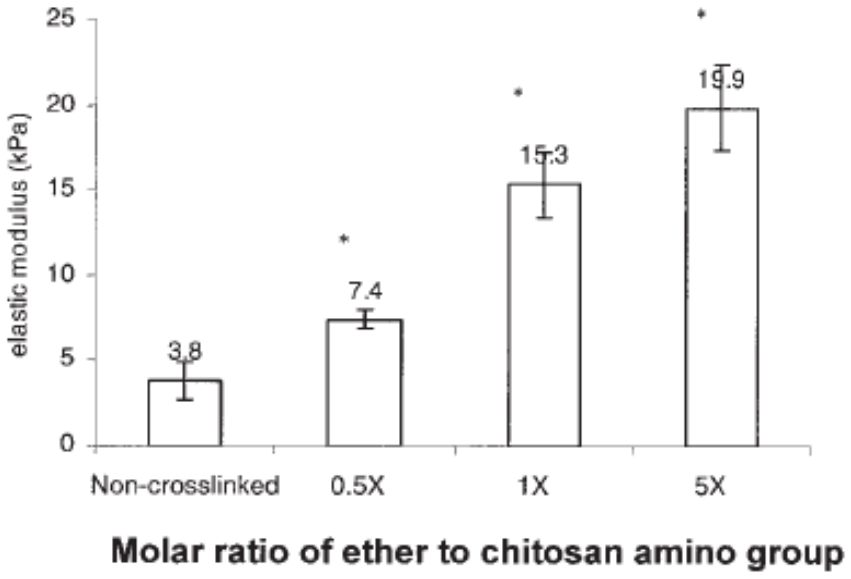

Figure 4.

Elastic modulus of scaffolds prepared by the FDL method in hydrated state. The test was done by an Instron using a 50-kgf load cell with crosshead moving at 2 mm/ min.

Seeded scaffolds have undergone a 10–30% reduction in diameter.16,17 The diameter of unseeded substrates (no cells) based on chitosan changed less than 2% during the three-week culture period. The present results (data not included) show that the spreading and adhesion of chondrocytes on films and scaffolds prepared by the FDL method did not deform the gels or shrink the surfaces. This may be explained by the difference in the Young's modulus of these materials. The substrates used in these studies had higher elastic modulus when compared to the flexible collagen substrates with reported Young's modulus of 100–150 Pa. This result is supported by the observation that the morphology of the scaffold, as judged by SEM, and the porosity of the scaffolds did not undergo a significant change upon crosslinking (data not included).

Degradation with chitosanase

The degradation results of uncrosslinked and crosslinked chitosan scaffolds are shown in Table I. The noncrosslinked scaffolds were completely digested in 24 h. The initial (<4 h) degradation of the more crosslinked samples was slower than the less- or non-crosslinked ones. However, by the end of 24 h, the amount of chitosan digested was not different between different degrees of crosslinked chitosan scaffolds. All chitosan samples were degraded over the 24-h period by chitosanase. More crossed chitosan had a slower initial (<4 h) degradation rate than the less crosslinked ones. Chitosanase-assisted hydrolysis requires that beta-1,4-linkages between *N*-acetyl-dglucosamine and d-glucosamine residues in chitosan be presented.41 Chemical modification of chitosan at the amino group may render the glucosamine residues inaccessible to chitosanase, or the crosslinked segments may form a conformation that hinders chitosanase from recognizing other active moieties. As no significant differences in the rate or extent of degradation were observed between uncrosslinked and crosslinked chitosan films, it is concluded that the method of crosslinking did not alter the enzymatic susceptibility. It is estimated that at the highest ratio of the functional diepoxide to the ONH2 groups on the chitosan molecule, only about 25–35% of the available amino groups were modified, and that at this level of modification the enzymatic susceptibility was not altered.

Cytotoxicity of crosslinked chitosan surfaces

The *in vitro* biocompatibility of crosslinked films and scaffolds prepared in the laboratory were evaluated to ascertain the impact, if any, of chemical crosslinkers used. The ability

of crosslinked substrates to support growth and proliferation of chondrocytes is presented in Table II. The absorbance from the cell viability test (MTT assay) on various degrees of crosslinked films was calculated into the percentage of the control (cells on tissue-culture polystyrene, TCP). The viability of cells on the crosslinked chitosan films was not significantly different from the cells on the TCP. There was no significant difference in viability between different degrees of crosslinked films. The results show that the modified surface was not cytotoxic to the chon drocyte cells, because the cell viability remained the same as the control after 3 days in culture. Cell viability in the range of 90–97% was routinely attained, indicating that modification is not cytotoxic and the crosslinked chitosan scaffold maintained biocompatibility.

TABLE I
Degradation of Chitosan Scaffolds in Chitosanase Acetate Solution (0.1 U/mL)

Sampling Time	Samples (% Degradation)			
	Control	0.5×	1×	5×
1 hour	42.3 ± 2.0	45.6 ± 8.7	44.0 ± 7.1	27.9 ± 5.6
2 hours	72.7 ± 4.7	66.4 ± 7.7	54.6 ± 0.6	32.4 ± 1.7
4 hours	95.4 ± 3.7	81.5 ± 0.9	70.1 ± 0.8	59.8 ± 0.2
24 hours	100.0 ± 0.0	89.3 ± 5.8	95.3 ± 3.2	94.1 ± 2.1

Unit is in percentage of weight loss based on initial dry weight. Numbers are presented in average ± standard deviation (Ave ± SD).

TABLE II
Cell Viability on Crosslinked Chitosan Films

Surface	TCP	0×	0.5×	1×	5×
Viability (% of control)	100.0 ± 12.0	95.4 ± 2.7	94.2 ± 6.7	90.9 ± 12.4	96.8 ± 6.2

Unit is in % TCP control [$=(A_{595\ \text{of samples}}/A_{595\ \text{of TCP}}) \times 100\%$]. 0.5×, 1×, and 5× are the molar ratio of diepoxide to amino groups of the film. Numbers are presented in average ± standard deviation (Ave ± SD).

Cell morphology and proliferation

Mechanical forces can arise from two sources—the environment and contractile forces generated by the cells, wherein cells provide the initial input and the substrate reacts to it by deforming passively. It is hypothesized that chondrocytes can sense their intrinsic mechanical environment and that substrate or scaffold rigidity/stiffness can also serve as an intrinsic mechanical stimuli. It is expected that chondrocyte will possess different morphologies when seeded on flexible substrates as opposed to stiffened substrates. Crosslinked films may be able to direct the chondrocytes to possess morphology that resembles Type IV classification42 when compared to uncrosslinked surfaces. The SEM images of chondrocytes grown on free swelling films, after 1 week of culture, are shown in Figure 5. When seeded on noncrosslinked and crosslinked chitosan films and observed 1 week after seeding, the cells possessed a flattened and spread morphology. This result agrees with the previous finding, where seeded chondrocytes possessed Type IV morphology on planar surfaces. 26,38 At the end of a week of culture, cell proliferation appeared to be higher on crosslinked films (9.2 < 104 to 12.6 < 104 cells per film) when compared to noncrosslinked films (6.5 < 1.1 < 104 cells per film). The SEM images of chondrocytes growing on FDL scaffolds are shown in Figure 6.

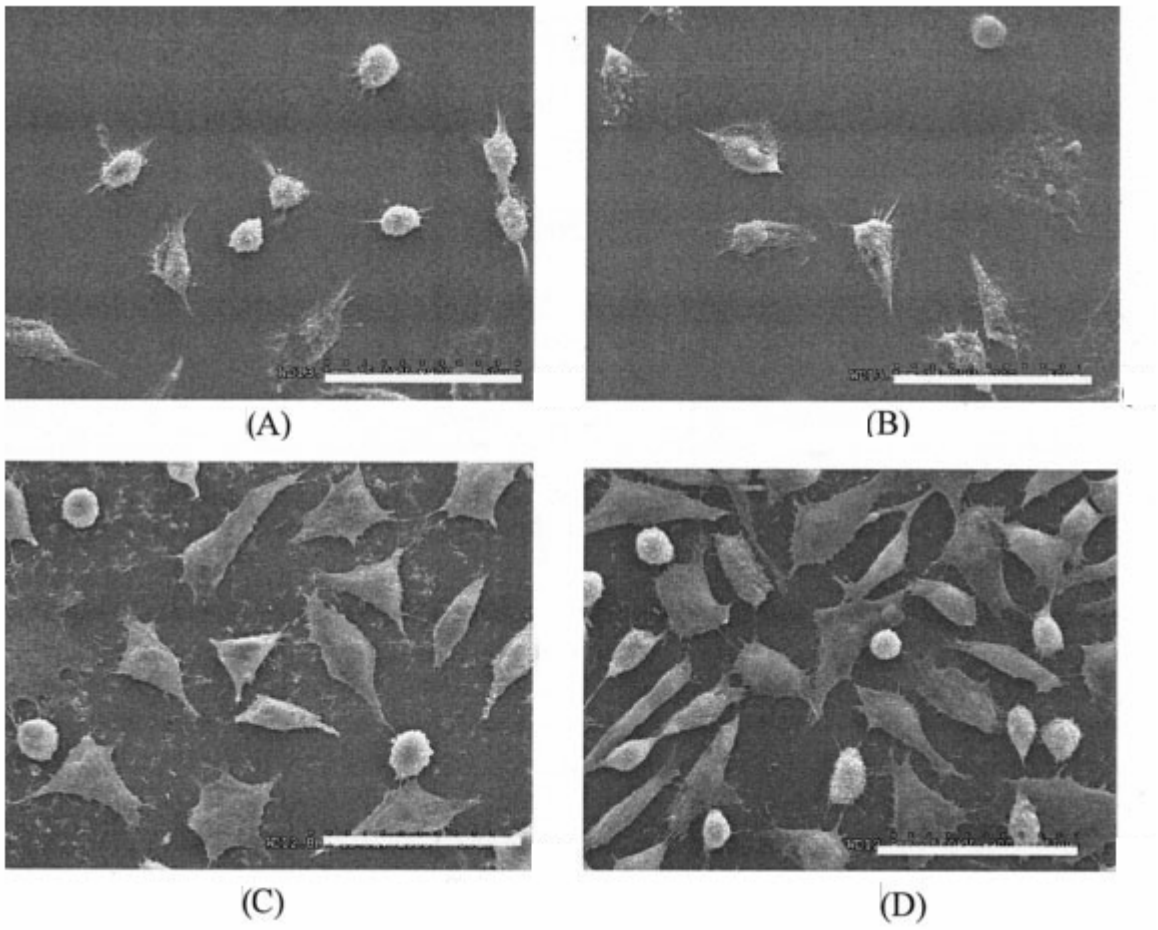

Figure 5.

SEM micrograph of chondrocytes seeded on uncrosslinked and crosslinked films. Representative SEM pictures of canine chondrocyte cells (passage 2) on (A) noncrosslinked (0<), (B) 0.5<, (C) 1<, and (D) 5< crosslinked chitosan films. SEM micrographs were taken after 1 week of culture. The initial seeding density was 1 < 104 cells/cm2. The scale bar is 50 <m.

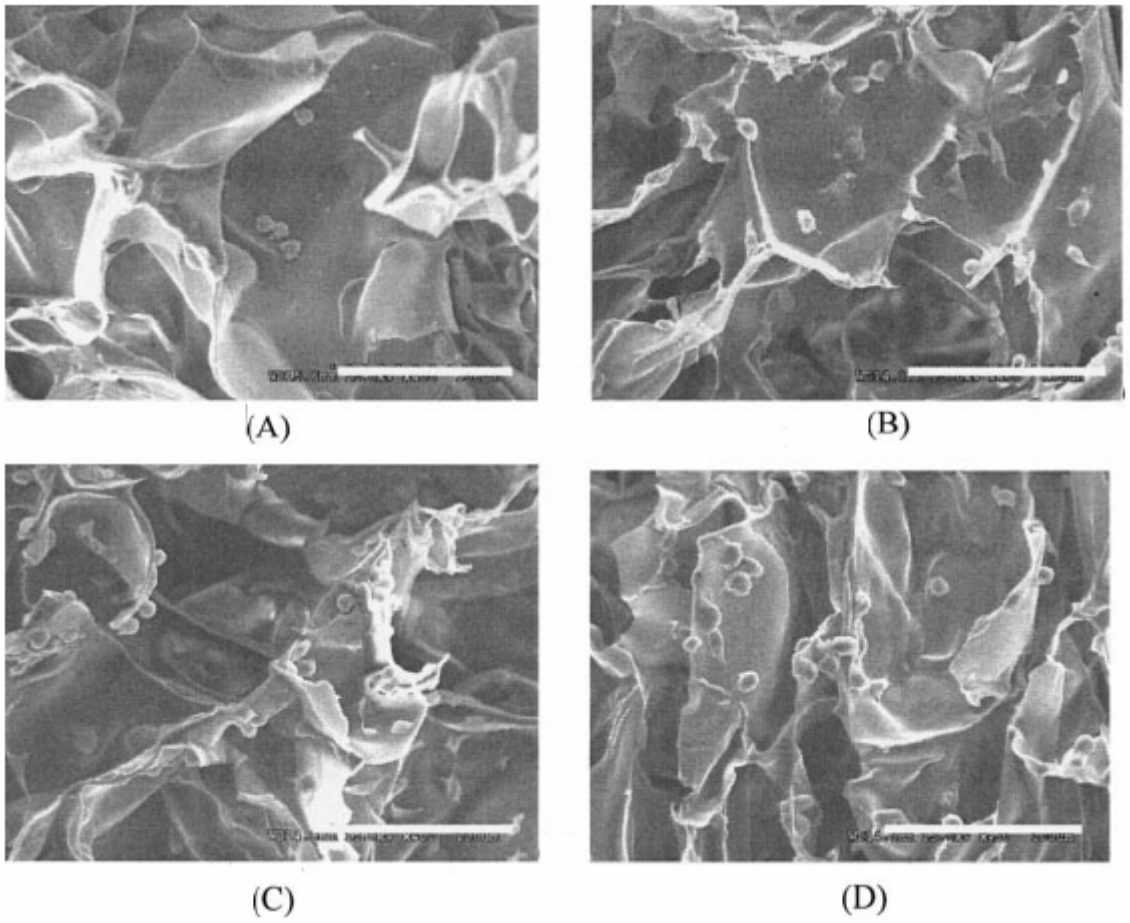

Figure 6. *SEM micrograph of chondrocytes seeded on noncrosslinked and crosslinked scaffolds. Representative SEM pictures of canine chondrocyte cells (passage 3) on (A) noncrosslinked, (B) 0.5<, (C) 1<, (D) 5< crosslinked chitosan scaffolds are shown. SEM micrographs were taken after 1 week of culture. The scale bar is 100 <m. Cells appeared to have rounded chondrocytic morphology.*

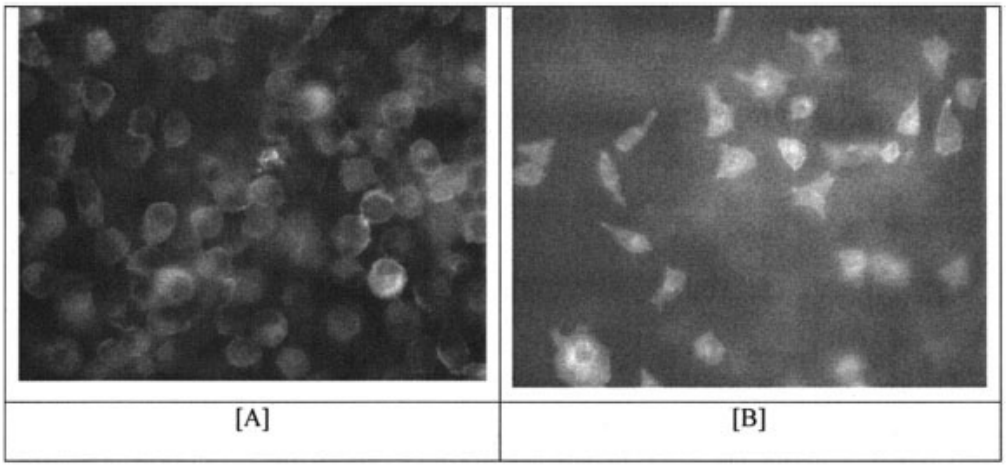

Figure 7. *Morphologies of chondrocytes seeded on uncrosslinked and crosslinked chitosan films. Representative fluorescent pictures of canine chondrocyte cells (passage 3) of (A) on a noncrosslinked film and (B) on a 0.5< film. Cell and cytoskeletal morphology were assessed with the use of rhodamine-conjugated phalloidin to visualize filamentous actin. The initial seeding density was 1 < 104 cells/cm2, and cultures were maintained for 2 weeks. Actin filaments were detected by staining with TRITC conjugated phalloidin, and cells were observed by epifluorescence microscopy. The scale bar is 50 <m. Cells on uncrosslinked surfaces were nebulous, and cells on crosslinked surfaces appeared to have Type IV, flattened morphology.*

Chondrocyte cells seeded on chitosan scaffolds proliferated, possessing a rounded chondrocytic morphology that is indicative of cells retaining their phenotypes. From the SEM pictures, it is concluded that at the end of a week of culture, crosslinked chitosan scaffolds [Fig. 6(B–D)] had a higher cell proliferation when compared to the non-crosslinked surfaces [Fig. 6(A)] as determined by the total amount of doublestranded

DNA (dsDNA) detected in the digests. Uncrosslinked, 0.5<, 1<, and 5< crosslinked scaffolds gave 57.0 < 7.7, 68.7 < 0.8, 103.2 < 4.6, and 110.8 < 6.6 ng of dsDNA/mL, respectively. Measurement of the cell count (data not included) showed a similar trend. The present observations suggest that chondrocytes growing on a 3D hydrogel scaffold have proper cell– cell contact with neighboring chondrocytes, whereas the chondrocytes growing on two dimensional films express flat and spread morphology. These results agree with previous studies, where chondrocytes appear to be rounded in three dimensional scaffolds while maintaining their phenotypes. 44–46 Previous studies have shown that it is necessary for chondrocytes to assume a spherical morphology in order to express the chondrocytic phenotype (Type II collagen and GAGs).45,46 This spherical morphology is also an indication of chondrocyte differentiation.46 In an extended experiment, chondrocytes, when maintained on non crosslinked scaffold for 21 days, gave 0.15 < 0.05 <g of GAG/mL and chondrocytes maintained on 0.5<, 1<, and 5< crosslinked scaffolds for a month gave 0.52 < 0.13, 0.52 < 0.07, and 0.69 < 0.04 <g of GAG/mL, respectively.

Chondrocytes were seeded onto crosslinked and uncrosslinked films and scaffolds, and the cytoskeletal morphologies were observed upon rhodamine– phalloidin staining of actin filaments of seeded chondrocytes. Representative morphologies obtained on noncrosslinked and crosslinked chitosan films membranes are shown in Figure 7. About 90% of the cells on uncrosslinked films had a spherical morphology and nebulous, punctate actin. Cells on the crosslinked films were flattened with stress fibers and 40–50% of cells surveyed had a Type-IV flattened morphology.42 Thus it appears that stiffer substrates directed the cells to possess a flattened morphology. Representative morphologies obtained on noncrosslinked and crosslinked chitosan scaffolds are shown in Figure 8. As observed by the fluorescent staining; a higher rate of proliferation was

seen on crosslinked scaffolds [Fig. 8 (B,C)] when compared to noncrosslinked scaffolds [Fig. 8(A)].

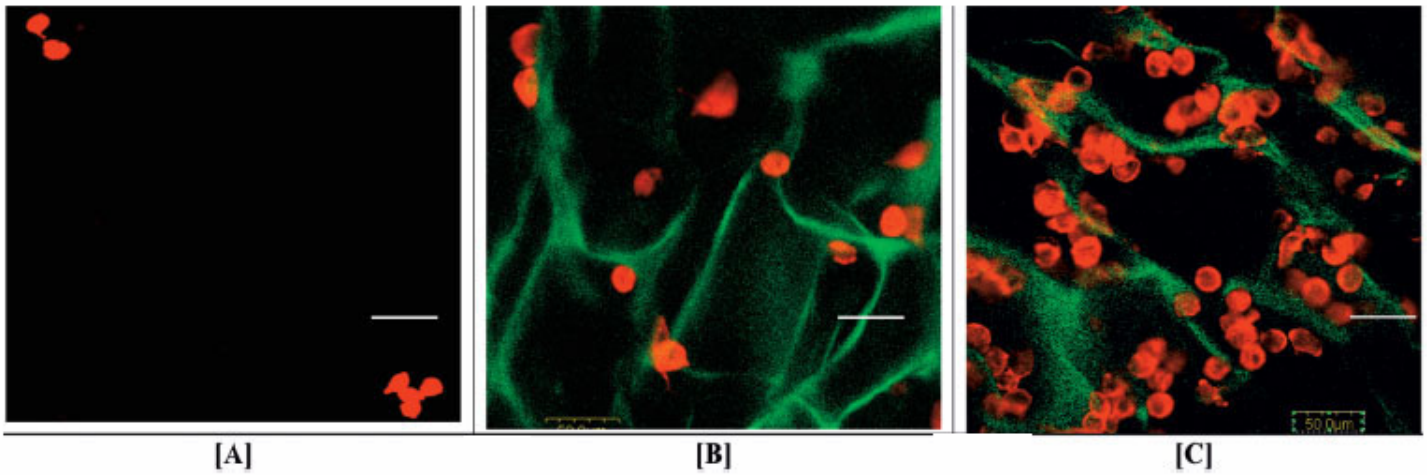

Figure 8. *A rhodamine–phalloidin stain of chondrocytes seeded on uncrosslinked and crosslinked scaffolds. Representative fluorescent pictures of canine chondrocyte cells (passage 3) (A) on a noncrosslinked scaffold; (B) on a 0.5< crosslinked scaffold, and (C) on a 5< crosslinked chitosan scaffolds. The initial seeding density was 3 < 105 cells/mL (2 mL of cell suspension added per well) and cultures were maintained for 2 weeks. Actin filaments were detected by staining with TRITC conjugated phalloidin, and cells were observed by epifluorescence microscopy. The scale bar is 50 <m.*

CONCLUSION

The images of cells from SEM and the fluorescent stain indicate that stiffer surfaces promote cell proliferation. The elastic moduli of crosslinked scaffolds were 100– 400% more than that of the noncrosslinked scaffold. In the absence of any external stimuli, it is postulated that the rigidity of the films might be the factor enhancing chondrocyte cell growth

and biosynthetic activity. In conclusion, it has been shown that the chemical crosslinking strategy used imparts additional matrix stiffness without impacting the biocompatibility or the cell adhesive properties of the films or scaffolds. The augmented mechanical strength may provide additional resistance to cell-mediated scaffold shrinkage and pore size change. Finally, the increase in substrate stiffness appears to serve as an intrinsic stimulus that promotes cell proliferation and preservation of morphology. The slower enzyme degradation rate of crosslinked scaffolds can be viewed as an advantage for a degradable material, because the degradation rate can thus be further modified to suit various tissue recovery time *in vivo*. This study provides an alternative way to control the physical properties of implant materials and to improve chondrocyte proliferation. Continuing studies aim to assess the phenotype and biosynthetic activity of chondrocytes on the surfaces evaluated in the present study.

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