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Three-Dimensional Porous Collagen/Chitosan Complex Sponge for Tissue Engineering

Sung Eun Kim[1,2], Yong Woo Cho[1], Eun Jung Kang[1], Ick Chan Kwon[1], Eunhee Bae Lee[1], Jung Hyun Kim[1,2], Hesson Chung[1], and Seo Young Jeong[1,*]

[1]*Biomedical Research Center, Korea Institute of Science and Technology, Seoul 136-791, Korea*
[2]*Department of Chemical Engineering, Yonsei University, Seoul 120-749, Korea*
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Abstract: A three-dimensional, porous collagen/chitosan complex sponge was prepared to closely simulate basic extracellular matrix (ECM) constitutes, collagen and glycosaminoglycan. The complex sponge was prepared by a lyophilization method and had the regular network with highly porous structure, suitable for cell adhesion and growth. The pores were well interconnected, and their distribution was fairly homogeneous. The complex sponge was crosslinked using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to increase its biological stability and enhance its mechanical properties. The crosslinking medium had a great effect on the inner structure of the sponge. The homogeneous, porous structure of the sponge was remarkably collapsed in an aqueous crosslinking medium. However, the morphology of the sponge remained almost intact in a water/ethanol mixture crosslinking milieu. Mechanical properties of the collagen/chitosan sponge were significantly enhanced by EDC-mediated crosslinking. The potential of the sponge as a scaffold for tissue engineering was investigated using a Chinese hamster ovary cell (CHO-K1) line.

Keywords: Collagen, Chitosan, Scaffold, Sponge, Pore, Mechanical properties, CHO cell, Tissue engineering

Introduction

Tissue engineering is a promising therapeutic strategy designed to regenerate natural tissues or to create biological substitutes for defective or lost tissues and organs. A polymeric scaffold plays a critical role on tissue engineering. It guides the development and organization of new tissues or organs with appropriate structure and function. If a cell suspension is directly injected into injured tissues without a cell-adhesion substrate, it is impossible to control localization of transplanted cells. A wide variety of biomaterials encompassing natural polymers (e.g., collagen[1,2], alginate[3,4], hyaluronic acid[5,6], and chitosan[7-10]) and synthetic polymers (e.g., polylactide[11-14]) were extensively studied in tissue engineering.

Collagen is the major and ubiquitous structural protein in mammalian, accounting for about 30% of all body proteins [15]. It has a great potential as a scaffolding material for tissue engineering due to its low antigenicity, its biodegradability and cell-binding properties[1,2,15,16]. It has been reported that collagen may assist in the expression of the phenotype and the retention of activity of various type of cells, including chondrocyte[1] and hepatocyte[2].

Chitin is the second most abundant natural polysaccharide, found in exoskeleton of insects and marine invertebrates. Chitosan, the principal derivative of chitin, is obtained commercially by the chemical N-deacetylation of chitin. It has been well known to be non-toxic and bioresorbable[17]. In addition, its biodegradability can be modulated according to its degree of acetylation[17-19]. Chitosan is a linear copolymer of N-acetyl D-glucosamine and glucosamine. Human connective tissues do not actually contain chitosan. However, it has a structural similarity to glycosaminoglycan (GAG), mostly components of extracellular matrix (ECM). GAG attached to the core protein of proteoglycan consists of repeating disaccharide unit, usually include an uronic acid component (e.g., D-gluconic acid and L-gluconic acid) and a hexoamine component (e.g., N-acetyl-D-glucosamine and N-acetyl-D-galactosamine). GAG is involved in cell-cell and cell-matrix interaction, and probably acts as a modulator of cell morphology, differentiation, movement, synthesis, and function[20].

In this study, collagen and chitosan were used as scaffolding materials to mimic the basic, crucial constituents of ECM. Three dimensional, porous collagen/chitosan complex sponges were prepared by freeze-drying of collagen/chitosan blended solutions in dilute acetic acid. To prolong the durability and enhance the mechanical properties, the complex sponge was crosslinked using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). The morphology of the scaffold was observed by scanning electron microscopy (SEM), and the effect of crosslinking on the mechanical properties of the sponge was examined. The potential of the collagen/chitosan complex scaffold for tissue engineering was evaluated through Chinese hamster ovary cell (CHO-K1) culture.

Experimental

Materials

Collagen type I extracted form calf skins was purchased from Sigma Chemical Co. Chitosan (degree of deacetylation

*Corresponding author: syjeong@kist.re.kr

95%, molecular weight 100,000) was kindly supplied from Synyoung Chitosan Co., Korea. EDC and NHS were purchased from Sigma Chemical Co. All other reagents used were of a reagent grade.

Preparation of Collagen/Chitosan Complex Sponge

Collagen and chitosan were separately dissolved in 2% aqueous acetic acid solution at a concentration of 3 g/dl. Two solutions were sufficiently mixed under gentle stirring for 6 h at room temperature. The blended solution was poured into polystyrene molds, frozen at −70°C, and freeze-dried. The obtained collagen/chitosan sponge was neutralized with 0.5 N NaOH/ethanol mixture, washed with water/ethanol mixture, and freeze-dried.

Chemical crosslinking was performed using EDC and NHS. The complex sponge was immersed in water/ethanol mixture containing EDC (10% with respect to the sponge weight) and NHS (molar equivalent to EDC). After incubation under gentle shaking for 6 h at room temperature, the sponge was thoroughly washed with water/ethanol mixture. Finally, the matrices were lyophilized, resulting in crosslinked collagen/chitosan sponges.

Morphology of Collagen/Chitosan Complex Sponge

The porous structure of the sponge was assessed by SEM (S2460N, Hitachi, Japan). The scaffold sections were attached to aluminum stubs and coated with an ultra-thin layer of gold using an ion sputter (E-1010, Hitachi, Japan).

Mechanical Properties of Collagen/Chitosan Complex Sponge

The mechanical properties of the sponge were evaluated using a tensile machine (Instron 8511, Instron Corp., USA). The complex sponge was sectioned to yield a cylindrical sample with approximately 12 mm of diameter, 8 mm of length. It was uniaxially compressed in a dry state at a crosshead speed of 1 mm/min, and a strain-stress curve was recorded out. Samples were run in triplicate. The compressive modulus, compressive strength at yield, and maximum compressive strength were calculated from the strain-stress curve.

Cell Culture

CHO-K1 cells (KCLB 10061) were cultured in Ham F-12 medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. The culture medium was changed every 3 days. At confluence, the cells were retrieved from the flasks by trypsinization with 0.05% trypsin and 0.02% EDTA solution. The cells were seeded at 1×10^6/ml on the sponge with 12 mm diameter and 3 mm thickness, and

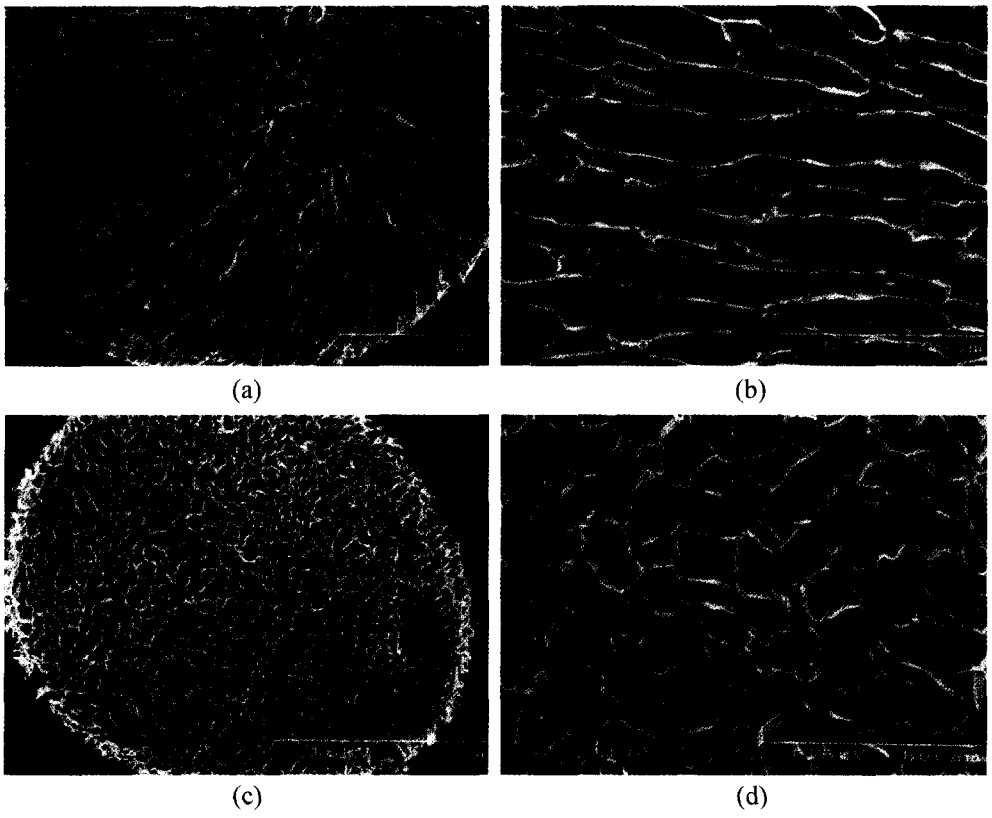


Figure 1. Scanning electron micrographs of collagen (a, b) and chitosan (c, d) sponges.

cultured for 4 days at 37°C and 5% CO_2 atmosphere.

Cell Morphology

After washing with phosphate-buffered saline (PBS) solution, cells on the scaffolds were fixed in PBS containing 2.5% glutaraldehyde, dehydrated in ethanol with sequential concentrations of 50, 70, 80, 90, 95, 99% for 10 min each, and then freeze-dried. The cell morphology was observed through SEM.

Results and Discussion

3-D Porous Collagen and Chitosan Scaffolds

Tissue engineering has rapidly developed in biotechnology fields in recent years. Scaffolds for tissue engineering should provide an environment to shape and guide the tissue development *in vitro* and *in vivo*. Therefore, the 3-D porous structure of a scaffold is prerequisite in many cell culture systems. Some cells, like chondrocyte, loose their phenotypic expression in monolayer culture[5,6]. For regeneration of natural tissues or organs, the quality of a three-dimensional scaffold has a great effect on cell adhesion and growth, and eventually determines the success of tissue healing. An optimal scaffolding material should elicit minimal inflammatory and antigenic response, have an adequate biodegradation rate, and promote cell binding, proliferation, expression of cell-specific phenotype, and activity of cells. The physico-chemical characteristics of scaffolds have a crucial role on tissue engineering such as the texture of the scaffold (e.g., non-woven and sponge), the presence of pore, the pore size and distribution, the surface hydrophobicity, and the presence of ionic charges[2-6,21,22].

A three-dimensional collagen scaffold was prepared by lyophilization and its porous structure was visually confirmed as shown in Figure 1(a) and (b). The scaffold had the regular, interconnected network structure with large porosity. The pores were oriented, rectangular-shaped and its distribution was fairly homogeneous. The size of the pores was from 300 to 500 μm in a horizontal direction and from 100 to 200 μm to a vertical direction.

Chitosan, GAG analog, is a potential scaffolding material with good biocompatibility and cell-adhesion capability. A positive charge of a substrate has been known to enhance cell adhesion[22,23]. It was reported that chitosan with cationic nature promoted cell adhesion and cellular differentiation, and supported the expression of extracellular matrix proteins in various cells, including fibroblast[24,25], chondrocyte[10], osteoblast[8,10], and hepatocyte[7].

Porous chitosan sponges were prepared by a freeze-drying technique, as well. Inner structure of the chitosan scaffold was shown in Figure 1(c) and (d). The chitosan scaffold showed a highly homogeneous, porous morphology. The round-shaped pores with a diameter from 100 to 200 μm were well interconnected. Both collagen and chitosan sponges seemed to have very suitable morphology as scaffolds for tissue engineering.

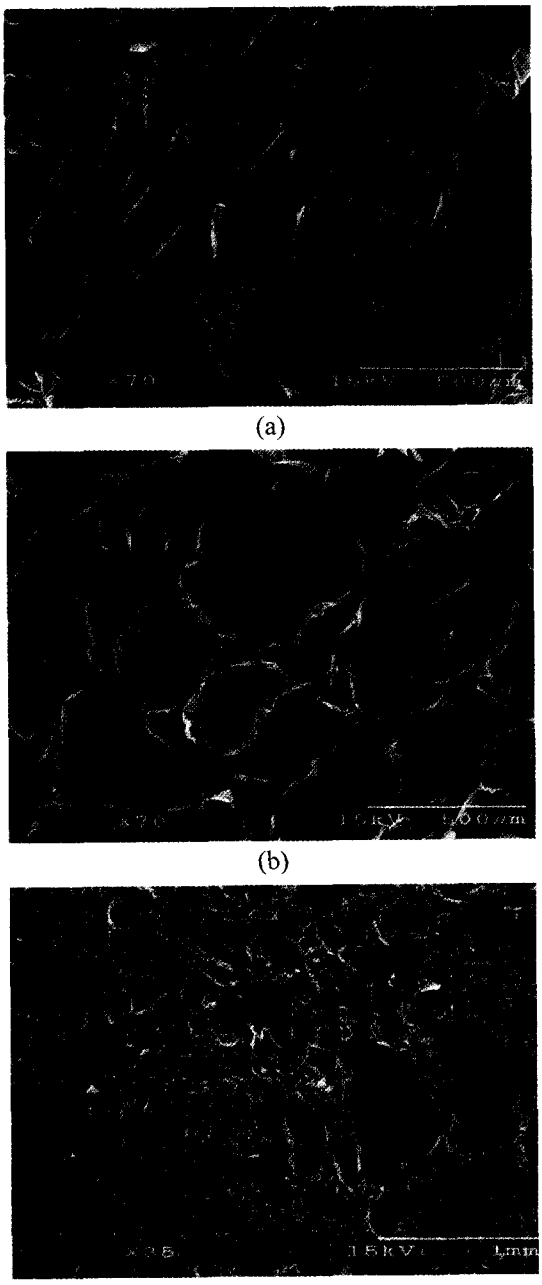


Figure 2. Scanning electron micrographs of collagen/chitosan complex sponges: (a) non-crosslinked collagen/chitosan complex sponge, (b) crosslinked collagen/chitosan complex sponge using EDC in water/ethanol (1/1) mixture, (c) crosslinked collagen/chitosan complex sponge using EDC in water.

Collagen/Chitosan Complex Scaffolds and Their Crosslinking by EDC

Collagen/chitosan complex scaffolds were prepared by

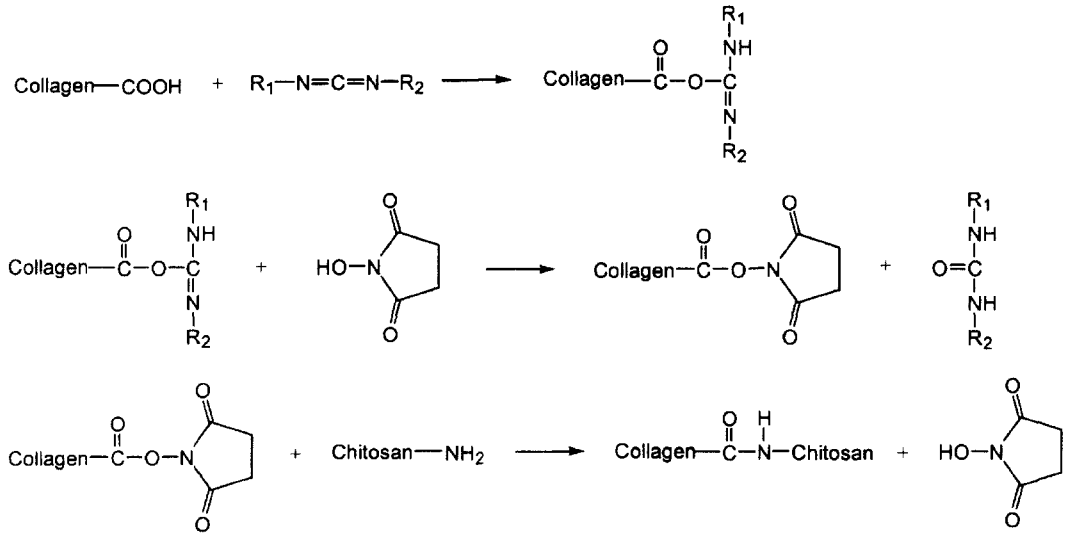
lyophilization of blended solutions in dilute acetic acid. They are designed not only to mimic the basic, crucial constituents of ECM but also to enhance biological durability of collagen matrices. The scaffold for tissue engineering has to provide a temporary physical support for tissue regeneration. It should maintain a potential space to guide the development and organization of new tissues. The mechanical strength of the scaffolds should be sustained until the regenerated tissue has sufficient mechanical integrity to support itself. Although collagen has numerous advantages as a scaffolding material, non-crosslinked collagen matrices are too rapidly degraded *in vivo* situations, and have poor mechanical properties, especially being hydrated.

Chitosan, a polycationic natural polymer, was incorporated into collagen matrices to enhance the mechanical strength of the scaffold. Chitosan fibers, films, and sponges have better mechanical properties than those of collagen. In addition, it is important to note that the biodegadation rate of chitosan can be regulated by degree of acetylation. Chitosan forms a stable complex with collagen[26,27]. It possesses a large quantity of amine groups to be able to ionically interact with carboxylic groups of glutamic and aspartic acid residues in collagen. Figure 2(a) displays the inner structure of collagen/chitosan complex scaffolds. The complex sponge possessed appropriate morphology with high porosity and high interconnectivity of pores. The homogeneity of pore size and distribution of the complex sponge was slightly lower than that of single collagen or chitosan sponge.

The collagen/chitosan complex sponge was crosslinked using EDC and NHS. Different methods for stabilization of collagen matrices have been studied: physical treatments including heat, ultraviolet, and gamma irradiation[28] and the chemical crosslinking using reagents such as glutaraldehyde [29], hexamethylene diisocyante[30], 1,4-butanediol diglycidyl ether[31], and EDC[29,32,33]. It was reported that EDC-mediated crosslinking resulted in relatively low toxic biomaterials[32,33]. Scheme 1 shows the mechanism of EDC-mediated crosslinking in collagen/chitosan complex sponges. A carboxyl groups in collagen is activated to form an ester intermediate, which reacts a primary amino group of chitosan via reaction of NHS to form an amide bond. The addition of NHS in EDC-mediated crosslinking reaction increases the rate and degree of crosslinking. EDC is not incorporated into crosslinked structure but transformed to a low cytotoxic, water-soluble urea derivative. Therefore, EDC are called as "zero length crosslinker".

Figure 2(b) and (c) compares the inner morphology of the collagen/chitosan complex sponge crosslinked by EDC and NHS in two different reaction media, water and water/ethanol (1/1) mixture. The homogeneous, porous structure of the sponge was remarkably collapsed in an aqueous crosslinking medium. However, the morphology of the sponge remained almost intact in a water/ethanol mixture. It is considered that the presence of ethanol in reaction media keeps collagen from being swelled or dissolved by water and prevents the breakdown of porous structure.

As mentioned previously, 3-dimensional scaffolds have to possess appropriate mechanical properties for physical support during tissue development despite their high porous structure. The effect of ionic complex and EDC-mediated crosslinking on the mechanical properties of the scaffold was evaluated by measuring its compressive modulus, compressive strength at yield and maximum compressive strength. A typical strain-stress behavior of the collagen/chitosan complex sponge and definitions of three mechanical properties were represented in Figure 3. The strengths and the moduli of four kinds of the sponges were shown in Figures 4-6. The compressive modulus was calculated from



Scheme 1. Mechanism of crosslinking between collagen and chitosan using EDC and NHS ($R_1 = -CH_2-CH_3$, $R_2 = -(CH_2)_3-NH^+-(CH_3)_2Cl^-$).

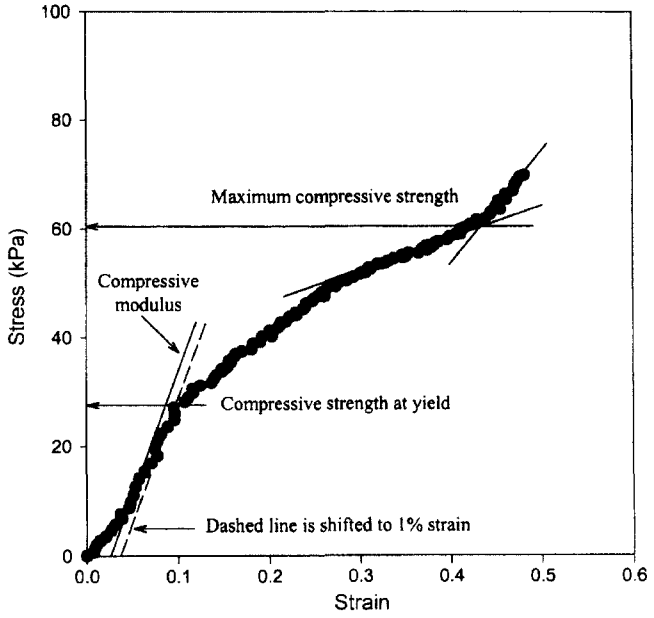


Figure 3. A typical strain-stress curve of the crosslinked collagen/chitosan sponge and definitions of three mechanical properties.

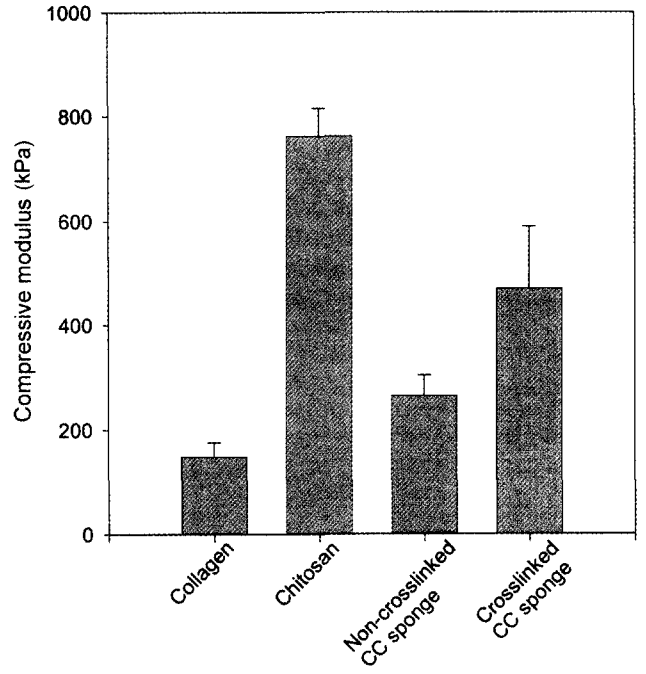


Figure 4. Compressive moduli of four kinds of sponges (CC sponge, collagen/chitosan sponge).

a slope of an initial strain-stress curve[34]. The compressive strength at yield was determined by drawing a line parallel to slope of the initial modulus, beginning at 1% strain[34]. The compressive strength at yield was calculated from the stress at the intersection of this line with strain-stress curve. The

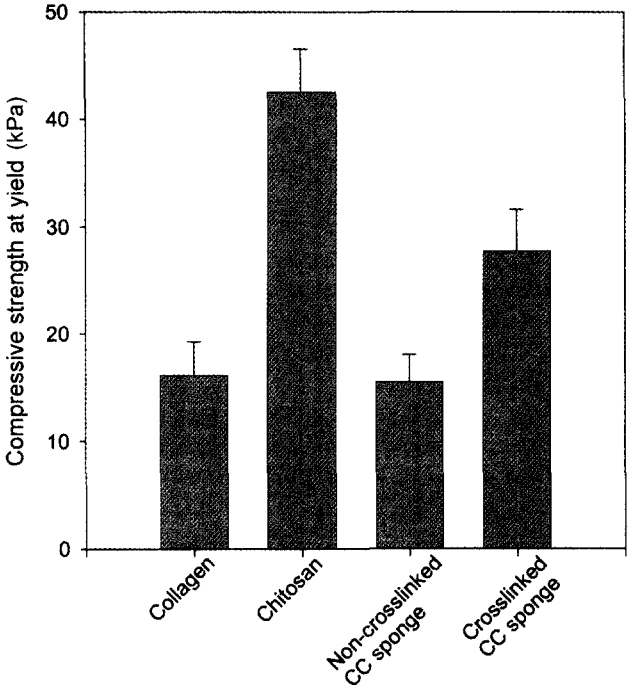


Figure 5. Compressive strengths at yield of four kinds of sponges (CC sponge, collagen/chitosan sponge).

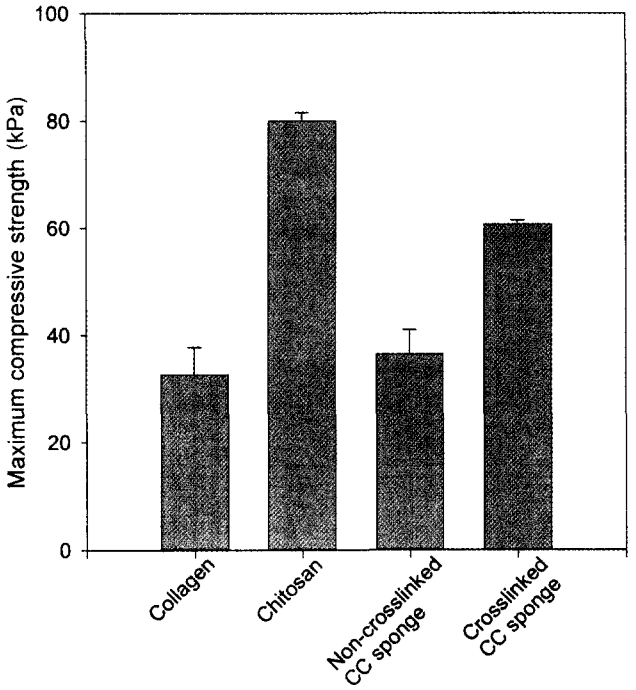
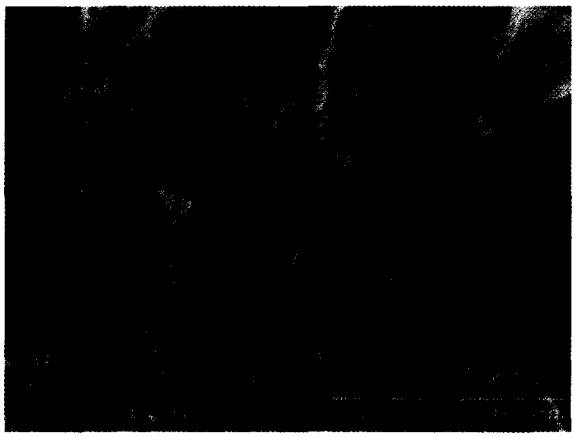
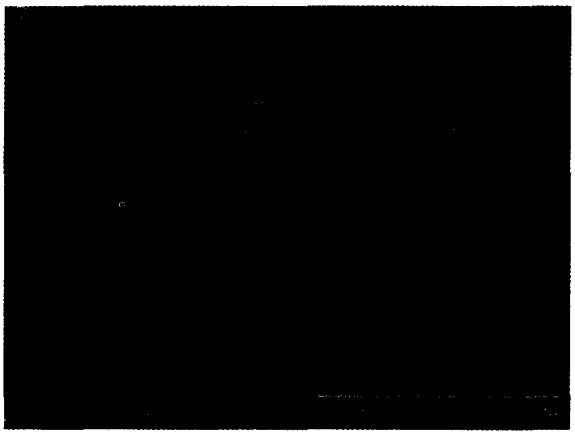


Figure 6. Maximum compressive strengths of four kinds of sponges (CC sponge, collagen/chitosan sponge).

maximum compressive strength was defined as the stress just before the steep ascent of the stress in the latter half of



(a)



(b)

Figure 7. Scanning electron micrographs of CHO-seeded sponges after 4 days in culture.

the mechanical tests. The chitosan sponge had the highest values in all three mechanical properties. Mechanical properties of the collagen sponge were scarcely increased by ionic complex with chitosan. The compressive strength at yield and the maximum strength of the collagen/chitosan complex sponge were almost equal to those of the collagen sponge, while the modulus of the complex sponge was higher than that of the collagen one. However, EDC-mediated crosslinking had a significant effect on compressive strengths and modulus. All three mechanical properties of the complex scaffold were considerably increased by EDC-mediated crosslinking. This suggested that EDC-mediated crosslinking should be very efficient for enhancing mechanical properties of complex scaffolds, maintaining their porous inner structure.

Cell Culture

Chinese hamster ovary cells were seeded on the crosslinked collagen/chitosan complex sponge and cultured in Ham F-12 medium containing 10% FBS under 5% CO_2 atmosphere at 37°C. The cell morphology in the sponge was visualized by SEM at 4 days post-seeding, as shown in Figure 7. The cells penetrated deeply the sponge and adhered to pore surface in the sponge. The round or flattened cells were surrounded by newly synthesized ECM and their metabolism seemed to be very prosperous.

Conclusions

The present study describes the preparation and characterization of collagen/chitosan complex sponges to be used as a scaffold for cell growth and tranplantation. The collagen/chitosan complex sponges had an appropriate morphology for a scaffold with high porosity and homogeneous pore distribution. Mechanical properties of the sponge were considerably enhanced by EDC-mediated crosslinking in the presence of ethanol. The porous, regular structure of the complex sponge remained intact in spite of chemical crosslingking. *In vitro* studies using a CHO cell line implied that the collagen/chitosan complex sponge might have a potential as a scaffold for cell adhesion and growth in tissue engineering.

Acknowledgement

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