Polysaccharide-Based Artificial Extracellular Matrix: Preparation and Characterization of Three-Dimensional, Macroporous Chitosan, and Heparin Composite Scaffold

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ABSTRACT: Scaffold-guided tissue engineering based on synthetic and natural occurring polymers has gained many interests in recent year. In this study, the development of a chitosan-heparin artificial extracellular matrix (AECM) is reported. Three-dimensional, macroporous composite AECMs composed of heparin (Hep) and chitosan (Chito) were prepared by an interpolyelectrolyte complex/lyophilization method. The Chito-Hep composite AECMs were, respectively, crosslinked with glutaraldehyde, as well as cocrosslinked with *N*,*N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide (EDC/NHS) and *N*-hydroxysuccinimide (NHS). The crosslinking reactions were examined by FT-IR analysis. In physiological buffer solution (PBS), the EDC/NHS-crosslinked Chito-Hep composite AECM showed a relative lower water retention ratio than its glutaraldehyde-crosslinked counterparts. The EDC/NHS-crosslinked Chito-Hep composite AECMs showed excellent biocompatibility, according to the results of the *in vitro* cytotoxic test. This result suggested that the EDC/NHS-crosslinked Chito-Hep composite AECMs might be a potential biomaterial for scaffold-guided tissue engineering applications. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 3639–3644, 2008

Key words: chitosan; heparin; artificial extracellular matrix; glutaraldehyde; scaffold

INTRODUCTION

The tissue regeneration involves the interaction of cell with their extracellular matrix (ECM). ECMs are composed of glycoproteins, collagen and glycosaminoglycans (GAGs).[1] Cells can attach to the ECM by means of transmembrane glycoproteins called integrins. Biodegradable polymers have been extensively used in scaffold-guided tissue engineering to construct porous three-dimensional scaffolds for directing the growth of new tissue.[2,3] This method has been used to create various tissue analogs including skin, cartilage, bone, liver, nerve, vessels, etc.[4–6]

Heparin and chitosan are both biopolymers widely used in biomedical applications. Heparin is a mucopolysaccharide with a molecular weight ranging from 6,000 to 40,000 Da. The polymeric chain is composed of repeating disaccharide unit of D-glucosamine and uronic acid linked by 1– –>4 interglycosidic bond. The uronic acid residue could be either D-glucuronic acid or L-iduronic acid. The hydroxyl groups on the uronic acid residues and the amino groups on the glucosamine residues were sulfated giving rise to a polymer with that is highly negatively charged. Heparin stimulates proliferation in some cell types and also modulates several phases of wound healing.[7–8] Chitosan is a copolymer of glucosamine and *N*-acetylglucosamine obtained by *N*-deacetylation of chitin.[9] In the previous studies, chitosan has been studied for drug delivery and biomedical applications.[10–14] Glucosamine is an intermediate substrate in the synthesis of the ground substance (noncollagen portion) of cartilage and is helpful to enhance proteoglycan synthesis. Therefore, the chitosan-based biomaterials have been noted for its cartilage repairing ability.[15–16] It is believed that the combination of chitosan with heparin will improve the wound-healing ability of chitosan.[17]

Several methods have been employed for the fabrication of scaffolds with large pores.[18–21] In the present study, we designed a macroporous, artificial ECM based on the combination of heparin and chitosan. Heparin (Hep) and chitosan (Chito) were cocrosslinked, respectively, with *N*,*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and *N*-

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hydroxy-succinimide (NHS), or crosslinked with glutaraldehyde. The polymeric properties of the Chito-Hep composite scaffolds were examined by FTIR, X-ray, SEM, and water retention ratio. Furthermore, cell cytotoxicity was performed to evaluate their potential as artificial ECM.

EXPERIMENTAL

Materials

Chitosan was purchased from Fluka (uchs, Switzerland). Heparin was purchased from Sigma (USA), respectively. All other reagents and solvents used were of reagent grade.

Preparation of Chito-Hep composite AECMs

The Chito-Hep composite AECMs with different Chito-to-Hep blend ratios (Chito/Hep = 1/1; CH11 and Chito/Hep = 2/1; CH21) were prepared using a homogenizing interpolyelectrolyte complex method. Chitosan solution (1.0% by weight) was prepared by dissolving chitosan powder (2 g) in 200 mL of deionized water containing acetic acid (1.0% by weight) at room temperature. Heparin solution (1.0% by weight) was prepared by dissolving powder of heparin (2 g) in 200 mL of deionized water at room temperature. The dissolved chitosan solution was then homogenized with the slowly dropped heparin using a homogenizer (IKA, T25) until an almost opaque aqueous solution was derived. The solution was sonicated to remove the trapped air bubbles. The air-bubble-free solution was poured into a glass disk in a dust-free atmosphere to be lyophilized by Eyela, FD-5N (Japan) freeze-drier for the preparation of Chito-Hep AECMs.

Crosslinking

The prepared Chito-Hep composite AECMs were reacted with EDC (24 m*M*)/NHS (5 m*M*) in 2-morpholinoethane sulfonic acid (MES) buffer, and glutaraldehyde (0.1 wt %) aqueous solution for crosslinking, respectively. The crosslinked AECMs were washed with deionized water and relyophilized to prepare chemically crosslinked Chito-Hep composite AECMs.

FT-IR analysis

The Chito-Hep composite AECMs and their crosslinking reaction were characterized by FT-IR analysis. FT-IR analysis was conducted by firstly mixing the powder forms of crosslinked or uncrosslinked Chito-Hep composite AECMs with KBr (1 : 100). The mixed powder then was pressed into a disk and analyzed by a FT-IR spectrometer (Perkin–Elmer Spectrum RXI FT-IR System, Buckinghamshire, England).

X-ray diffraction

The X-ray diffraction patterns were determined from original and crosslinked Chito-Hep composite films by using a Bruker D8 diffractometer. These dense films were prepared by the method as the aforementioned process for the preparation of Chito-Hep composite AECMs. However, the dense Chito-Hep composite films were prepared by dried in oven at 45°C, but were not lyophilized by a freeze-drier.

SEM study

The prepared Chito-Hep composite AECMs were attached onto a double-sided adhesive tape and fixed to an aluminum stage, respectively. The sponges were cut by a razor, then were sputter-coated with gold in a thickness of 500×10^{-8} cm using a Hitachi coating unit (IB-2 coater). Subsequently, the morphologies of cross section of the composite AECMs were examined using a Hitachi S-2300 scanning electron microscopy (SEM).

Water retention ratio

The water retention ratio of each Chito-Hep composite AECM was determined by swelling the membranes in the physiological buffer saline (PBS) at room temperature. The Chito-Hep composite AECM (200 mg) was placed in the PBS solution for a required period of time. Subsequently, the swollen Chito-Hep composite AECM was taken out and the wet weight of the Chito-Hep composite AECM was determined by first blotting the porous AECM with a filter paper to remove the adsorbed water on the surface, then weighed immediately on an electronic balance. The percentage water retention of the Chito-Hep composite AECM in the medium was calculated as follows:

$$E_{st} = [(W_t - W_0)/W_0] \times 100$$

where E_{st} is the water retention ratio of the Chito-Hep composite AECM at predetermined time. W_t denotes the weight of the Chito-Hep composite AECM at predetermined time and W_0 is the initial weight of the Chito-Hep composite AECM. Each water retention experiment was repeated three times and the average value was taken as the water retention ratio.

Cytotoxicity

In the study, the cellular compatibility of each test sample was evaluated by an MTT assay. In the cell-culture assay, 3T3 fibroblasts at 5×10^4 cells per well

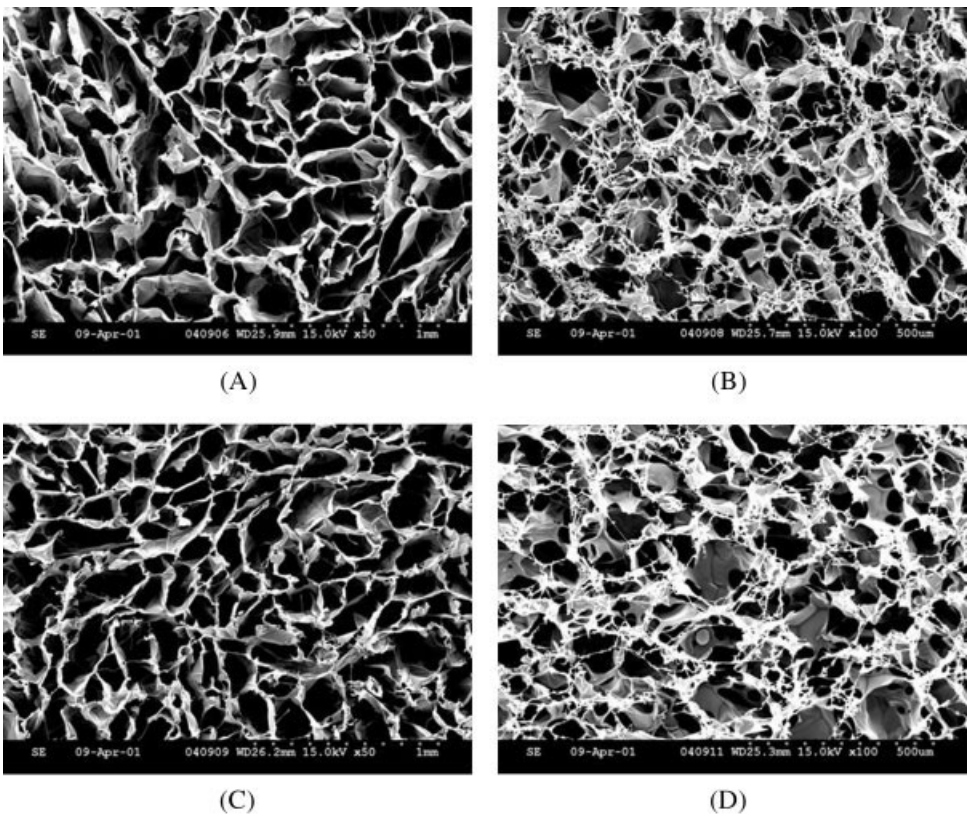

Figure 1 SEM micrographs of the crosslinked Chito-Hep composite AECMs: (A) CH21; surface (B) CH11; surface (C) CH21; cross section (D) CH11; cross section.

were seeded evenly in each well with Dulbecco's modified eagle medium (DMEM, Gibco 430-2800EG, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Hyclone Laboratories, Logan, UT). The culture dish used was 24 wells. The cell culture was maintained in a humidified incubator at 37°C with 10% CO_2 in air. After 24 h of culture, the media was replaced with fresh media and the Chito-Hep composite film was placed into the well. The cells were subsequently cultured for 24 h. The control group was cell cultured without adding any Chito-Hep composite film. Afterward, the media and Chito-Hep composite film were removed, and the well was washed with PBS. The number of attached cells was determined by MTT assay. The 200-μL MTT solution was added to each well. After 3 h incubation at 37°C, the media was removed DMSO (dimethyl sulfoxide) of 200 μL was added to dissolve the formazan crystals. The optical density of the solution was read on an enzyme-linked immunosorbent assay (ELISA) plate reader (Multiskan ascent-345) at 570 nm.

RESULTS AND DISCUSSION

Morphologies

The morphologies of Chito-Hep composite AECMs were characterized using SEM. Figure 1 shows the SEM micrographs of Chito-Hep composite AECMs (CH11 and CH21). The Chito-Hep composite AECMs showed a three-dimensional porous structure with a pore size of 100–200 μm.

FT-IR analysis

The covalent crosslinking reactions for the preparation of glutaraldehyde- and EDC/NHS-crosslinked Chito-Hep composites were analyzed by FT-IR. The results were shown in Figure 2–4. The spectra of chitosan displayed peaks around 905 cm^{-1} and 1153 cm^{-1} of assigned saccharine structure, and a strong amide characteristic peak at 1650 cm^{-1} as well as a characteristic peak assigned to protonated amine ($—NH_3^+$ groups), at around 1570 cm^{-1}. Heparin displayed a strong characteristic peak at 1235 cm^{-1} assigned to sulfate groups as well as another strong peak at around 1650 cm^{-1} attributed to carboxylic ions. The intensity of characteristic absorption of $—NH_3^+$ in the Chito-Hep composites decreased with the decrease of Chito-to-Hep blend ratio, accompanied with the increased intensity of characteristic absorptions of sulfate and carboxylic ions (Fig. 2).

After crosslinking with glutaraldehyde, the characteristic absorption of amino groups at 1570 cm^{-1}

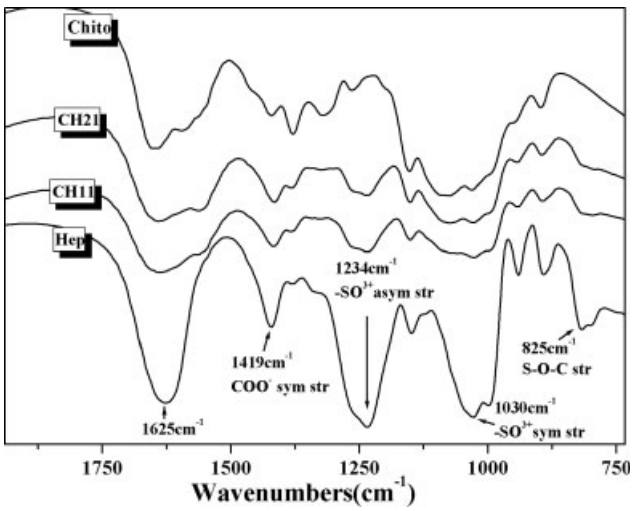

Figure 2 FT-IR spectra of the Chito-Hep composite AECMs with different Chito-to-Hep blend ratios (CH11 and CH21).

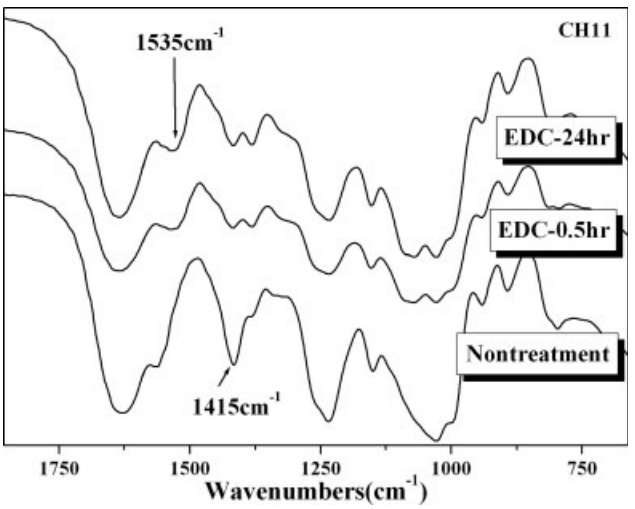

Figure 4 FT-IR spectra of the EDC/NHS-crosslinked Chito-Hep composite AECMs (CH11).

decreased, accompanied with the increase of characteristic absorption at 1600 $\sim$ 1650 cm^{-1} due to the formation of imine bonds (C=N) (Fig. 3). Glutaraldehyde crosslinks at the C-2 amine of glucosamine units in chitosan via imide bonds formation. However, the functional groups on heparin are not easy to react with glutaraldehyde. The peak intensity of amino groups at 1560 cm^{-1} decreased accompanied with the increase of amide characteristic peak at 1650 cm^{-1}, after crosslinking with EDC/NHS (Fig. 4). As EDC/NHS-crosslinking results in the formation of amide bonds between carboxylic acid groups on heparin and amine groups on chitosan, heparin will be crosslinked with the chitosan macromolecular chain.

X-ray diffraction

Figure 5 shows the X-ray diffraction patterns of chitosan, heparin and the Chito-Hep composite films. Pure chitosan showed a strong reflection at 19.7° and another reflection at 15.4°. The heparin showed relatively weak and broad reflections among 10° $\sim$ 40°, which suggested that its macromolecular chain was randomly arranged. In the Chito-Hep composite films, the intensity of reflection peaks for chitosan was diminished. This indicated that there was a significant decrease in chitosan crystallization upon Chito-Hep complex. It was probably due to the effect of the stiff Chito-Hep complex on mobility in the overall mixture, which inhibited the crystal growth of chitosan. After crosslinking, the reflection peaks further decreased by inhibiting the crystallization of chitosan and heparin.

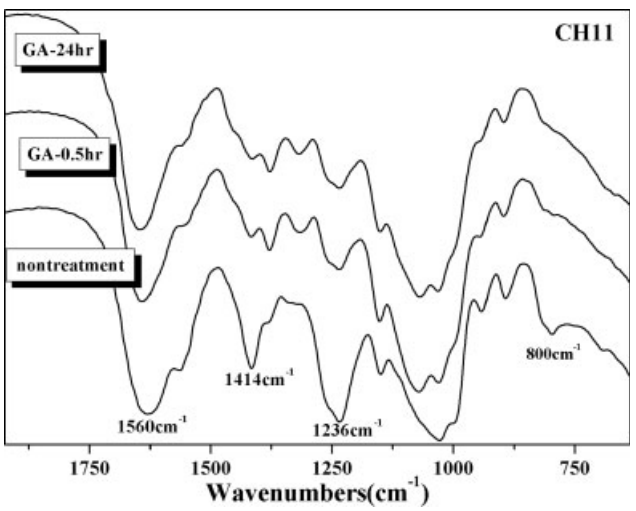

Figure 3 FT-IR spectra of the glutaraldehyde-crosslinked Chito-Hep composite AECM (CH11).

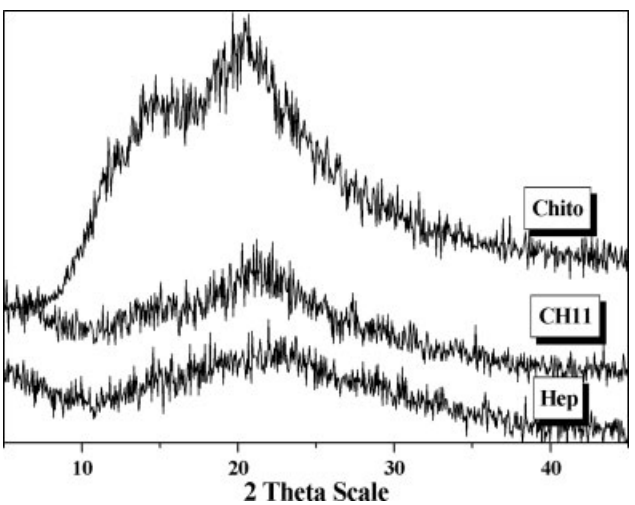

Figure 5 X-ray diffraction patterns of Chito-Hep composite AECMs (CH11).

Water retention ratio

Figure 6 shows the water retention ratios of Chito-Hep composite AECMs crosslinked by glutaraldehyde and EDC/NHS. As shown, the water retention ratios of the EDC/NHS-crosslinked Chito-Hep composite AECMs were significantly lower than those of the glutaraldehyde-crosslinked ones. This might be attributed to the differences in the crosslinked structures of the glutaraldehyde- and EDC/NHS-crosslinked Chito-Hep composite AECMs. Glutaraldehyde-crosslinked Chito-Hep composite AECM may have stronger ability to keep the scaffold shape, thus brings larger water retention ratio. In contrast, the water retention ratio of EDC/NHS-crosslinked Chito-Hep composite AECM was relatively lower as compared with its glutaraldehyde-crosslinked counterpart.

Cytotoxicity

Figure 7 shows the relative cell proliferation 48 h after cell seeding, which was determined from the MTT assay. The MTT value thus obtained is directly proportional to the cell number and the activity in each well. It is reasonable to hypothesize that chemical reagents can generate cytotoxicity. Therefore, the cell cultured with EDC/NHS- and glutaraldehyde-crosslinked Chito-Hep composite films might have a lower relative proliferation than their un-crosslinked counterpart. Indeed, relatively lower cell proliferation is observed from the cell cultured with glutaraldehyde-crosslinked Chito-Hep composite films as compared with that of EDC/NHS-crosslinked ones. Several studies have reported the cytoxicity of glutaraldehyde. However, the cytotoxicity arisen from EDC/NHS-crosslinked Chito-Hep composite films are not clearly found.

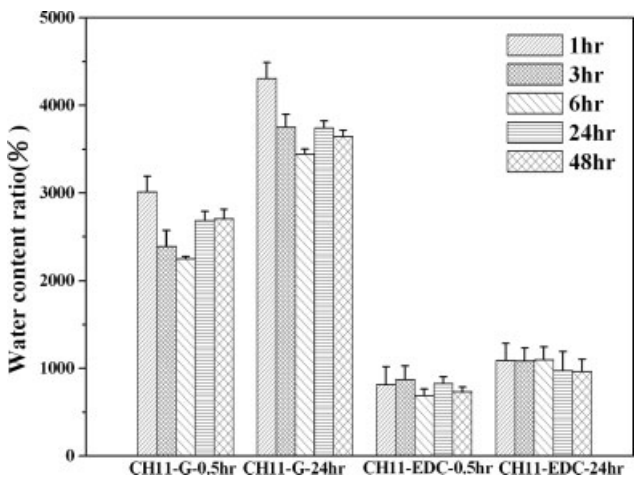

Figure 6 Swelling characteristics of EDC/NHS- and glutaraldehyde-crosslinked Chito-Hep composite AECMs (CH11). GA-0.5, glutaraldehyde crosslinked for 0.5 h; GA-24, glutaraldehyde crosslinked for 24 h; EDC-0.5, EDC crosslinked for 0.5 h; EDC-24, EDC crosslinked for 24 h.

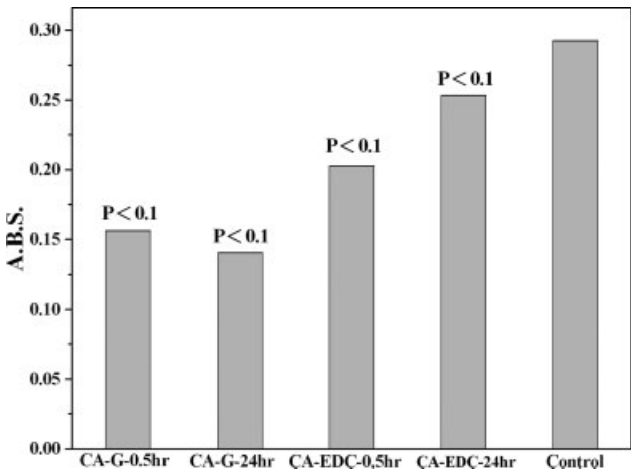

Figure 7 Cytotoxicity of EDC/NHS- and glutaraldehyde-crosslinked Chito-Hep composite AECMs (CH11). GA-0.5, glutaraldehyde crosslinked for 0.5 h; GA-24, glutaraldehyde crosslinked for 24 h; EDC-0.5, EDC crosslinked for 0.5 h; EDC-24, EDC crosslinked for 24 h.

CONCLUSIONS

We have prepared the Chito-Hep composite AECMs for the application of scaffold-guided tissue engineering in future. The three-dimensional, macroporous AECMs were prepared by the methods of interpolyelectrolyte complex and lyophilization, followed by the crosslinking of Chito-Hep composite AECMs with glutaraldehyde, and EDC/NHS. These covalent-crosslinked Chito-Hep composite AECMs showed more stability in PBS solution as compared with their noncrosslinked counterpart. The EDC/NHS-crosslinked Chito-Hep composite AECMs showed low cytotoxicity, according to the results of the *in vitro* cytotoxicity test. This result suggested that the EDC/NHS-crosslinked Chito-Hep composite AECMs might be a potential biomaterial for tissue-engineering application.

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