Polysaccharide-Based Artificial Extracellular Matrix: Preparation and Characterization of Three-Dimensional, Macroporous Chitosan and Chondroitin Sulfate Composite Scaffolds

Chih-Kang Peng,[1] Shu-Huei Yu,[2,3] Fwu-Long Mi,[4] Shin-Shing Shyu[2]

[1]*Department of Chemical and Materials Engineering, National Central University, Chung-Li, Taiwan 320, Republic of China*
[2]*Center of Polymer Materials Research, Vanung University, Chung-Li, Taiwan 320, Republic of China*
[3]*Department of Textile Science, Vanung University, Chung-Li, Taiwan 320, Republic of China*
[4]*Department of Applied Science, Chinese Naval Academy, 669 Jiun Shiaw Road, Kaohsiung, Taiwan 813, Republic of China*

Received 6 December 2004; accepted 19 April 2005
DOI 10.1002/app.22730
Published online 6 December 2005 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Scaffold-guided tissue engineering based on synthetic and natural occurring polymers has gained much interest in recent years. In this article, the development of a polysaccharide-based artificial extracellular matrix (AECM) is reported. Three-dimensional, macroporous composite AECMs composed of chondroitin sulfate (ChS) and chitosan (Chito) were prepared by an interpolyelectrolyte complex/lyophilization method. The ChS–Chito composite AECMs were crosslinked with glutaraldehyde and calcium ions (Ca^{2+}) and cocrosslinked with *N*,*N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The crosslinking reactions were examined with Fourier transform infrared analysis. Glutaraldehyde and Ca^{2+} crosslinked with Chito and ChS, respectively, to produce different types of ChS–Chito semi-interpenetrated networks. In contrast, EDC/NHS crosslinked with both Chito and ChS to produce ChS–Chito connected networks. In physiological buffer solutions, the Ca^{2+}-crosslinked ChS–Chito composite AECMs showed a lower swelling ratio than their EDC/NHS- and glutaraldehyde-crosslinked counterparts. The ChS–Chito composite AECMs showed excellent antibacterial capability and biocompatibility according to the results of the in vitro antibacterial test and cytotoxic assay. This result suggested that the ChS–Chito composite AECMs might be a potential biomaterial for scaffold-guided tissue-engineering applications. J Appl Polym Sci 99: 2091–2100, 2006

Key words: chitosan; chondritin sulfate; artificial extracellular matrix

INTRODUCTION

Scaffold-guided tissue regeneration is a newly developed specialty involving seeding highly porous, biodegradable scaffolds with donor cells and then culturing and implanting the scaffolds to induce and direct the growth of new tissue.[1,2] The tissue regeneration involves the interaction of cells with their extracellular matrix (ECM). ECMs are composed of glycoproteins, collagen, and glycosaminoglycans (GAGs).[3] Cells can attach to ECMs by means of transmembrane glycoproteins called integrins. Recently, artificial ECMs based on biopolymers such as collagen and GAGs have gain increased interest for tissue engineering.[4–6]

Chondroitin sulfate (ChS) and chitosan (Chito) are both biopolymers widely used in biomedical applications. ChS belongs to a family of GAGs composed of alternating sequences of differently sulfated residues of uronic acid (β-D-glucuronic) and α-D-*N*-acetylgalactosamine linked by β(1→3) bonds.[7] ChSs are found in humans in cartilage, bone, corneas, skin, and arterial walls.[8] They are usually employed as chondroprotective drugs with applications in the therapy of tibiofibular osteoarthritis of the knee and in articular cartilage osteoarthritis by intramuscular and oral routes. Chito is a copolymer of glucosamine and *N*-acetylglucosamine obtained by N-deacetylation of chitin.[9] In previous studies, Chito has been studied for drug delivery and biomedical applications.[10–14] Glucosamine is an intermediate substrate in the synthesis of the ground substance (non-collagen portion) of cartilage and is helpful for enhancing proteoglycan synthesis. Therefore, Chito-based biomaterials have been noted for their cartilage-repairing ability.[15,16] Chito-based composite materials prepared from polyelectro-

Correspondence to: S.-S. Shyu.
Contract grant sponsor: National Science Council of Taiwan (Republic of China); contract grant number: NSC 92-2313-B-238-002.

lyte complexes and polymer blends have been studied for drug delivery and biomedical purposes.[17–20] We believe that the combination of ChS and Chito will be suitable for the preparation of artificial extracellular matrices (AECMs) for cartilage tissue engineering.

In this study, we designed an AECM based on a combination of ChS and Chito. ChS and Chito were cocrosslinked with *N*,*N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) or crosslinked with glutaraldehyde and calcium ions, respectively. The polymeric properties of the ChS–Chito composites were examined with Fourier transform infrared (FTIR), X-ray, scanning electron microscopy (SEM), and swelling studies. Furthermore, cell cytotoxicity and antibacterial assays were performed to evaluate their potential as AECMs.

EXPERIMENTAL

Materials

Chito was purchased from Fluka (Buchs, Switzerland). ChS was purchased from Sigma (St. Louis, MO). All other reagents and solvents were reagent-grade.

Preparation of the ChS–Chito composite AECMs

The ChS–Chito composite AECMs were prepared with a homogenizing interpolyelectrolyte complex method. A Chito solution (1.0 wt %) was prepared by the dissolution of Chito powder (2 g) in 200 mL of deionized water containing acetic acid (1.0 wt %) at room temperature. A ChS solution (1.0 wt %) was prepared by the dissolution of a powder of ChS (2 g) in 200 mL of deionized water at room temperature. The dissolved Chito solution then was homogenized with slowly dropped ChS with a homogenizer (T25, IKA-LABORTECHNIK, Staufen, Germany) 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 an FD-5N freeze drier (Eyela, Japan) for the preparation of ChS–Chito AECMs.

Crosslinking

The prepared ChS–Chito composite AECMs were reacted with EDC (24 m*M*)/NHS (5 m*M*) in a 2-morpholinoethane sulfonic acid (0.05*M*) buffer and glutaraldehyde (0.1 wt %) and calcium sulfate (0.1 wt %) aqueous solutions for crosslinking, respectively. The crosslinked AECMs were washed with deionized water and relyophilized to prepare chemically and ionically crosslinked ChS–Chito composite AECMs, respectively.

FTIR analysis

The ChS–Chito composites and their crosslinking reactions were characterized with FTIR analysis. The FTIR analysis was conducted by the mixing of the powder forms of crosslinked or uncrosslinked ChS–Chito composites with KBr (1 : 100). The mixed powder then was pressed into a disk and analyzed with an FTIR spectrometer (Spectrum RXI FTIR system, PerkinElmer, Buckinghamshire, England).

X-ray diffraction

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

SEM study

The prepared ChS–Chito composite AECMs were attached onto double-sided adhesive tape and fixed to an aluminum stage. The sponges were cut with a razor and then were sputter-coated with gold in a thickness of 500×10^{-8} cm with a Hitachi IB-2 coater. Subsequently, the morphologies of the cross sections of the composite AECMs were examined with a Hitachi S-2300 scanning electron microscope.

Swelling ability

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

$$E_{st} = [(W_t - W_o) / W_o] \times 100 \quad (1)$$

where E_{st} is the swelling percentage of the ChS–Chito composite AECM at a predetermined time, W_t denotes the swollen weight of the ChS–Chito composite AECM at a predetermined time, and W_0 is the initial weight of the ChS–Chito composite AECM. Each

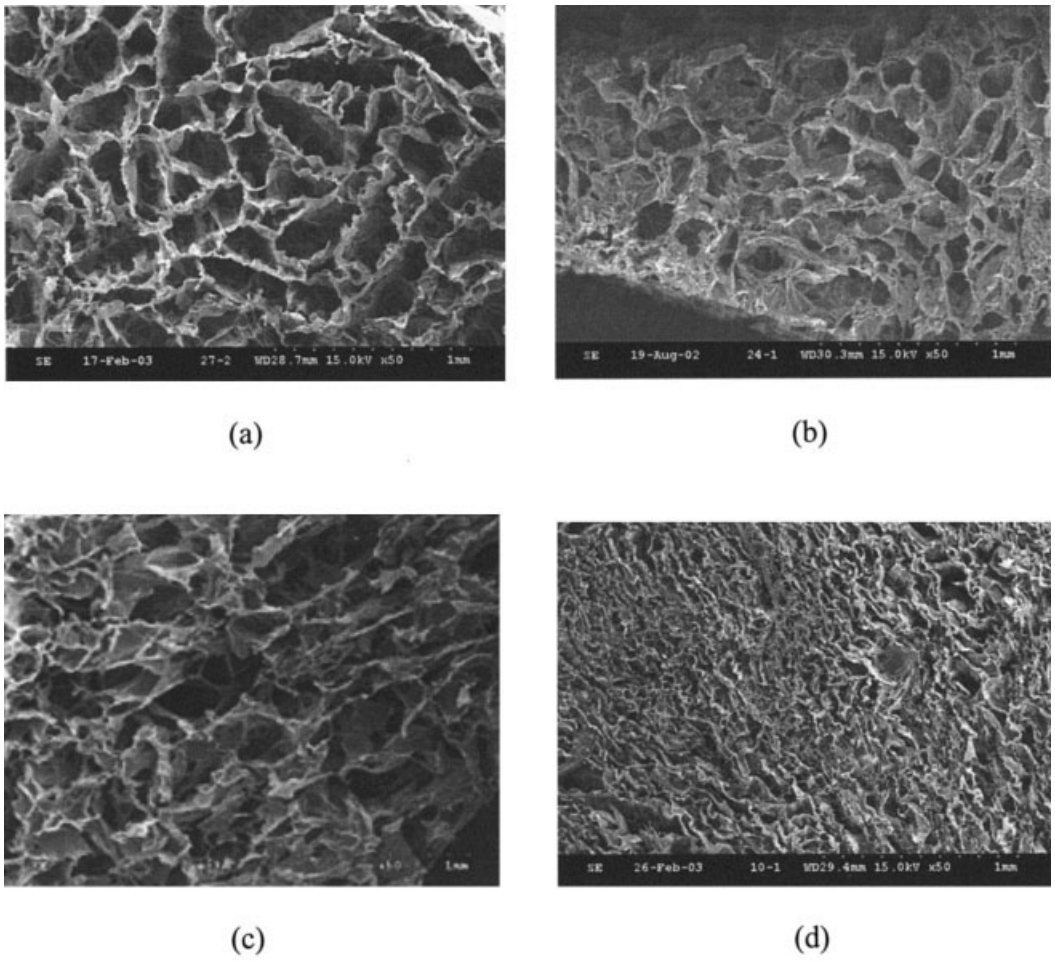


Figure 1 SEM micrographs of crosslinked ChS–Chito composite AECMs: (a) uncrosslinked ChS–Chito composite AECMs, (b) glutaraldehyde-crosslinked ChS–Chito composite AECMs, (c) EDC-crosslinked ChS–Chito composite AECMs, and (d) Ca^{2+}-crosslinked ChS–Chito composite AECMs

swelling experiment was repeated three times, and the average value was taken as the swelling percentage.

Antibacterial test

The testing of the antibacterial activity of the ChS–Chito composite films was performed according to the method described by Grzybowski et al.[21] In the antibacterial assay, 16-mm-diameter test samples cut from UV-light-sterilized ChS–Chito composite films were placed on the bottoms of wells in a 24-well plate (the diameter of each well was ca. 16 mm). Subsequently, 50 μL of a bacterial broth culture was seeded onto the ChS–Chito composite films (10^6 CFU/mL). Bacterial broth cultured in a well not containing any ChS–Chito composite film was used as a control. The bacteria used for the test was *Escherichia coli*. Subsequently, the films were put in a moisture incubator and incubated at 37°C. After 1,2, or 4 h of incubation, each ChS–Chito composite film was placed in a test tube containing 1 mL of PBS and was sonicated for 75 s in an ultrasonic washer (64 kHz). Subsequently, 50 μL of the incubated medium taken from each tube was seeded on agar plates containing nutrient broth and incubated at 37°Cfor 24 h. Finally, the units of colony formation in each agar plate were calculated to examine the antibacterial ability of each ChS–Chito composite film.

Cytotoxicity

In this study, the cellular compatibility of each test sample was evaluated with an MTT (agent for cytoxicity) assay. In the cell-culture assay, fibroblasts at 5 $\times$ 10^4 cells per well were seeded evenly in each well with Dulbecco's modified eagle medium (430-2800EG, Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT). The cell culture was maintained in a humidified incubator at 37°C with 10% CO_2 in air. After 24 h of culturing, the medium was replaced with fresh medium, and the ChS–Chito composite film was placed in the well. The cells were subsequently cultured for

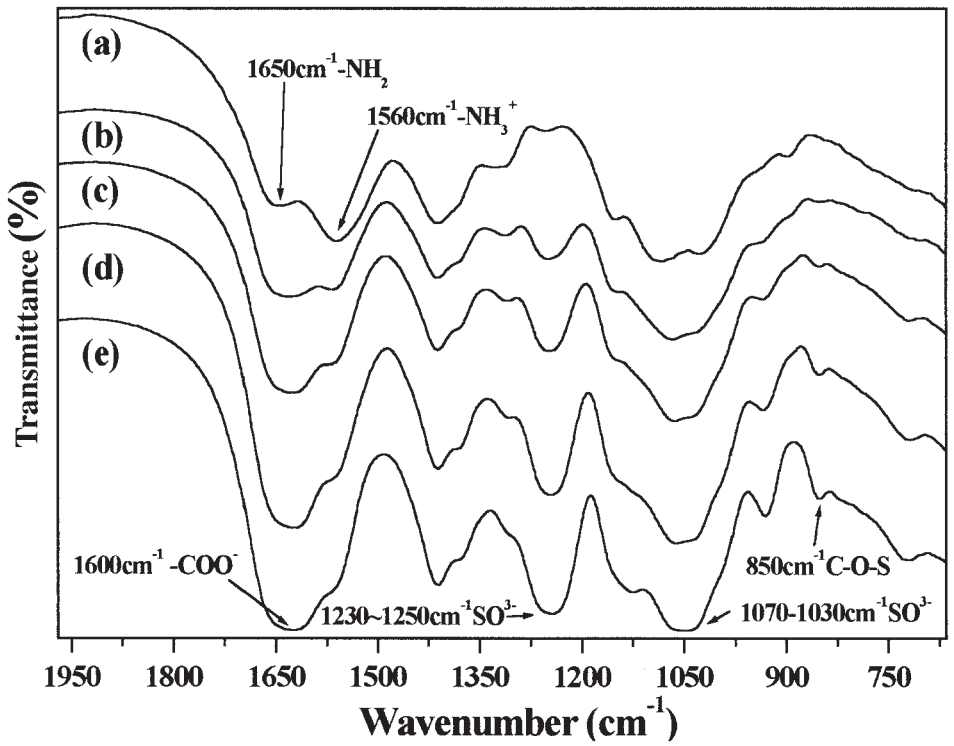


Figure 2 FTIR spectra of ChS–Chito composite AECMs with different ChS-to-Chito blend ratios: (a) pure Chito, (b) ChS/Chito = 2/1, (c) ChS/Chito = 1/1, (d) ChS/Chito = 1/2, and (e) pure ChS.

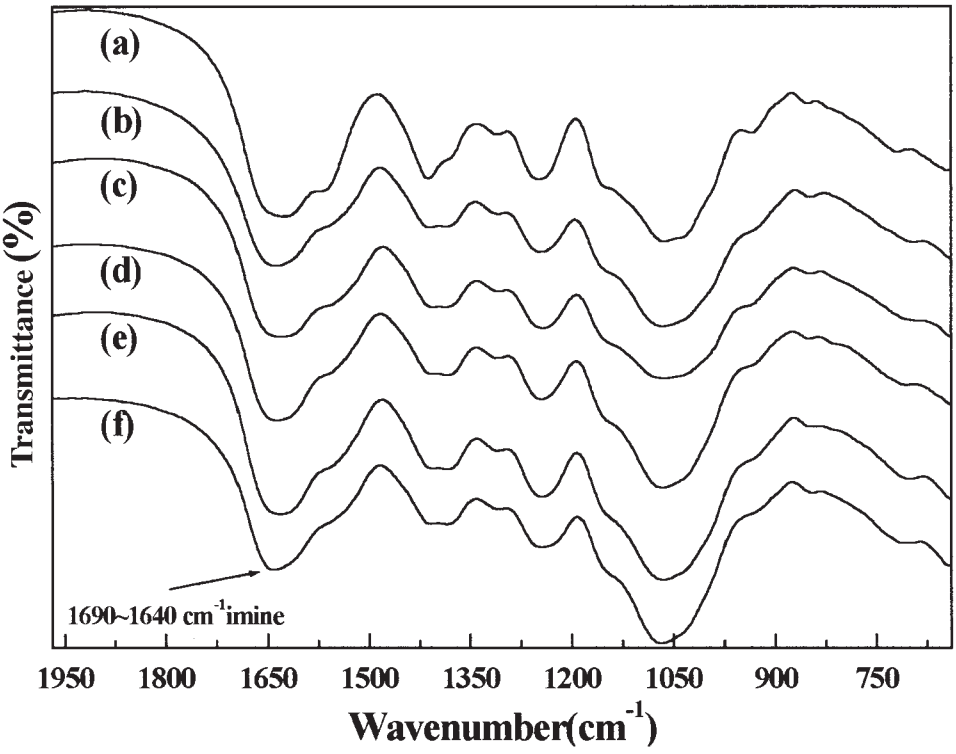


Figure 3 FTIR spectra of glutaraldehyde-crosslinked ChS–Chito composite AECMs: (a) 1/1 ChS/Chito, (b) 0.5 h, (c) 1 h, (d) 2 h, (e) 6 h, and (f) 24 h.

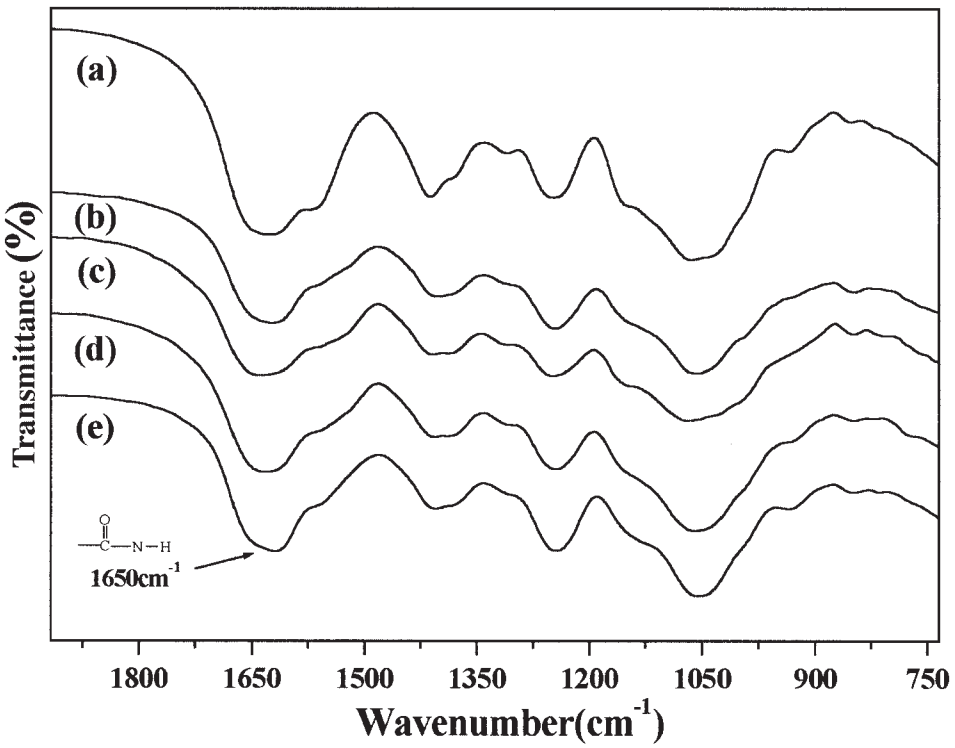


Figure 4 FTIR spectra of EDC-crosslinked ChS–Chito composite AECMs: (a) 1/1 ChS/Chito, (b) 1 h, (c) 2 h, (d) 6 h, and (e) 24 h.

24 h. Afterward, the medium and ChS–Chito composite film were removed, and the well was washed with PBS. The number of attached cells was determined by an MTT assay. The 200-μL MTT solution was added to each well. After 3 h of incubation at 37°C, the medium was removed. Dimethyl sulfoxide (200 μL) was added to dissolve the formazan crystals. The optical density of the solution was read on an enzyme-linked immunosorbent assay plate reader (Multiskan Ascent 345) at 570 nm.

RESULTS AND DISCUSSION

Morphologies

The morphologies of the ChS–Chito composite AECMs were characterized with SEM. Figure 1 shows the SEM micrographs of the glutaraldehyde-, EDC/NHS-, and Ca^{2+}-crosslinked ChS–Chito composite AECMs (ChS/Chito = 1/1). The ChS–Chito composite AECMs showed a three-dimensional, porous structure with a pore size of 150–400 μm [Fig. 1(a)]. The crosslinking of the ChS–Chito composite AECMs with glutaraldehyde and EDC/NHS demonstrated no obvious change in their morphology [Fig. 1(b,c)]. However, the crosslinking of ChS–Chito composite AECMs with Ca^{2+} produced a significant change in their porous structure [Fig. 1(d)]. The Ca^{2+}-crosslinked ChS–Chito composite AECMs had a smaller pore size (<100 um) than their glutaraldehyde- and EDC/NHS-crosslinked counterparts, possibly because of the establishment of Ca^{2+}-coordinated ChS networks.

FTIR analysis

The covalent crosslinking reactions for the preparation of glutaraldehyde- and EDC/NHS-crosslinked ChS–Chito composites were analyzed by FTIR. The results are shown in Figures 2–4. The spectra of Chito displayed peaks around 905 and 1153 cm^{-1} of the 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) around 1570 cm^{-1}. ChS displayed a strong characteristic peak at 1250 cm^{-1} assigned to sulfate groups as well as another strong peak around 1650 cm^{-1} attributed to carboxylic ions. The intensity of the characteristic absorption of —NH_3^+ in the ChS–Chito composites decreased with an increase in the ChS-to-Chito blend ratio, and this was accompanied by an increased intensity of the characteristic absorptions of sulfate and carboxylic ions (Fig. 2).

After crosslinking with glutaraldehyde, the characteristic absorption of amino groups at 1570 cm^{-1} decreased, and this was accompanied by an increase in the characteristic absorption at 1650 cm^{-1} due to the

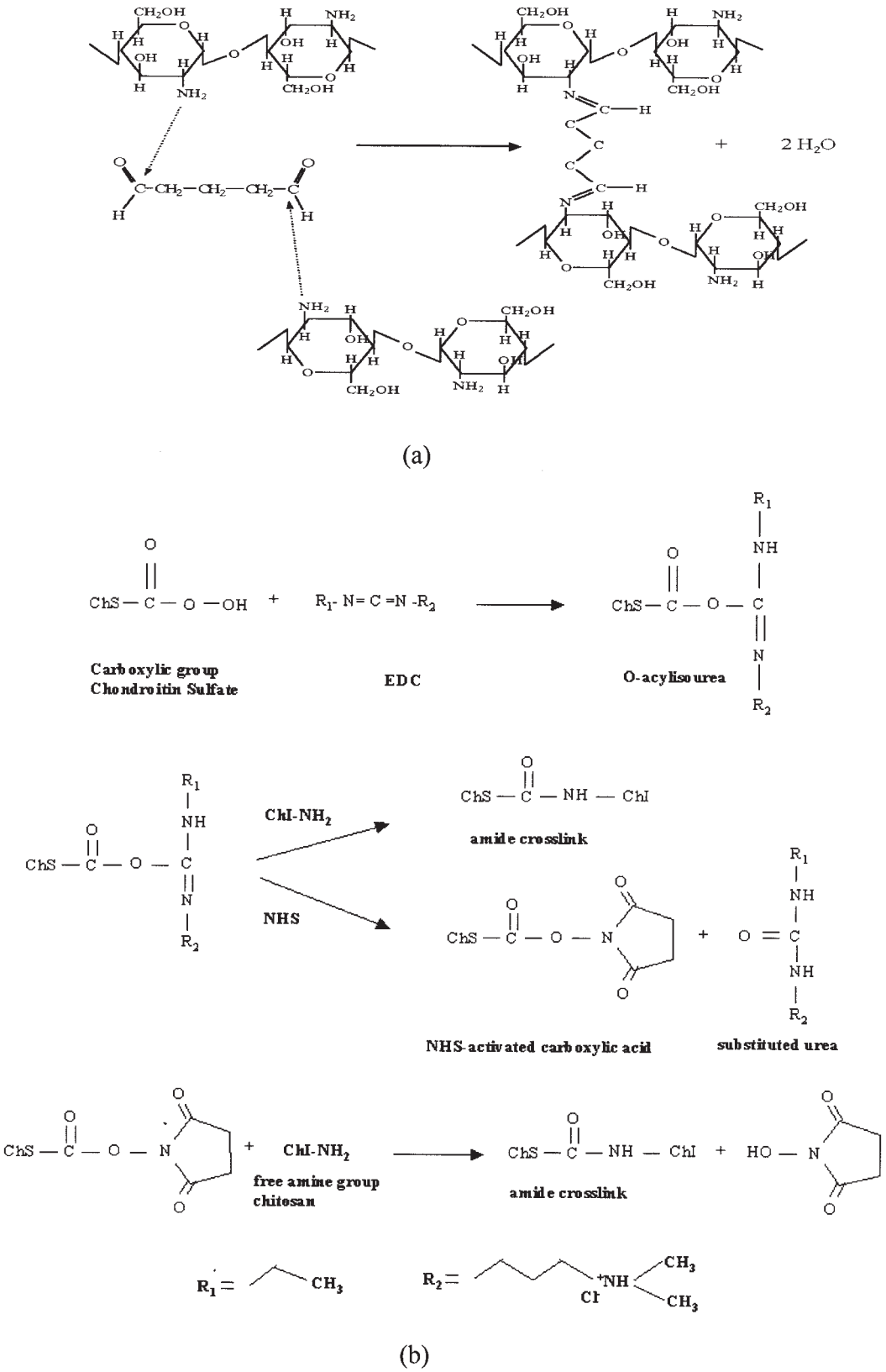
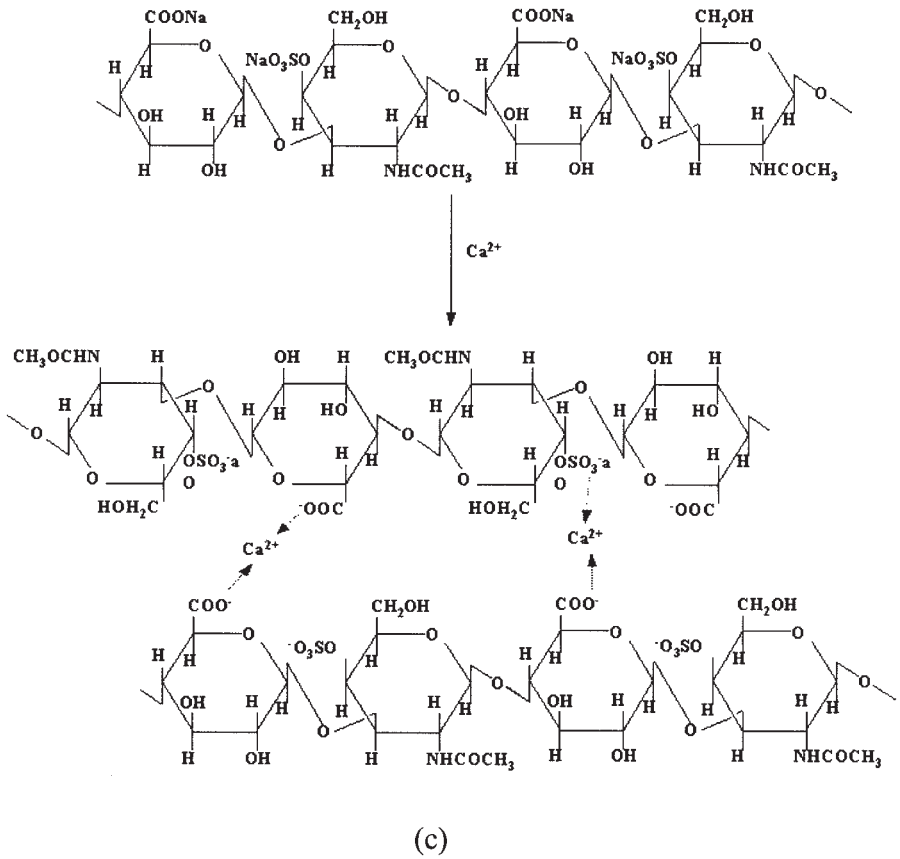


Figure 5 Schematic crosslinking reactions for the preparation of (a) glutaraldehyde-, (b) EDC-, and (c) Ca^{2+}-crosslinked ChS–Chito networks.



(c)

Figure 5 (Continued)

formation of imine bonds (C═N; Fig. 3). Glutaraldehyde crosslinked at the C-2 amine of glucosamine units in Chito via imide-bond formation. However, the functional groups on ChS were not easy to react with glutaraldehyde. This fact suggests that the crosslinking of ChS–Chito composites with glutaraldehyde could produce a structure like semi-interpenetrated networks (semi-IPNs). The peak intensity of amino groups at 1560 cm^{-1} decreased, and this was accompanied by an increase in the amide characteristic peak at 1650 cm^{-1}, after crosslinking with EDC/NHS (Fig. 4). As EDC crosslinking resulted in the formation of amide bonds between carboxylic acid groups on ChS and amine groups on Chito, ChS became crosslinked with the Chito macromolecular chain to form Chito–ChS connected networks.

Calcium ions are known to prepare alginate networks via ionic crosslinking. Ionic-crosslinking-induced gelation has been demonstrated to result from specific and strong interactions between calcium ions and blocks of galacturonic and guluroonic acid residues for alginate.[17] This interacting leads to an efficient association with numerous van der Waals contacts and provides cavities of appropriate size for binding calcium ions. The sulfate residues of uronic acid (β-D-glucuronic) for ChS, like galacturonic and guluroonic acid residues for alginate, are supposed to interact with calcium ions. The association of sulfated uronic acid chains with calcium ions may result in the formation of Ca^{2+}-bridged ChS networks. Chito in Ca^{2+}-crosslinked ChS–Chito networks supposedly binds to ChS via a polyelectrolyte complex, but it is not fixed with the calcium ions. Accordingly, the ionic crosslinking of ChS–Chito composites with calcium ions will lead to another type of ChS–Chito semi-IPN. However, no spectral variation could be observed in Ca^{2+}-crosslinked ChS–Chito networks in comparison with an uncrosslinked ChS–Chito composite. The schematic crosslinking reactions for the preparation of glutaraldehyde-, genipin-, and Ca^{2+}-crosslinked ChS–Chito networks are shown in Figure 5.

X-ray diffraction

Figure 6 shows the X-ray diffraction patterns of Chito, ChS, and ChS–Chito composite films. Pure Chito showed a strong reflection at 19.7° and another reflection at 15.4°. ChS showed relatively weak and broad reflections at 10–40°, which suggested that its macromolecular chain was randomly arranged. In the ChS–Chito composite films, the intensity of the reflection peaks for Chito was diminished (ChS/Chito = 1/1). This indicated that there was a significant decrease in Chito crystallization upon ChS–Chito complexation. It

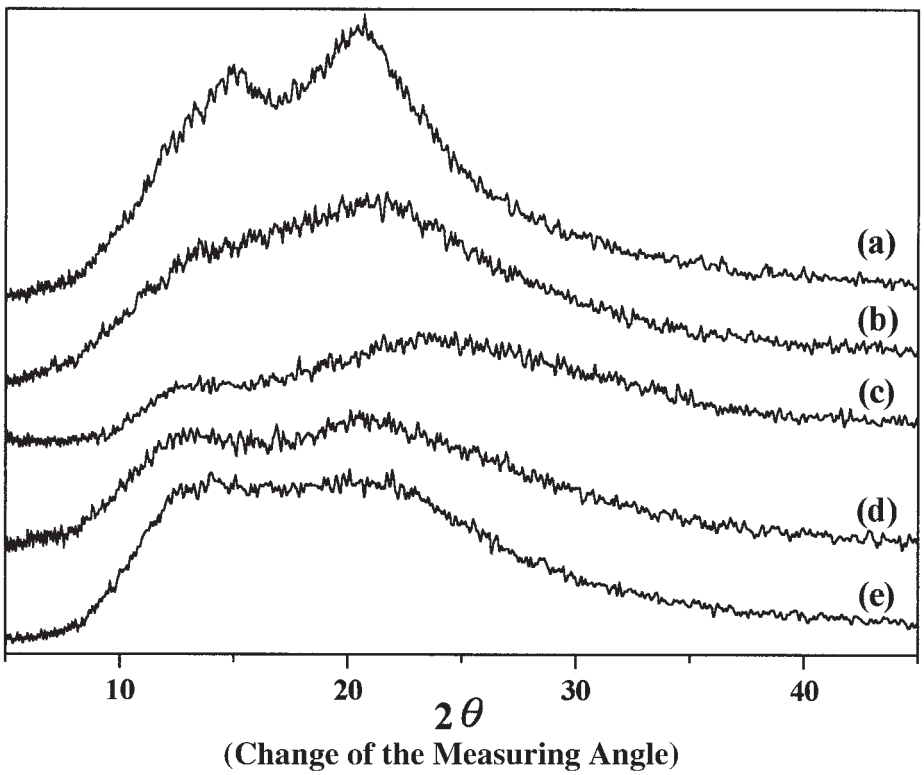


Figure 6 X-ray diffraction patterns of glutaraldehyde- and EDC-crosslinked ChS–Chito composite AECMs: (a) pure Chito, (b) 1/1 ChS/Chito, (c) pure ChS, (d) glutaraldehyde for 24 h, and (e) EDC/NHS for 24 h.

was probably due to the effect of the stiff ChS–Chito complex on mobility in the overall mixture, which inhibited the crystal growth of Chito. After crosslinking, the reflection peaks further decreased because of the inhibition of the crystallization of Chito and ChS.

Swelling properties

Figure 7 shows the swelling characteristics of ChS–Chito composite AECMs crosslinked by glutaraldehyde, EDC/NHS, or Ca^{2+}. As shown, the swelling ratios of the Ca^{2+}-crosslinked ChS–Chito composite AECMs were significantly lower than those of the glutaraldehyde-crosslinked ones. This might be attributed to the differences in the crosslinking structures of the glutaraldehyde-, EDC-, and Ca^{2+}-crosslinked ChS–Chito composite AECMs. Because the sulfate and carboxylic acid groups were easily ionized in PBS solutions, ChS was the most important factor dominating the swelling properties of the ChS–Chito composite AECMs. Chito, in contrast to ChS, was not easy to swell in PBS solutions. As mentioned previously, glutaraldehyde only crosslinks with Chito via imide-bond formation. Therefore, the chain relaxation of ChS macromolecules resulted in a high swelling ratio of the glutaraldehyde-crosslinked ChS–Chito composite AECM. In contrast, the association of sulfated uronic acid chains with Ca^{2+} resulted in the formation of stably crosslinked ChS networks. Therefore, the swelling ratio of the Ca^{2+}-crosslinked ChS–Chito composite AECM was significantly reduced. The swelling ratio of the EDC-crosslinked ChS–Chito composite AECM was higher than the ratio of the Ca^{2+}-crosslinked one but lower than that of the glutaraldehyde-crosslinked one. ChS was covalently conjugated with Chito in the EDC-crosslinked ChS–Chito composite AECM, and this led to the formation of a structure less compact than its Ca^{2+}-crosslinked counterpart.

Antibacterial properties and cytotoxicity

In this study, *E. coli* was used as the test bacteria to examine the antibacterial properties of ChS–Chito composite films. The colony numbers of these bacteria formed on the test films are shown in Figure 8. The colony numbers of the test bacteria formed on the ChS–Chito composite films increased with an increase in the ChS-to-Chito weight ratios. The amino groups on Chito were responsible for the inhibition of bacterial growth. The results indicated that the ChS–Chito composite AECMs might serve as biomedical materials with antibacterial properties.

Figure 9 shows the relative cell proliferation 48 h after cell seeding, which was determined from the MTT assay. The MTT value thus obtained was directly proportional to the cell number in each well. It is

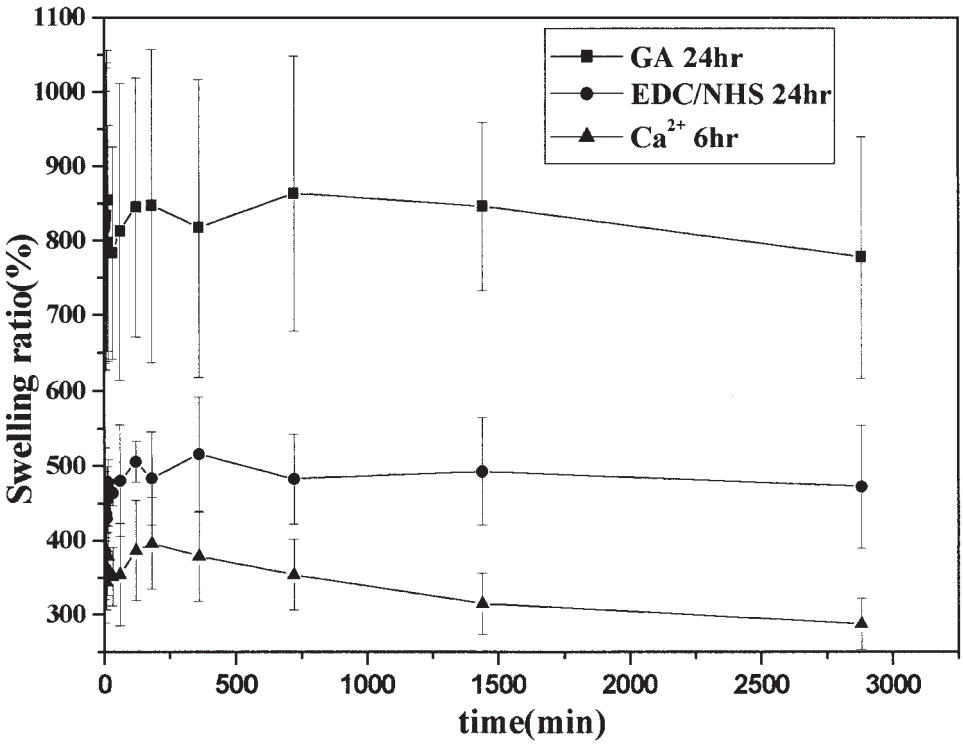


Figure 7 Swelling characteristics of glutaraldehyde (GA)-, EDC-, and Ca^{2+}-crosslinked ChS–Chito composite AECMs.

reasonable to hypothesize that chemical reagents can generate cytotoxicity. Therefore, cells cultured with EDC-, glutaraldehyde-, and Ca^{2+}-crosslinked ChS–Chito composite films might have a lower proliferation than their uncrosslinked counterparts. Indeed, lower cell proliferation was observed for the cell cultured with glutaraldehyde-crosslinked (crosslinking for 24 h) and Ca^{2+}-crosslinked ChS–Chito composite

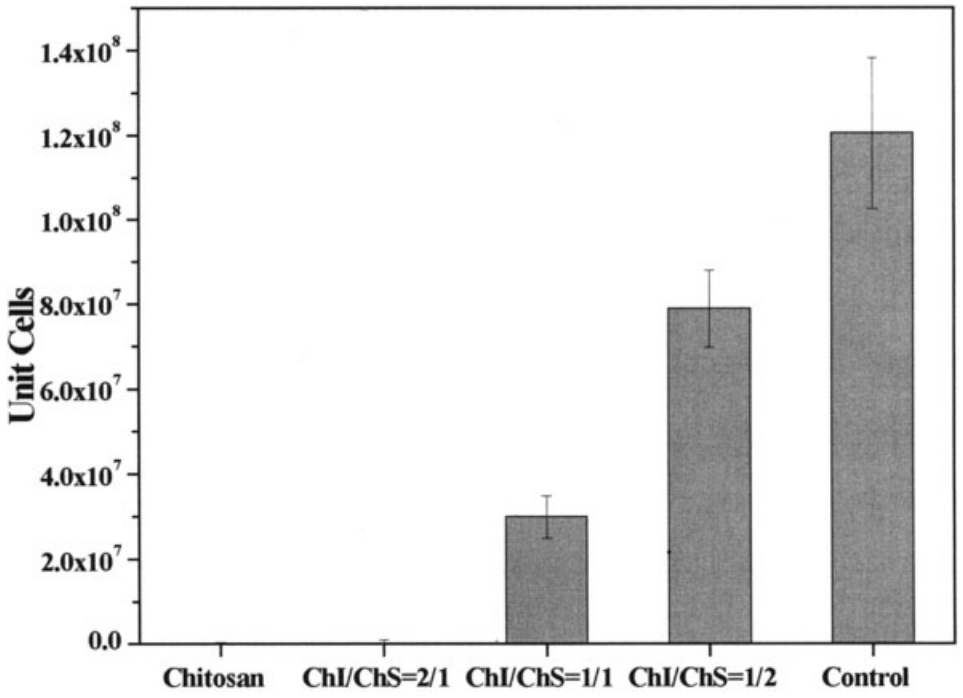


Figure 8 Antibacterial properties of ChS–Chito composite AECMs with different ChS-to-Chito blend ratios.

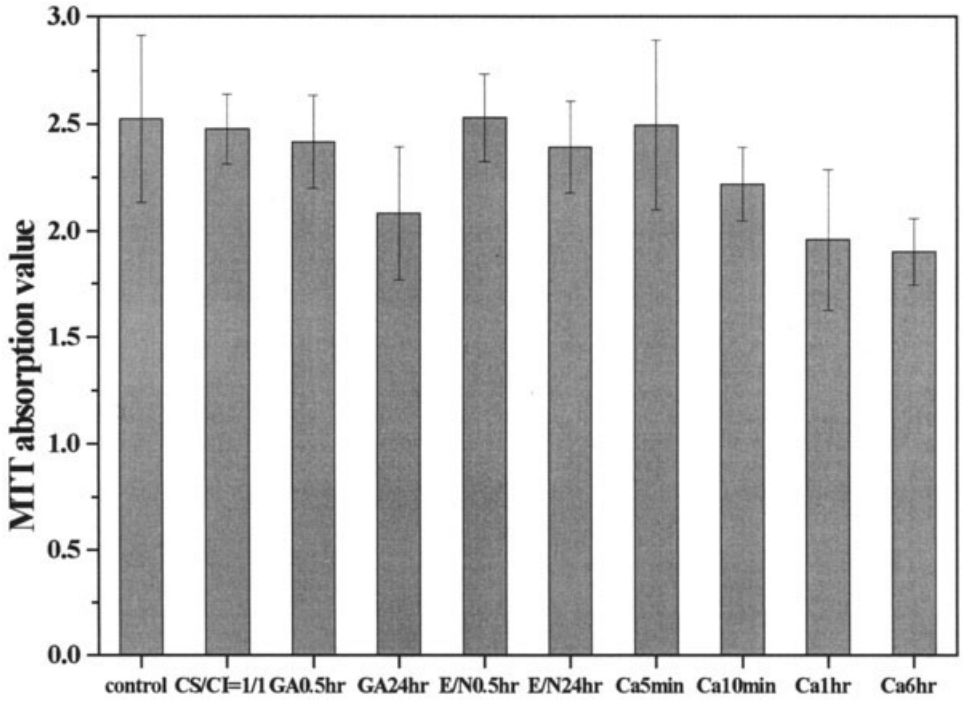


Figure 9 Cytotoxicity of glutaraldehyde (GA)-, EDC (E/N)-, and Ca^{2+} (Ca)-crosslinked ChS–Chito (CS/CI) composite AECMs.

films (crosslinking for 1 h and 6 h). Several studies have reported the cytotoxicity of glutaraldehyde. However, the cytotoxicity, arising from residual calcium ions, is not clearly understand. It may be due to the calcification effect arising from the residual calcium ions that inhibit the cell proliferation.

CONCLUSIONS

We prepared ChS–Chito composite AECMs for the application of scaffold-guided tissue engineering in future. The three-dimensional, macroporous AECMs were prepared by the methods of interpolyelectrolyte complexes and lyophilization, followed by the crosslinking of these ChS–Chito composite AECMs with glutaraldehyde, EDC/NHS, and calcium ions. These covalently and ionically crosslinked ChS–Chito composite AECMs showed more stability in PBS solutions than their noncrosslinked counterparts. The ChS–Chito composite AECMs showed antibacterial capability and low cytotoxicity according to the results of the in vitro antibacterial test and MTT assay. This result suggested that the ChS–Chito composite AECMs might be potential biomaterials for tissue-engineering applications.

References

1. Langer, R.; Vacanti, J. P. Science 1993, 260, 920.
2. Vacanti, J. P.; Langer, R. Lancet 1999, 354, 32.
3. Shin, H.; Jo, S.; Mikos, A. G. Biomaterials 2003, 24, 4353.
4. Lee, K. Y.; Mooney, D. J. Chem Rev 2001, 101, 1869.
5. Mann, B. K.; Gobin, A. S.; Tsai, A. T.; Schmedlen, R. H.; West, J. L. Biomaterials 2001, 22, 3045.
6. Gilbert, T. W.; Stolz, D. B.; Biancaniello, F.; Simmons, B. A.; Badylak, S. F. Biomaterials 2005, 26, 1431.
7. Bryant, S. J.; Davis-Arehart, K. A.; Luo, N.; Shoemaker, R. K.; Arthur, J. A.; Anseth, K. S. Macromolecules 2004, 37, 6726.
8. Mourao, P. A. S.; Rozenfeld, S.; Laredo, J.; Dietrich, C. P. Biochim Biophys Acta 1976, 428, 19.
9. Muzzarelli, R. A. A. Carbohydr Polym 1993, 20, 7.
10. Mi, F. L.; Sung, H. W.; Shyu, S. S. J Appl Polym Sci 2001, 81, 1700.
11. Mi, F. L.; Shyu, S. S.; Wu, Y. B.; Lee, S. T.; Schoung, J. Y.; Huang, R. N. Biomaterials 2001, 22, 165.
12. Mi, F. L.; Wu, Y. B.; Shyu, S. S.; Schoung, J. Y.; Huang, Y. B. J Biomed Mater Res 2002, 59, 438.
13. Mi, F. L.; Shyu, S. S.; Lin, Y. M.; Wu, Y. B.; Peng, C. K.; Tsai, Y. H. Biomaterials 2003, 24, 5023.
14. Chen, S. C.; Wu, Y. C.; Mi, F. L.; Lin, Y. H.; Yu, L. C.; Sung, H. W. J Controlled Release 2004, 96, 285.
15. Suh, J. K. F.; Matthew, H. W. T. Biomaterials 2000, 21, 2589.
16. Muzzarelli, R. A. A.; Mattioli-Belmonte, M.; Tietz, C.; Zucchini, C.; Biagini, R.; Ferioli, G.; Brunelli, M.; Fini, M.; Giardino, R.; Ilari, P.; Biagini, G.; Castaldini, C. Biomaterials 1994, 15, 1075.
17. Jeong, K. S.; Jung, L. K.; Sun, K. J Appl Polym Sci 2004, 93, 1097.
18. Zhang, L.; Guo, J.; Peng, X.; Jin, Y. J Appl Polym Sci 2004, 92, 878.
19. Li, L.; Ding, S.; Zhou, C. J Appl Polym Sci 2004, 91, 274.
20. Braccini, I.; Pérez, S. Biomacromolecules 2001, 2, 1089.
21. Grzybowski, J.; Antos, M.; Trafny, E. A. J Pharmacol Toxicol 1996, 36, 73.