Photocrosslinked Electrospun Chitosan-Based Biocompatible Nanofibers

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ABSTRACT: The electrospun fibers chitosan/polyvinyl alcohol (CS/PVA) were crosslinked by the incorporation of photocrosslinking agent poly(ethyleneglycol)-600-dimethacrylate (PEGDMA) and photoinitiator 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (HEPK) into the spinning solutions and a subsequent irradiation by UV light. The photocrosslinking process of CS-based nanofibers was studied by the Fourier transform infrared spectra, and the water resistance was investigated by scanning electron microscopy (SEM) and water swelling measurement. The results indicated that the water resistance of photocrosslinked CS-based nanofibers was improved obviously and the swelling ratio decreased with the increasing content of PEGDMA/HEPK. When the concentration of crosslinking agent was 20 wt %, the swelling ratio value decreased to half of that in none crosslinking CS/PVA nanofiber membranes, further increasing the concentration of crosslinking agent to 50 wt %, the swelling ratio value was one eighth of the none crosslinking CS/PVA nanofiber membranes. Cytotoxicity evaluation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay indicated that the photocrosslinked CS-based fiber membranes were nontoxic to L929 cells. Results from cell culture SEM imaging showed that cells which exhibited the spindle shape could grow properly on the surface of nanofibrous structure of the CS/PVA. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 3337–3343, 2008

Key words: electrospinning fibers; chitosan; crosslinking; water resistance; biocompatibility

INTRODUCTION

Electrospinning provide a simple and versatile method to generate ultrathin fibers from various materials that include polymers, composites, and ceramics.[1] The membranes made of nanofibrous polymer and prepared by electrospinning have drawn great interest in multiple biomedical applications because their properties are unique when compared with conventional methods of fiber production, such as larger specific surface areas and smaller pores. It has been suggested that very high surface area-to-volume ratio and high porosity can be achieved for better cell incorporation and perfusion.[2]

Chitosan (CS) is a N-deacetylated derivative of chitin, which is the second most abundant natural polysaccharide in the world. It is widely studied because of its biological properties, such as biodegradability, bioactivity, and antibacterial properties.[3–5] The preparation of electrospun CS-containing nanofibers can be achieved from pure CS[6] or blend of CS with other polymers, such as poly(ethylene oxide) (PEO),[7] silk fibroin,[8] poly(vinyl pyrrolidone) (PVP),[9]and poly(vinyl alcohol) (PVA).[6] Until now, there are a lot of reports on the electrospinning of CS due to its potential use in wound dressing[9] and various tissue-engineering applications[10,11]; however, water solubility of CS-based fibers limited a variety of promising applications. Generally, a solute to the nonwaterproof is taken such that the CS-based electrospun membrane was crosslinked by glutaraldehyde vapor to maintain its mechanical properties and fiber-shape in wet stage. However, the glutaraldehyde crosslinking resulted in brittleness and a color change of the electrospun membranes.[12] Ignatova et al.[9,13] used triethylene glycol diacrylate (TEGDA)/2,2-dimethoxy-2-phenylacetophenone (DMPA) as photocrosslinking agents for the nanofibers containing quaternized chitosan (QCh) to prepare QCh/PVP or QCh/PVA electrospun fibers with stability to water.

In previous work,[14] our group prepared chemically crosslinked CS-based nanofibers by using triethylene glycol dimethacrylate (TEGDMA) as crosslinking agent with heat mediated. The crosslinking procedure had to be undertaken under 80°C, however, for a variety of applications it would be highly desirable to gain control on water resistance of CS-based fibers by photoinduced crosslinking without the requirement

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of heat treatment. In the present work, the preparation of electrospun CS-containing fibers were achieved from mixed CS/PVA solutions, because it was difficult to obtain continuous fibers from acetic acid solution of pure CS, and PVA used as the nonionogenic partner facilitating the electrospinning was good for fiber forming and had biocompatibility. Then the CS/PVA electrospun membranes were crosslinked by photoinduced polymerization at room temperature using poly(ethyleneglycol)-600-dimethacrylate (PEGDMA) and 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (HEPK) as photocrosslinking agent and photoinitiator. Water-resistance of photocrosslinked CS-based nanofibrous membranes was compared with the noncrosslinked fiber membranes. Furthermore cytotoxicity and cell adhesion on electrospun membranes were investigated.

EXPERIMENTAL

Materials

CS (molecular weight = 2×10^5, degree of deacetylation = 82.5%) was purchased from Zhejiang Golden-Shell Biochemical, China. PVA (degree of polymerization = 3500, 88% hydrolyzed) was kindly supplied from Kuraray, Japan. Acetic acid was from Beijing chemical reagents (China). PEGDMA was donated by Sartomer. HEPK was donated by Ciba specialty chemicals (Basel, Switzerland). The chemicals were of analytical grade and used without further purification. Mouse fibroblasts (L929) were obtained from Department of Microbiology, Peking University Health Science Center (China).

Preparation of the spinning solutions and electrospinning process

A 10 wt % PVA solution was prepared by dissolution of 10 g PVA in 90 mL distilled water at 80°C with vigorous stirring for a period about 4 h. CS (7.0 g) was dissolved at a concentration of 7 wt % in 93 g aqueous acetic acid solution(0.0157 mol/L). The PVA solution was mixed with the CS solution at weight ratio of CS/PVA 90/10. Crosslinking solution was prepared by mixing 95 wt % PEGDMA and 5 wt % HEPK. Electronspinning solution was prepared by mixing above crosslinking solution and CS/PVA polymer solution. The contents of PEGDMA/HEPK were 0, 20, 35, 50 wt % (all in weight percentage to total polymer content of CS/PVA solution).

The above mixed solution was placed into a plastic syringe (5 mL) with a metal hypodermic needle with flat-filed tip with inner diameter of 0.87 mm. The positive electrode of a high voltage power supply (BMEI, China) was connected to the metal capillary by copper wires. A grounded aluminum foil was used as the collector. Nanofibers were electrospun at 25 kV with a tip-to-collector distance of 10 cm at room temperature in dark. Then the electrospun membranes were irradiated by a 2000 kw medium pressure mercury lamp with light intensity of 50 mW/cm^2 (Lantian UV system, Zhuozhou, China) for 5 min. The crosslinked membranes were dried at 45°C in vacuum for 12 h.

Characterization of the electrospun nanofiber membranes

Fiber morphology observation

The morphology and diameter of the electrospun fibers were observed by using a scanning electron microscopy (SEM) (S-450, Hitachi, Japan) at an accelerating voltage of 20 kV. A small piece of the fiber membranes was placed on the sample holder and sputtercoated with gold. The diameters of nanofibers were measured by using image analyzer. Thirty fibers were statistic in image.

Fourier transform infrared spectra

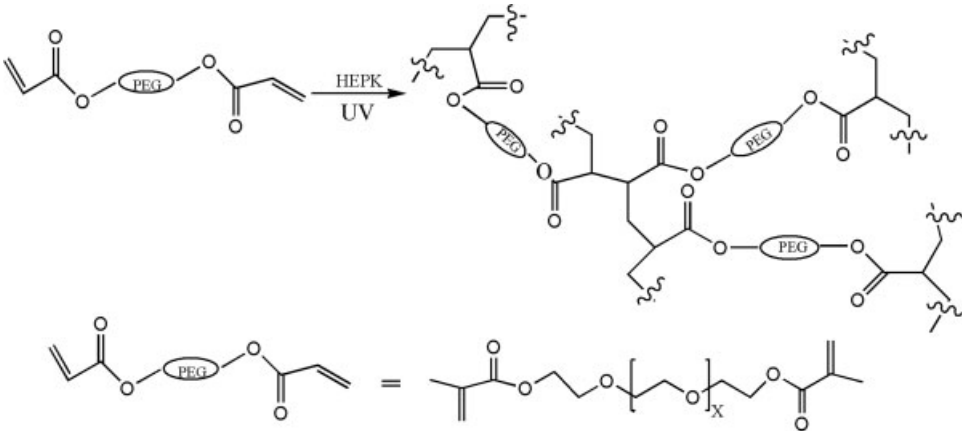
The photocrosslinking process of CS-based nanofibers was analyzed by Fourier transform infrared spectra (FTIR) (Nicolet 5700 instrument, Thermo Electron Corp, Madison, WI). The FTIR spectras of nanofiber membranes before and after UV irradiation were recorded.

Thermal analysis

Thermal analysis was conducted on a differential scanning calorimeter (DSC) (Q100, TA instruments) under a nitrogen atmosphere. About 4 mg of sample was sealed in an aluminum pan for the measurement. First, to remove thermal history, the samples were heated to 250°C, held at this temperature for 3 min, and then quenched into liquid nitrogen. Secondly, the samples were reheated from −100 to 250°C at a heating rate of 10°C/min. Glass transition temperature (T_g) was determined from the second heating run.

Water resistance of photocrosslinked fiber membranes

Stability in water of the crosslinked CS/PVA fibers was tested by immersing fiber membranes in distilled water for 1 h, followed by observation of fiber morphology by SEM. The swelling specimens for measurement were immersed into water at room temperature. After 24 h of incubation, gel samples were carefully taken out from water, blotted with a filter paper to remove free water from the surface, and then weighed. The swelling ratio (SR, g/g) was evaluated by using the following equation:



Scheme 1 Reaction scheme of the crosslinking.

SR = $(m - m_0)/m_0$, where m_0 and m was the weight of sample before and after swelling in water.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The MTT assay is the method used to quantify the amount of viable cells on the basis of the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from active cells. The level of the reduction of MTT into formazan can reflect the level of cell metabolism.

Mouse fibroblasts (L929) were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, together with 1.0% penicillin-streptomycin, and 1.2% glutamine. Culture was maintained at 37°C in a wet atmosphere containing 5% CO_2. When the cells reached 80% confluence, they were trypsinized with 0.25% trypsin containing 1 m*M* EDTA.

For the MTT assay, the photocrosslinked membranes with 1.5 cm in diameter were sterilized with highly compressed steam for 15 min and placed in wells of 24-well culture plate respectively. The samples were then incubated in 1 mL RPMI1640 medium at 37°C for 24 h. At the end of this period, the membranes were removed, and the so-called extracts were obtained. L929 cells were seeded in wells of 96-well plate at a density of 10^3 cells per well. After incubated for another 24 h, the culture medium was removed and replaced with the as-prepared extraction medium and incubated for another 24 h, and then 100 μL MTT solution was added to each well. After 3 h incubation at 37°C, 200 μL of dimethyl sulfoxide was added to dissolve the formazan crystals. The dissolved solution was swirled homogeneously about 10 min by the shaker. The optical density of the formazan solution was detected by an ELISA reader (Multiscan MK3, Labsystem, Finland) at 570 nm. Results are depicted as mean ± standard error of the mean. Significance between the mean values was calculated using ANOVA one-way analysis (Origin6.0 SRO, Northampton, MA). Probability values $P < 0.05$ were considered as significant ($n = 6$).

Cell culture and adhesion

The photocrosslinked electrospun nanofibrous membranes (20 wt %PEGDMA/HEPK) were then sterilized and extensively washed three times with sterile phosphate buffer saline (PBS) prior to transfer to individual 24-well tissue culture plates. Aliquots (1 mL) of L929 suspension with 1.5×10^4 cell/mL were seeded on the sample membranes. After 48 h of culture, cellular constructs were harvested, rinsed twice with PBS to remove nonadherent cells, and subsequently fixed with 3.0% glutaraldehyde at 4°C for 4 h. After that, the samples were dehydrated through a series of graded ethanol solutions and air-dried overnight. Dry samples were sputtered with gold for observation of cell morphology on the surface of the scaffolds by SEM.

RESULTS AND DISCUSSION

Photocrosslinking of electrospun nanofiber membranes

During UV irradiation, the HEPK produced two free radicals by photocleavage, the free radical could induce photopolymerization of PEGDMA (see Scheme 1). FTIR spectra of CS/PVA with different content of PEGDMA/HEPK fiber membranes before and after UV irradiation were shown in Figure 1. The carbon–carbon double bond of PEGDMA had absorption peaks at around 1640 and 810 cm^{-1}. However, CS exhibited the characteristic bands at 1640 cm^{-1} (amide I), 1540 cm^{-1} (amide II), and 1320 cm^{-1} (amide III).[15] The peak at around 1640 cm^{-1} due to carbon–carbon double bond and the peaks due to amide I and II overlapped, thus the peak at 808cm^{-1} was chosen to indicate the occurrence of photocrosslink-

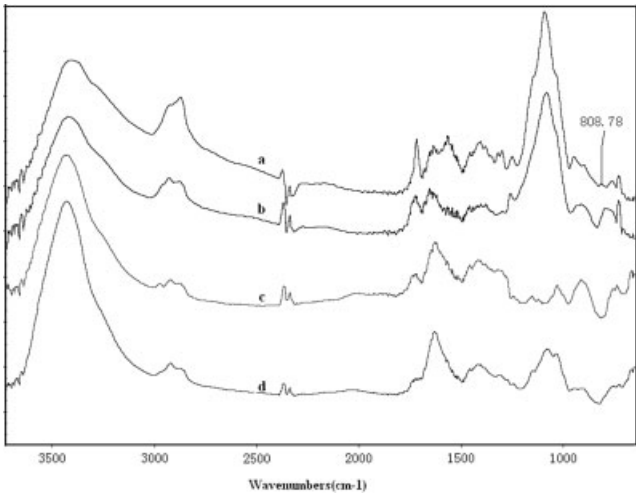


Figure 1 FTIR spectra of electrospun fibers of CS/PVA with different content of PEGDMA/HEPK. (a) 50 wt % PEGDMA/HEPK before UV irradiation; (b) 50 wt % PEGDMA/HEPK after UV irradiation; (c) 35 wt % PEGDMA/HEPK after UV irradiation; (d) 20 wt % PEGDMA/HEPK after UV irradiation.

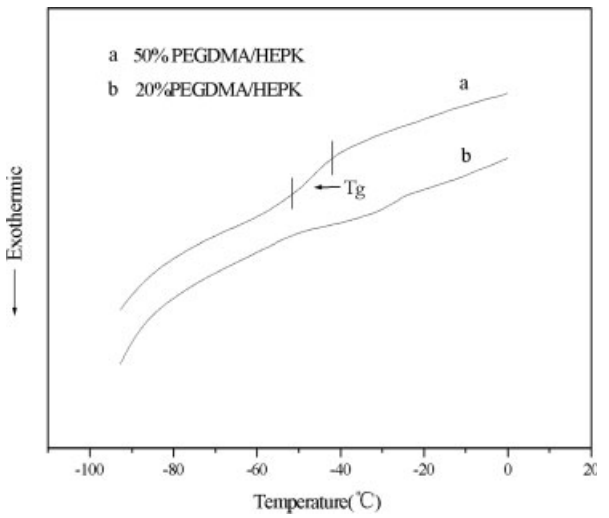


Figure 2 DSC thermogram of photocrosslinked electrospun fibers.

ing. For different concentration of crosslinking agent system, after UV irradiation for 5 min, the carbon–carbon double bond absorption peak at 808 cm^{-1} disappeared, indicating the occurrence of photocrosslinking. The results indicated that even in solid state, the photocrosslinking process could still occur.

Thermal behavior of photocrosslinked electrospun fiber membranes

It was both theoretically and experimentally shown that the T_g was a function of the crosslink-density in crosslinked systems.[16,17] When the crosslink-density was increased, molecular motions of crosslinked polymers were more restricted, and thus more energy was required for the polymer chains to move.[16,17] Figure 2 showed the DSC curves of photocrosslinked electrospun fiber membranes, when the concentration of crosslinking agent was 50 wt %, the T_g at $-46.9°C$ was obtained for the photocrosslinked electrospun fiber membranes, which belonged to the segmental movement of poly(PEGDMA), but when the concentration of crosslinking agent was 20 wt %, it was very difficult to find the T_g from the DSC curve, which indicated that at lower concentration of crosslinking agent, the crosslinking density was low. The T_g of samples with low crosslinking density

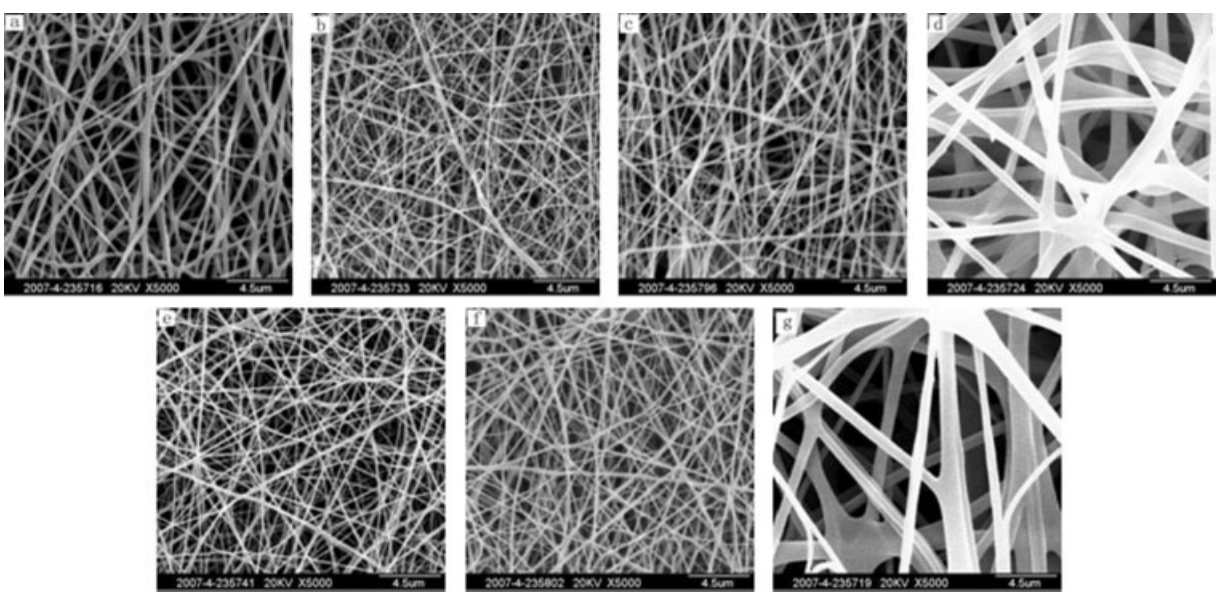


Figure 3 SEM images of electrospun fibers of CS/PVA with different content of PEGDMA/HEPK: (i) after UV irradiation (a) 0 wt %, (b) 20 wt %, (c) 35 wt %, (d) 50 wt %; (ii) before UV irradiation (e) 20 wt %, (f) 35 wt %, (g) 50 wt %.

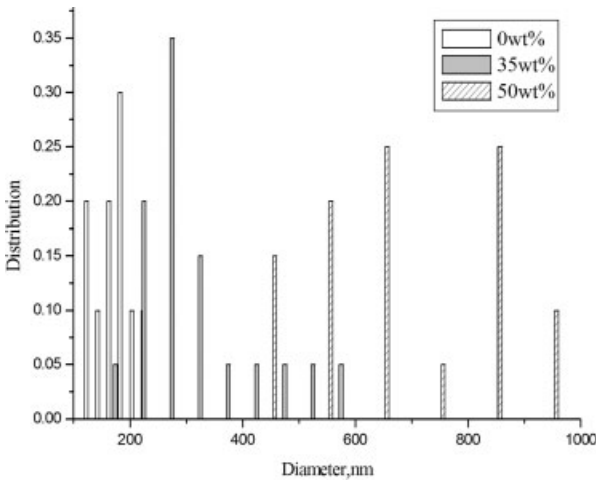


Figure 4 Diameter distributions of electrospun fibers of CS/PVA with different content of PEGDMA/HEPK after UV irradiation.

might be lower than −100°C, thus it was difficult to find the T_g from the DSC curve within measurement range.

Morphology of photocrosslinked electrospun fiber membranes

It was found that adding PEGDMA/HEPK to CS/PVA mixed solution did not affect the electrospinning process and the nanofiber formation. Figure 3 showed the SEM images of the nanofibers noncrosslinked (a) and crosslinked by PEGDMA/HEPK with different contents (b–d). After UV irradiation, the morphology of electrospun fibers did not change in comparison with noncrosslinked fibers [see Fig. 3(e–g)]. The fibers were cylindrical and smooth with average diameters ranging from 200 to 800 nm. Diameter distributions of nanofibrous membranes were presented in Figure 4. The concentration of crosslinking agent had effect on the diameter of nanofibers, high crosslinking agent caused the increase of diameter of nanofibers.

When the nanofibers were immersed in distilled water for 1 h, fiber morphology observed by SEM was shown in Figure 5. Noncrosslinked CS/PVA fibers completely disappeared after immersed in water [see Fig. 5(a)], whereas photocrosslinked fibers

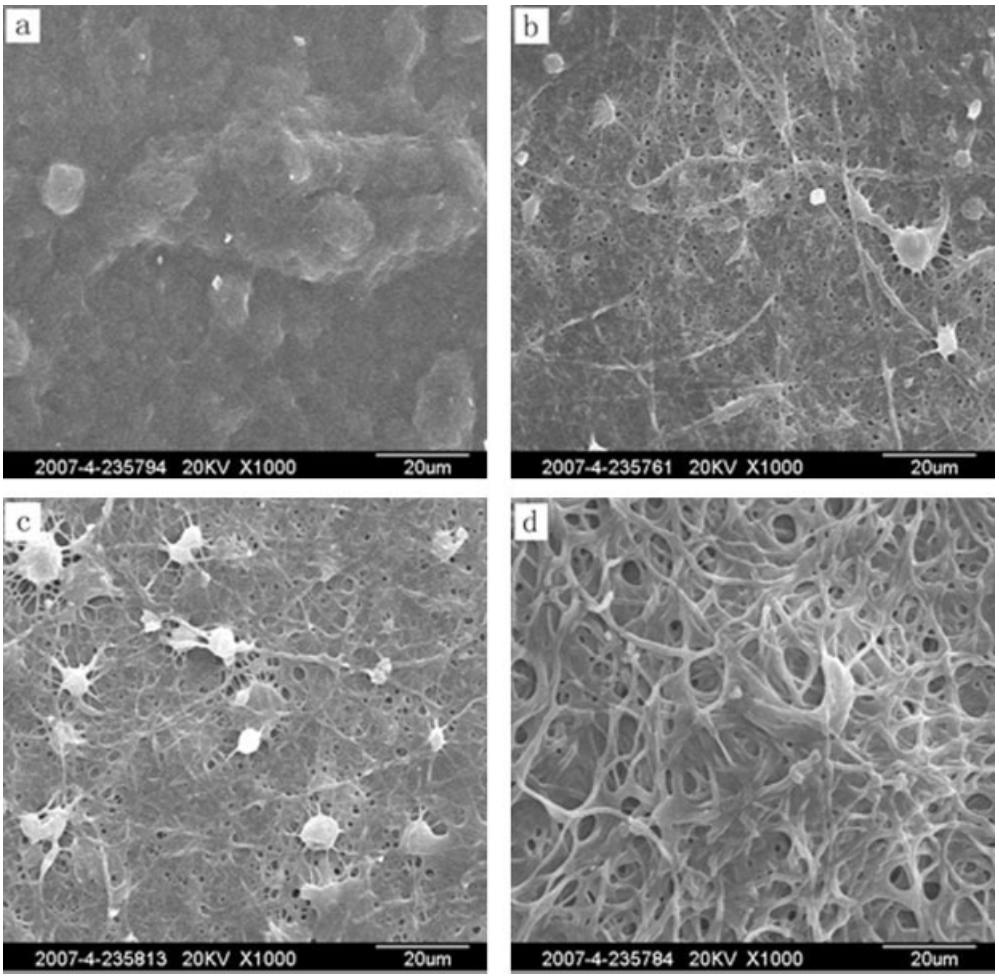


Figure 5 SEM images of nanofibers with different crosslinking agent content after water swelling. (a) 0 wt %; (b) 20 wt %; (c) 35 wt %; (d) 50 wt %.

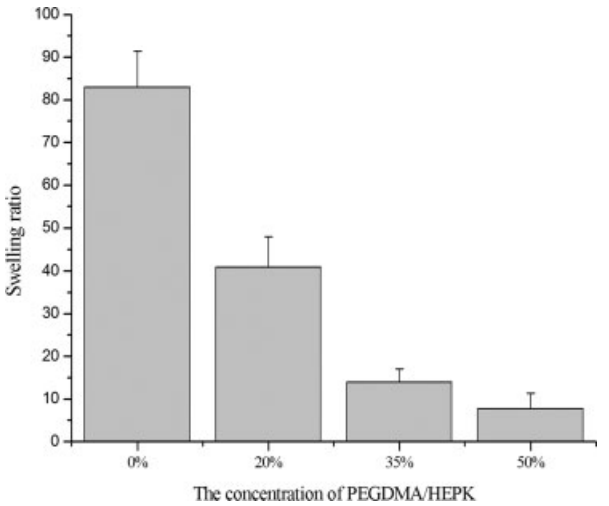


Figure 6 Swelling ratio of crosslinked nanofibrous membranes with different crosslinking agent content.

could endure the water and keep fibrous structure but swelled to certain degree [see Fig. 5(b–d)]. The swelling extent depended on the concentration of crosslinking agent, and increasing crosslinking density could cause a decrease in the swelling degree. The highly porous three-dimensional matrix of the photocrosslinked electrospun membranes might facilitate the migration and proliferation of cells for tissue-engineering scaffolds.[18]

Swelling test of photocrosslinked electrospun fiber membranes

The swelling ratio in water of the photocrosslinked nanofiber membranes is shown in Figure 6. It could be seen that degree of swelling decreased with the increasing of crosslinking agent concentration, which indicated that an increase in the crosslinking density of the CS-based nanofibers made network structure of blend polymers stable in water. After crosslinking, CS/PVA was trapped inside the highly crosslinked network of PEGDMA, which made the water molecule difficult to penetrate into the polymer network, decreased the solubility of PVA in the nanofiber membranes. The higher the concentration of crosslinking agent, the higher the crosslink density of polymer network, the lower the solubility of nanofibers. When the concentration of crosslinking agent was 20 wt %, the swelling ratio value decreased to half of the noncrosslinking CS/PVA nanofiber membranes, further increasing the concentration of crosslinking agent to 50 wt %, the swelling ratio value was one eighth of the none crosslinking CS/PVA nanofiber membranes.

Cytotoxicity evaluation and cell adhesion

Cytotoxicity is a basic testing for a biomaterial. Figure 7(a) showed the viability of the cells obtained from MTT assay after the cells had been cultured with extraction media from photocrosslinked CS/PVA fibers specimens. It could be seen that, although no statistically significant differences were observed in the cell activity of L929 culture for 24 h in the presence of different photocrosslinked CS/PVA fiber membranes extracts in comparison with control, the average absorbance values were lower than that of the control condition. Figure 7(b) showed appreciable adhesion of L929 cell on the nanofiber matrix after 48 h of seeding, indicating the cytocompatibility of the nanofiber matrices. The SEM

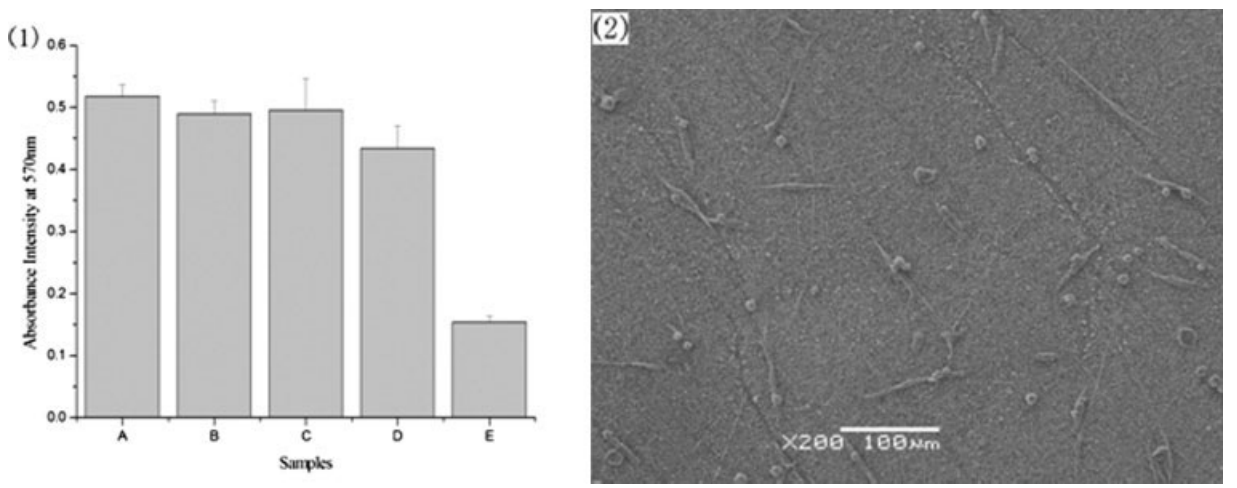


Figure 7 (a) Formazan absorption (A570 nm) in MTT assays L929 cells seeded on the samples for 24 h. A: Negative control; B: photocrosslinked CS/PVA fibers (20 wt % PEGDMA/HEPK); C: photocrosslinked CS/PVA fibers (35 wt % PEGDMA/HEPK); D: photocrosslinked CS/PVA fibers (50 wt % PEGDMA/HEPK); E: Positive ($P < 0.05$). The data represented mean and the standard error of the mean of six samples. (b) SEM image of L929 cells seeded on photocrosslinked electrospun nanofibrous membranes (20 wt %PEGDMA/HEPK) after 48 h culture.

images showed that L929 exhibited the spindle shape, typical of active fibroblastic cells, on all of the surfaces investigated. Results obtained from Figure 7 confirmed that the photocrosslinked CS/PVA fiber membranes were nontoxic to mouse fibroblasts (L929) and could provide a three-dimensional highly porous structure for cell attachment, migration, and proliferation in wet stage, which could be used as tissue engineering scaffolds.

CONCLUSIONS

The CS/PVA/PEGDMA/HEPK solution could be electrospun to form an ultrafine fiber with various diameters depending on the concentration of compositions. These fibers could be photocrosslinked even at solid state. The higher the concentration of crosslinking agent, the higher the crosslink density of polymer network, the lower the swelling ratio of nanofiber in water. Cytotoxicity evaluation and SEM imaging of cell-culture showed that the photocrosslinked CS/PVA fiber membranes were nontoxic to mouse fibroblasts (L929), and the cells on the surface of the nanofibrous scaffold appeared in their characteristic spindle shape. These results implied that the photocrosslinked electrospun fiber membranes could provide a highly porous structure for cell attachment and proliferation in wet stage.

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