Organic-Soluble Chitosan/Polyhydroxybutyrate Ultrafine Fibers as Skin Regeneration Prepared by Electrospinning

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ABSTRACT: In the present contribution, the ultrafine fiber membranes of polyhydroxybutyrate (PHB) and organic-soluble chitosan(O-CS) was prepared by electrospinning. The structure and thermal stability were studied by infrared (FTIR) and thermogravimetric analysis (TG). The surface properties of ultrafine fibers were estimated by contact angle measurements using water. The morphology was observed by scanning electron microscopy (SEM). The cytotoxicity assessment with mouse fibroblast cells (L929) was also investigated. Cell culture results showed that it benefits promoting the cell attachment and proliferation. The results showed it could be as tissue engineering for skin regeneration. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 3619–3624, 2010

Key words: biomaterials; thermogravimetric analysis (TGA); biological applications of polymers

INTRODUCTION

Chitosan is a partially N-deacetylated derivative of chitin, which is the second most abundant polysaccharide next to cellulose. Chitosan has been studied and used in a wide variety of biomedical application, such as drug delivery,[1,2] bone-healing materials,[3] and especially wound dressings,[4] mainly due to its biocompatible, biodegradable, antimicrobial, and nontoxic properties.[5-8] But its utilization is limited by its poor solubility in water and most organic solutions due to intermolecule or extramolecule hydrogen bond of chitosan. To improve the organic solubility of chitosan, and make it easy to process and suitable for use in the dry state, many efforts have done on the modification and process of chitosan.[9-11]

Poly-3-hydroxybutyrate (PHB) is a hydrophobic and thermoplastic polymer displaying physical and chemical properties similar to those of polypropylene. PHB can be biodegrade completely without any toxic by-products, and it is used as biomaterials. But, there are little research about electrospinning of PHB, which were used as bone scaffolds and skin regeneration.[12-14] Electrospinning is a recently explored simple, versatile, and effective fabrication technique for producing nano to microscale fibers with their own unique properties, such as average fiber diameters in the submicrometer range, high porosities, large surface areas, and high surface area to volume–mass ratio.[15,16] These outstanding properties make electrospun fibers attractive for a wide range of applications, including tissue engineering, wound dressing, military protective clothing, filter media, as well as nanosensor and electronics applications.[17-19]

Electrospinning of chitosan is limited by its poor solubility in water and most organic solvents. There have been several attempts to prepare a nonwoven fabric of pure chitosan system in the acids solvents by the electrospinning technique.[20-24] Electrospinning of two or several biopolymers has increasingly become an important technique to develop new biomaterials exhibiting combinations of properties that could not be obtained by individual polymers. There are much research on the blend nanofibers with chitosan and another polymer to improve the electrospinnability, such as Polyvinylalcohol (PVA), Polyethyleneoxide (PEO), Polyacrylonitrile (PAN), and Silk Fibroin (SF) in the organic or acids solution.[25-28] Recently, much attention has been paid to electrospinning of homogeneous chitosan and its derivative.[9,29-32] But, all of these chitosan based electrospun

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fibers are not free from the problems caused by acidic toxicity residue when it is applied as wound dressing or tissue engineering.

In this study, modification of organic-soluble chitosan (O-CS) and the O-CS/PHB blend electrospun fibers were prepared. Chemical and physical structure, thermal properties were examined by Fourier transform infrared (FTIR), thermogravimetric analysis (TG and DTG), respectively. The water contact angle of PHB/O-CS blend fibers was carried out as well. The morphological characteristics of the blend fibers were also investigated by Scanning Electron Microscope (SEM). The cytotoxicity test of the electrospun fiber membranes with mouse fibroblast cells (L929) was evaluated. The cell attachment and cell proliferation on the fiber membranes were also investigated in detail.

EXPERIMENTAL

Materials

Polyhydroxybutyrate (PHB; M_w = 300,000 g/mol^{-1}) were supplied by Sigma–Aldrich (Thailand, USA). The organic-soluble chitosan (O-CS) was prepared according to our previous report.[33] Chloroform and Triethylamine, were used as received from Beijing Chemical Reagent Company (Beijing, China).

Preparation of phb/o-cs composite ultrafine fibers by electrospinning

PHB was dissolved at a concentration of 4 wt % in $CHCl_3$ solvent, O-CS was dissolved in $CHCl_3$ to make a 6 wt % solution, and then mixed in the different weight ratio of mixed solution. The above mixed solution was placed into a plastic syringe (5 mL) with a metal capillary having an inner diameter of 0.57 mm. Typical electrospinning parameters were as follows: the applied electric voltage was 20 kV, the distance between the syringe tip and the grounded drum was 15 cm, and the solution feed rate was 20 mL/min.

FTIR spectroscopy

The Fourier transform infrared (FTIR) spectrum of the blend films containing KBr pellets were recorded by Nicolet 5700 instrument (Nicolet Instrument, Thermo Company, USA) with the wavenumber range 600–4000 cm^{-1}.

Water contact angle

The contact angles of PHB/O-CS membrane against water were measured on the Dataphysics OCA 20 instrument at room temperature. Firstly, a piece of film was laid on the sample holder of the instrument, then, uniform drops of liquids were deposited on the blend film surface by a micrometer syringe, and lastly, the instrument was adjusted until the contact angle could be read clearly. Each film was measured 10 times to calculate the average value of contact angle.

Thermogravimetric

TGA of the composite membrane was performed with a Netzsch TG 209 analyzer (Germany). All samples were examined at a heating rate of 10°C/min under a nitrogen stream in the temperature range of 25–500°C.

Scanning electron microscope

SEM was utilized to characterize the surface morphologies. The SEM images of the films were taken by using a Hitachi S-4700 scanning electron microscope (Hitachi company, Japan). Samples were pretreated by gold sputtering. According to the SEM images, the diameter and distributions of ultrafine fibers were measured by Image J analysis software.

Methylthiazolydiphenyl-tetrazolium bromide assay

The cytotoxicity of the electrospun fiber membranes was evaluated based on a procedure adapted from the ISO10993-5 standard test method. Mouse fibroblast-like cell line (L929) was 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* ethylenediamine tetraacetic acid (EDTA). The viabilities of cells were determined by the Methylthiazolydiphenyl-tetrazolium (MTT) (3-[4,-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; thiazolyl blue)assay. The level of the reduction of MTT into formazan can reflect the level of cell metabolism. For the MTT assay, the as-spun membranes were sterilized with highly compressed steam for 15 min and placed in wells of a 24-well culture plate, respectively. The samples were then incubated in 1 mL of RPMI1640 medium at 37°C for 24 h. The extraction ratio was 6 cm^2/mL. At the end of this period, the membranes were removed and the so-called extracts were obtained, and further were diluted to obtain extraction medium samples. L929 cells were seeded in wells of a 96-well plate at a density of 4×10^3 cells per well. After incubation for another 24 h, the culture medium was removed and replaced with the as-prepared extraction medium and incubated for another 24 h, then 150 μL of MTT solution was added to each well. After 24 h incubation at 37°C, 200 μL of

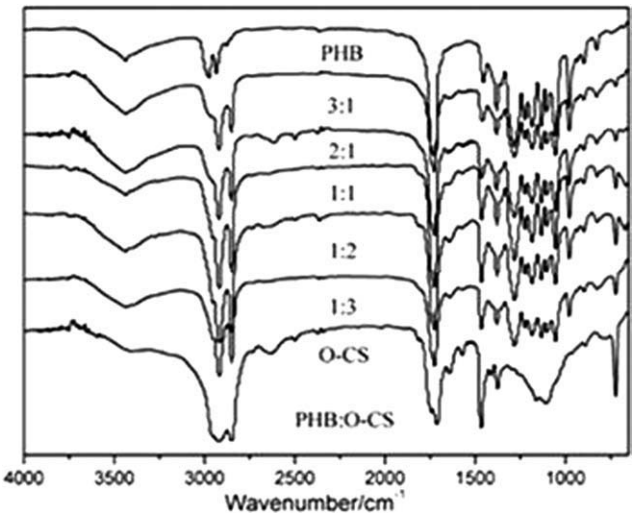

Figure 1 FTIR of electrospun composite membrane of different mass ratio of PHB and O-CS.

dimethyl sulfoxide was added to dissolve the formazan crystals. The dissolved solution was swelled homogeneously for about 10 min by the shaker. The optical density of the formazan solution was detected by an ELISA reader (Multiscan MK3, Labsystem Co. Finland) at 570 nm. For reference purposes, cells were seeded to medium a fresh culture medium (negative control) under the same seeding conditions, respectively.

Cell culture and adhesion

Mouse fibroblast cells (L929) was selected for all the biological assays to evaluate the effect of electrospun fiber membranes on cell culture, adhesion, and proliferation. The electrospun fiber membranes were fixed on the glass cover slips using copper tapes. The sample membranes were sterilized, rinsed three times with sterile phosphate buffer solution (PBS), then transfered to individual 24-well tissue culture plates. Aliquots (1 mL) of mouse fibroblast cells (L929) suspension with 1.5×10^4 cells/mL were seeded on the sample membranes. After 24 h of culture, cellular constructs were harvested, rinsed twice with PBS to remove nonadherent cells. The samples were dehydrated through a series of graded ethanol solutions and dried overnight at room temperature. The dry samples were coated with gold by sputtering for further analysis cell morphology on the surface of the scaffolds by SEM.

RESULTS AND DISCUSSION

FTIR analysis

Figure 1 shows the FTIR spectra of PHB/O-CS composite electrospun membranes with different mass ratios. There was a weak absorption broad band at 3400 cm^{-1} attributed to —OH stretching vibration of hydrogen bonding of PHB and O-CS. The characteristic absorption band at 2900 cm^{-1} from CH— stretch of PHB and reside of O-CS. The strong band at 1746 and 1726 cm^{-1} due to the asymmetrical and symmetrical stretching of NCO— and COO— group of O-CS, and COO— group of PHB. The FTIR spectrum of composite electrospun membrane contained the characteristics of both PHB and O-CS. The intensity of the characteristic peaks of O-CS functional groups was significantly changed when the amount of O-CS in the blend film was increased.

Themogravimetric analysis

Figure 2 shows the TG thermogram of the composite electrospun fiber membranes with different mass ratio of PHB and O-CS. TG analysis of the blend fibers showed the presence of both polymer fractions in the blend fiber membranes. For the blend films, the discrete weight losses occurred at approximately 100 and 500°C corresponding to loss of water, degradation of PHB chains, and degradation of ring of O-CS. The observed degradation peak value for all of the films was calculated according to TG curves. The composite electrospun membranes of PHB and O-CS showed two degradation peaks at about 270 and 320°C, respectively. The first strong degradation peak at 270°C was due to the degradation of PHB chains and the partial resides of O-CS, and the weak curves at 320°C attributed to the disintegration of intermolecular interaction and the breakage of the molecular ring structure. Obviously, the thermal stability of the blend films increased with the increase of O-CS content, which indicated that the thermal stability of O-CS was higher than that of PHB.

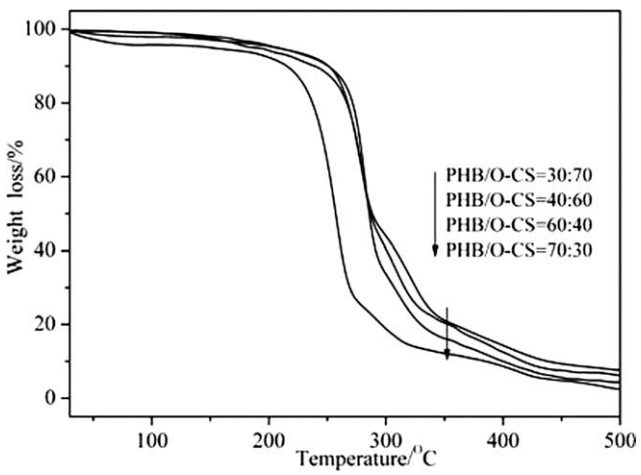

Figure 2 TG thermograms of electrospun composite membrane of different mass ratio of PHB and O-CS.

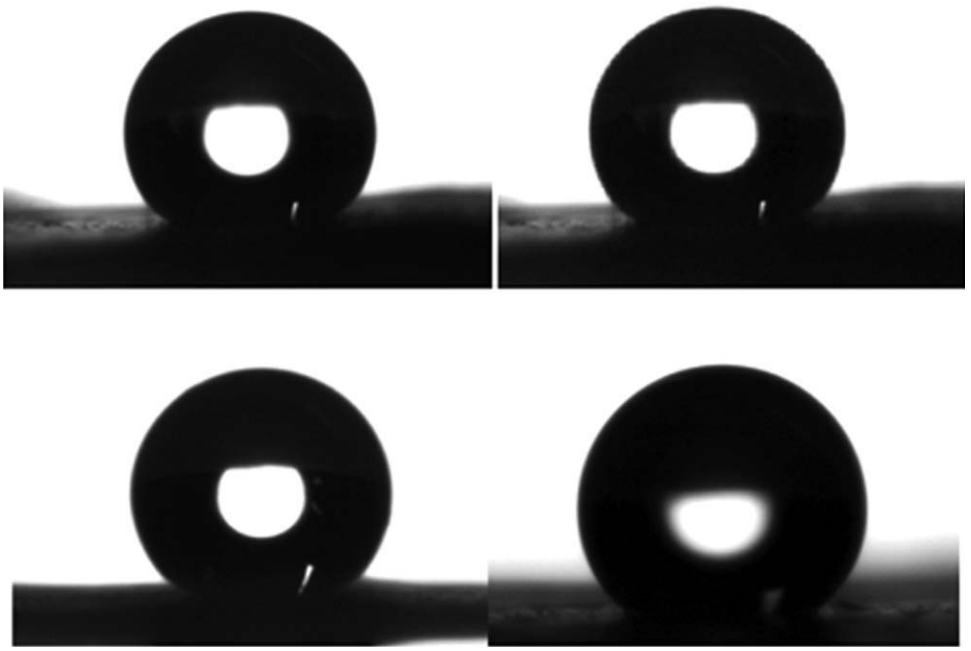

Figure 3 Water contact angle of different mass ratio of PHB and O-CS electrospun composite membrane.

Water contact angle of the membrane

Figure 3 shows the water contact angle of the PHB/O-CS fibrous composite membrane electrospun from the composite solution. PHB is chemically hydrophobic and its water contact angle was higher than that of other hydrophilic polymers. The water contact angle of the electrospun PHB fiber membranes was 140°.[14] In Figure 4, the water contact angle values of the composite electrospun membrane was higher than 140°, and it increased from 140 to 150° with the increase content of O-CS. This may be

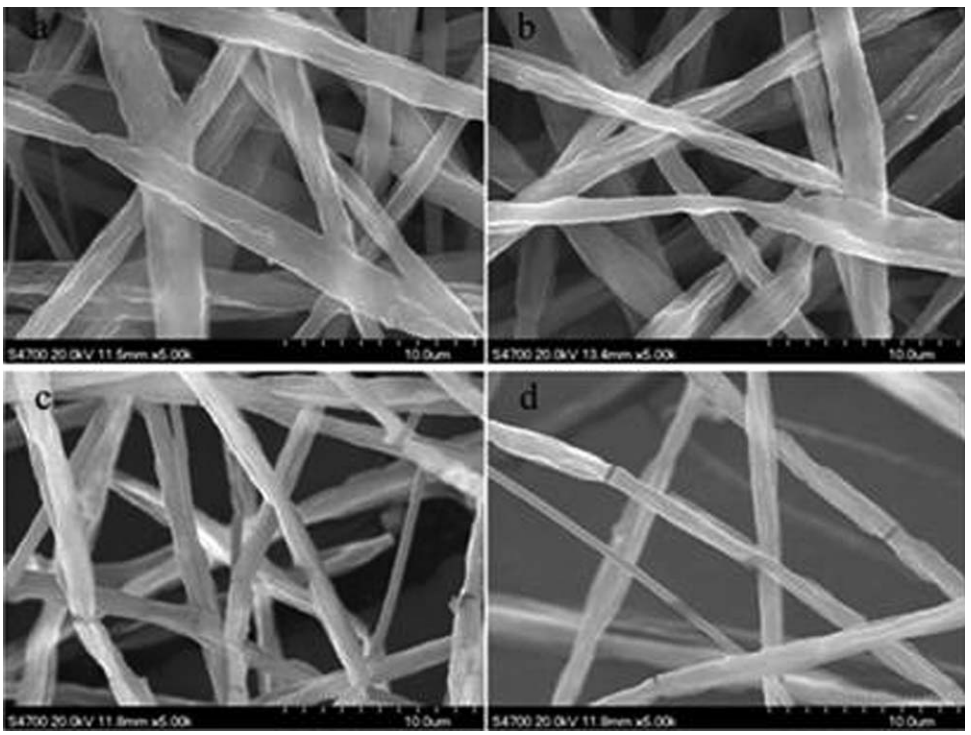

Figure 4 Magnified SEM images of electrospun PHB/O-CS fibers membranes at different electrospinning voltage.

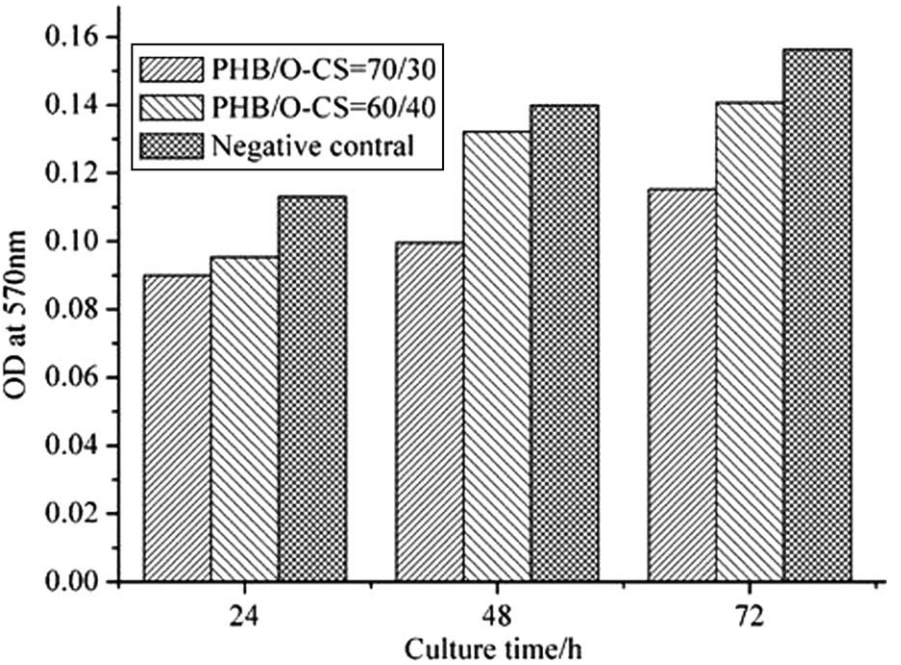

Figure 5 Cytotoxicity test of PHB/O-CS fiber membrane with negative controls ($p < 0.05$) *$p < 0.05$ when compared to the negative control of cytotoxicity. The data represented mean and standard deviations of six samples.

indicated that the water contact angle of the surface increased with increase of the surface roughness when the surface was composed of hydrophobic materials.[34] Hydrophobic surface, roughness will prevent water from contacting the solid surface completely by trapping air bubbles at the water–solid interface.[35] The result of the study is according to the recent report that superhydrophobic surfaces were made by controlling the surface chemistry energy or surface roughness of various materials.[36]

Scanning electron microscope

Mass ratio is important factor on the formation of fibers. O-CS could not be electrospun, but incorporation of PHB significantly improved the fiber-forming ability or electrospinnability of O-CS solutions. Bead-on-string structures with various sizes and shapes were obtained when the mass ratio of PHB/O-CS was 30/70 or lower than it. The number of bead defects was reduced and fibers could be formed when the mass ratio of PHB/O-CS increased owing to the viscosity of the composite solution increased. When the PHB/O-CS mass ratio reached up to 50/50, the bead-on-string morphology disappeared and rough, homogeneous fibers were obtained. The average diameter of the blend ultrafine fibers gradually decreased and the diameter distribution of fibers became slightly narrower, when continued to increase the mass ratio of to 70/30. It also could get the porous fibers at different electrospun voltage (Fig. 4). Increase the fraction of PHB in the composite polymer solution during the electrospining, which not only leaded to reduce the number of bead defects but also could get the porous and rough fibers.

MTT assay

An ideal wound dressing should not release toxic products or produce adverse reactions, which could be evaluated through cytotoxic tests. In the evaluation, mouse fibroblast cells (L929) was used as reference. Figure 5 shows the absorbance illustrating the viability of L929 with extraction medium that were cultured 24, 48, and 72 h. It could be seen that no statistically significant differences ($p < 0.05$) were observed in the cell activity of L929 culture for 72 in the presence of PHB/O-CS electrospun fiber membranes in comparison with negative control, although the average absorbance values were lower than that of the control condition. A similar result was observed in the PHB/O-CS (60/40) electrospun fiber membrane and it was better than PHB/O-CS (70/30). Especially the PHB/O-CS electrospun fiber membranes with ratio of 60/40 showed the highest absorbance value after culture for 72 h. All of the obtained results clearly suggested that the extraction media from the electrospun fiber membranes were unharmful to the cells and these materials could be use as wound dressing for further investigation.[37]

Cell adhesion and morphology

The composite electrospun fiber membranes (PHB/O-CS = 60/40) were used for cell adhesion,

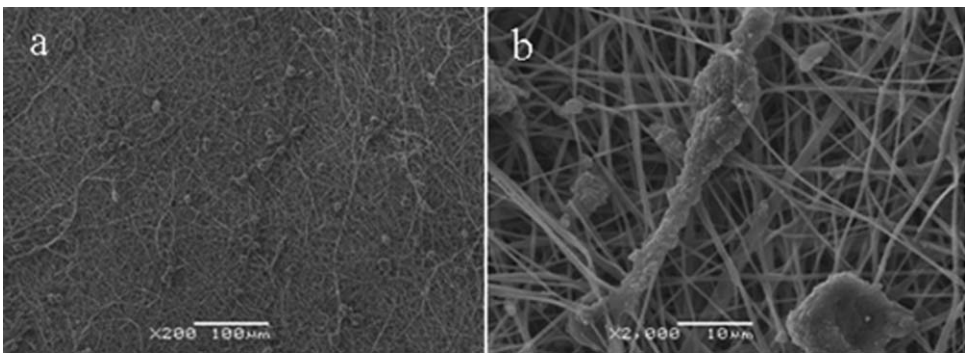

Figure 6 SEM images of L929 cell seeded on fiber membrane of PHB/O-CS (60/40) after 48 h culture.

spreading, and proliferation study. Figure 6 shows SEM images of L929 grown on the electrospun fiber membranes after 48 h cell culture. L929 cells appeared to attach and proliferate on the surface of fiber membrane in accordence to the result of MTT. The cells attached to the surfaces by discrete filopodia and exhibited short and numerous microvilli on the surfaces of the fiber membrane. It might be due to the porous and rough surface structure of the electrospun fibers, which benefit to the cell attachment and proliferation.[32]

CONCLUSION

The thermal stability of O-CS was higher than that of PHB, and the result of water contact angle of the composite electrospun membrane showed that it was hydrophobic. The cytotoxicity assessment of the electrospun fiber membranes with mouse fibroblast cells (L929) indicated that the materials were nontoxic and did not release substances harmful to living cells. Cell culture results showed that fiber membranes were good to attach, grow, and proliferate. The obtained results showed the potential use of the electrospun fiber membranes as tissue engineering for skin regeneration.

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