Preparation and Characterization of a Bacterial Cellulose/Chitosan Composite for Potential Biomedical Application

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ABSTRACT: Bacterial cellulose (BC)/chitosan (Ch) composite has been successfully prepared by immersing wet BC pellicle in chitosan solution followed by freeze-drying process. The morphology of BC/Ch composite was examined by scanning electron microscope (SEM) and compared with pristine BC. SEM images show that chitosan molecules can penetrate into BC forming three-dimensional multilayer structure scaffold. The scaffold has very well interconnected porous network structure and large aspect surface. The composite was also characterized by Fourier transform infrared spectrum (FTIR), X-ray diffraction (XRD), thermogravimetric analysis (TGA), and tensile test. By incorporation of chitosan into BC, crystallinity tends to decrease from 82% to 75%. According to TGA result, the thermal stability of BC in the composite has been improved by incorporating chitosan. At the same time, the BC/Ch composite displays 530% of improvement in tensile strength, 240% of decrease in elongation at break, and 4000% of increase in Young's modulus compared with pure chitosan. The biocompatibility of composite was preliminarily evaluated by cell adhesion studies. The tests were carried out using 3T3 fibroblast cells. The cells incubated with BC/Ch scaffolds for 48 h were capable of forming cell adhesion and proliferation. It showed much better biocompatibility than pure BC. So, the prepared BC/Ch scaffolds are bioactive and may be suitable for cell adhesion/attachment suggesting that these scaffolds can be used for wound dressing or tissue-engineering scaffolds. J Appl Polym Sci 121: 1488–1494, 2011

Key words: bacterial cellulose; chitosan; mechanical properties; cell adhesion

INTRODUCTION

Bacterial cellulose (BC), which is synthesized by *Acetobacter xylinum*, consists in the biogenous nanofiber network structure formed by self-assembling in an efficient way. BC possesses higher water holding capacity, higher crystallinity, higher tensile strength, and a fine web-like network. Compared with other natural biodegradable polymers such as collagen, chitin, and gelatin, BC presents much higher mechanical properties, which are required in most cases when used as scaffold in tissue engineering.

Several applications for BC in medical fields have already been reported such as artificial skin for humans with extensive burns,[1] artificial blood vessels for microsurgery,[2] scaffolds for tissue engineering of cartilage,[3] and wound-dressing.[4] BC shows high hydrophilicity, good sorption of liquids, nonallergenic, and can be safely sterilized without any change to its characteristics. However, BC has problems in some cases when it is used in its natural state. For instance, a study by Schonfelder et al. indicates that BC has no influence on the concentration of elastase *in vitro* and little antioxidant capacity against ROS.[5] BC itself has no antimicrobial activity to prevent wound infection. To improve its properties, one method is that BC is coated with another polymer. Another strategy is hybridizing BC with another polymer by mechanically mixing BC solution with the solution of another polymer.[6]

Scaffold made by BC can interact with the surroundings at the OH-functionalized outer surface, the surficial area of nanofiber architecture, and with large pore system. Water, synthetic macromolecules, and biopolymers as well as living cells can be accumulated by *in situ* or post modification forming a multitude of composites.[7]

Chitosan, like BC, has been recognized for its applications in various fields including the biomedical area. It has been known for its absorption of exudes, anti-fungal, anti-microbial, anti-viral, and wound-healing properties. Chitosan is useful as a wound management aid to reduce scar tissue.

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Chitosan has been also found to have beneficial biological properties including homeostasis,[8] antimicrobial activity stimulation of healing,[9] tissue-engineering scaffolds,[10] cell culture,[11] and drug delivery.[12]

In the present study, the author aims to prepare a composite of BC and chitosan for potential biomedical application by post modification in an attempt to use the synergic beneficial aspects of both polymers.

MATERIALS AND METHODS

Materials

Chitosan, medium molecular weight, Brookfield viscosity 200,000 cps, was purchased from Aldrich, Switzerland. Other chemicals of the highest purity available were used and were purchased from Sigma-Aldrich, New York, USA.

Preparation of bacterial cellulose pellicles

Gluconacetobacter xylinum BRC-5 was obtained from Yonsei University and used to produce the BC pellicles. The bacterium was cultured on Hestrin and Schramm (HS) medium, which was composed of 2% (w/v) glucose, 0.5% (w/v) yeast extract, 0.5% (w/v) bacto-peptone, 0.27% (w/v) disodium phosphate, and 0.115% (w/v) citric acid. All the cells precultured in a test tube containing a small cellulose pellicle on the surface of the medium were inoculated into a 500-mL Erlenmeyer flask containing 100 mL of the HS medium. The flasks were incubated statically at 30°C for 30 days. The cellulose pellicles were dipped into 0.25*M* NaOH for 48 h at room temperature to eliminate the cells and components of the culture liquid. The pH was then lowered to 7.0 by repeated washing with distilled water. The purified cellulose pellicles were stored in distilled water at 4°C to prevent drying.[13]

Preparation of BC/chitosan composite scaffold

The wet BC pellicle was placed between two sheets of filter paper to remove some of free water. Then, it was immersed in 1% chitosan-dissolved acetic acid solution for 6 h in room temperature. After that, it was withdrawn from the vessel, and the excess chitosan solution was removed using filter paper. Finally, it was dried by freeze-dryer (IP3 Jouan, France) at −40°C for 3 days.

BC/chitosan composite characterization

Scanning electron microscopy (SEM) images of BC and BC/chitosan (BC/Ch) composite were taken with a microscope (Hitachi S-4200, Japan) to study the morphology. The surface and cross-section of the samples were sputtered with gold and then observed and photographed. Fourier transform infrared (FTIR) spectra were obtained using a Perkin-Elmer System 2000 FTIR spectrophotometer. The samples were cut into very little particles for the evaluation of chemical structures using a KBr pellet. The obtained data were transferred to the PC for the line fitting. X-ray diffraction (XRD) pattern were recorded on an X-ray diffractometer (D/MAX-2500, Rigaku), by using Cu Kα radiation at 40 kV and 30 mA. Scattered radiation was detected in the angular range of 5–40° (2θ). Thermogravimetric analysis (TGA) was carried out with a NETZSCH STA 409 PC/PG system. All analyses were performed with a 10 mg sample in aluminum pans under a dynamic nitrogen atmosphere between 30 and 1000°C. The experiments were run at a scanning rate of 20°C/min. Tensile test specimens were prepared by cutting the membranes to 10-mm wide and 65-mm long strips using a precise cutter. Young's modulus of samples were found from the tensile test results conducted according to ASTM D-882-97 as a standard test method for tensile elastic properties of thin plastic sheeting. Tensile test was done on a universal testing machine. Two ends of the specimens were placed between the upper and lower jaws of the instrument, leaving a length of 50 mm of the film in between the two jaws. Extension speed of the instrument was 2 mm/min. The test was performed in ambient condition.

Cell attachment studies

The BC and BC/Ch cylinder scaffolds (20 mm diameter × 4 mm height) were used for the cell attachment studies. Prior to cell culture work, the samples were sterilized using ethylene oxide for 18 h. The samples were then pretreated by immersing in DMEM (Dulbecco's Modified Eagle Medium) for 24 h. After the pretreatment, the samples were carefully placed in 24-well plates, and the cells were seeded at a density of 2.5×10^4 cells/well. And the samples were incubated at 37°C/5% CO_2 for 48 h. The morphology of the cells seeded on the samples was investigated after 48 h of incubation with a scanning electron microscope. For preparing SEM analysis, the samples washed twice with PBS to remove non-adherent cells and the attached cells were fixed with 2.5% glutaraldehyde buffer solution (pH 7.4) at 4°C for 12 h. The samples were subsequently rinsed in distilled water and dehydrated by freeze-dryer at −40°C. The samples were sputter coated with platinum and the cell morphology was examined using SEM.

RESULTS AND DISCUSSION

The extended polyglucosan chains of BC produced by *A. xylinum* are surrounded by layers of water

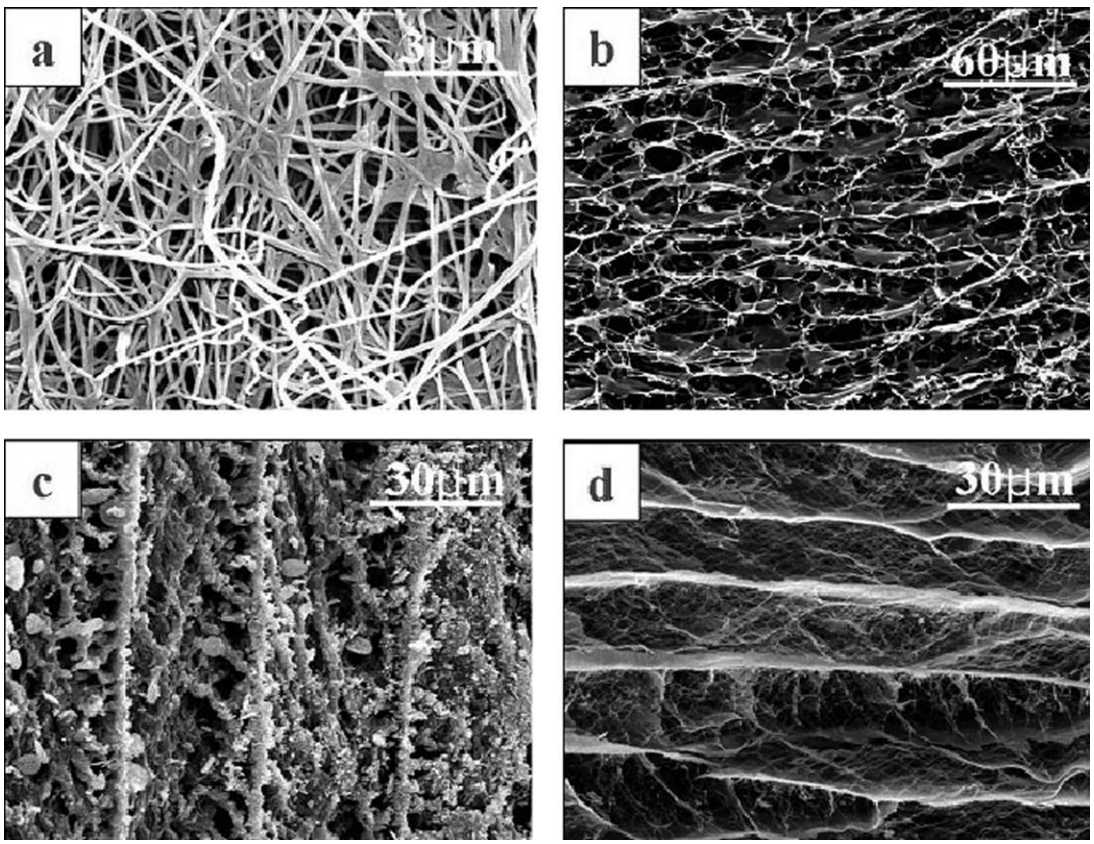

Figure 1 SEM images of BC (A: surface morphology; C: cross-section morphology) and BC/Ch composite (B: surface morphology; D: cross-section morphology).

molecules forming a soft gel. If this gel undergoes dehydration through evaporation of water, it is converted from a "gel" into a "film" by irreversible formation of new hydrogen bonds between cellulose hydroxyl groups.[14] In the present study, the soft gel obtained by *A. xylinum* was treated with chitosan acetic acid solution, instead of being dehydrated. Chitosan is widely employed in pharmaceutical and tissue engineering as a recipient in drug delivery and scaffolds for tissue culture. It is also used in the manufacture of wound dressing because of its antimicrobial activity. Thus, we expect that, by immersing the BC gel in chitosan solution, the multiple layers of water surrounding polyglucosan chains would be displaced, inducing the formation of bonds between cellulose and chitosan, with consequent marked structural modifications.

Figure 1 presents typical SEM images of freeze-dried BC and BC/Ch composite. For pure BC, as seen from Figure 1(A), BC nanofibrils can be observed on the surface. The mean diameter of this nanofibrils is about 40 nm. From cross sectional image [Fig. 1(C)], we can see this multiple layers structure with high aspect ratio. Evidently, a well-organized three-dimensional (3D) network structure is observed. The BC sample has porous morphology, which was subject of discussion in the last decade and is already established. Cellulose biosynthesis is characterized by unidirectional growth and crystallization, where glucose molecules are linear bonded by β(1→4)-glycosidic bond. The union of glycosidic chains forms oriented microfibrils with intramolecular hydrogen bonds.[15] The cellulose is crystallized outward the organisms, particularly in *A. xylinum* that synthesizes cellulose chains by introducing glucose units to the reducing ends of the polymer. The growth mechanism during bacterial activity determines the morphology of the final cellulose.

After treated by chitosan, the surface morphology changed. BC nanofibrils cannot be observed because of coverage by a thick layer of chitosan. Porous structure remains but the pore size becomes much bigger [Fig. 1(B)]. From cross sectional image [Fig. 1(D)], we can see that chitosan molecules can penetrate into BC and forms layers of BC/Ch composite. BC nanofibrils can be observed between layers. Note that the present nanofibrous BC and BC/Ch composite has well interconnected pore network structure and large surface area necessary for cellular attachment and vascularization. In other words, this BC/Ch composite, when used as tissue engineering scaffolds, can promote cellular ingrowths.

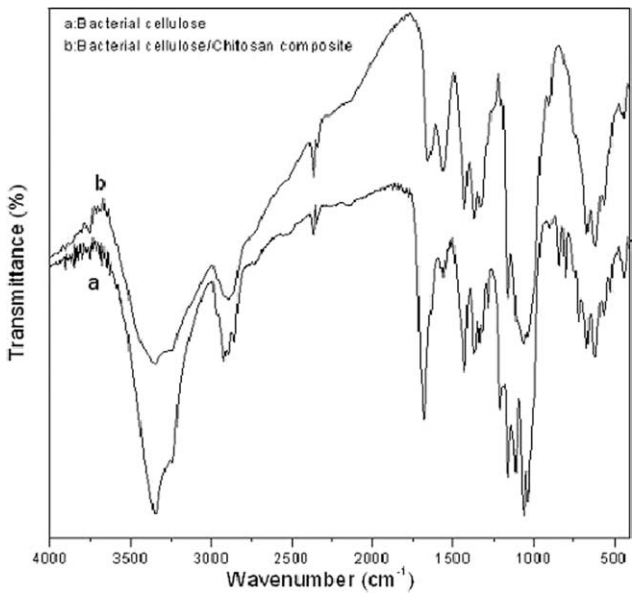

Figure 2 FTIR spectra of BC and BC/Ch composite.

As the molecular structure of BC and chitosan is very similar, it is expected that these two polymers have good compatibility and miscibility.

Figure 2 depicts the FTIR spectra of BC and BC/Ch composite samples. In the case of pure BC, a broad band at 3450 cm^{-1} is attributed to O—H stretching vibration. Band at 2820 cm^{-1} represents the aliphatic C—H stretching vibration. A sharp and steep band observed at 1080 cm^{-1} is because of the presence of C—O—C stretching vibrations. In the case of BC/Ch composite, N—H stretching vibration band is located at 3420 cm^{-1}, which is combined with O—H stretching vibration band. Three new bands observed at 1647 cm^{-1}, 1575 cm^{-1}, and 1375 cm^{-1} are attributed to amide-I, amide-II, and amide-III, respectively, which exist in chitosan molecules.

Regarding the crystalline structure of cellulose fibers, it is known to be classified into four crystallization types, viz. cellulose I, II, III, and IV, and their crystalline structure are able to be transformed form one type to another.[16] Figure 3 shows the X-ray diffraction pattern of the BC and BC/Ch composite samples. Three main peaks can be identified in both spectra, which are assigned to the (1 $\bar{1}$ 0), (1 1 0), and (2 0 0) reflexions planes of cellulose I.[17] For BC, the location of these three peaks is 14.2°, 16.6°, and 22.4°. For BC/Ch composite, characteristic peaks are remained in the same location, but the height of the (1 $\bar{1}$ 0) peak decreased and the ratio of the (1 $\bar{1}$ 0) peak to the (0 2 0) peak was also decreased from 0.50 for BC to 0.46 for BC/Ch composite. This result means that the presence of chitosan affected the preferential orientation of the (1 $\bar{1}$ 0) plane during the water removal from BC pellicle. Moreover, the XRD data were analyzed using specialized software (ANALYZE and DRXWin). The crystallite size was determined using the value of the full-width at half-maximum (FWHM) into the Scherrer eq. (1):

$$t = \frac{0.89\lambda}{\beta \cdot \cos\theta} \quad (1)$$

where λ is the wavelength of the radiation (0.1542 nm), β is the halfwidth at half-maximum (in radians), and θ is the half of the diffraction angle in the plane of analysis. The calculated crystallite size for BC and BC/Ch composite is around 10 nm. As cellulose biosynthesis is characterized by unidirectional growth and crystallization. The union of glycosidic chains forms BC microcrystal—oriented nanofibrils.[15] However, for chitosan incorporated BC, the full-width at half-maximum for all diffraction peaks tends to decrease, which means worse crystallinity compared with pure BC.[18,19]

The crystallinity index (CrI) of the samples was estimated following the method of literature method.[20] Briefly, the curve smoothing is performed using Savitzky-Golay processing to filter white noise. Then, it uses the height of (2 0 0) peak and the minimum between (2 0 0) and (1 1 0) peaks, assuming that intensity of (2 0 0) represents both crystalline and amorphous parts while the minimum intensity at the mentioned location is for amorphous part only.

$$\mathrm{CrI} = \frac{(I_{(200)} - I_{(\mathrm{am})})}{I_{\mathrm{am}}} \times 100\% \quad (2)$$

where CrI is the crystallinity index, $I_{(200)}$ is the intensity at (2 0 0) peak ($2\theta = 22.3°$), and $I_{(\mathrm{am})}$ is the intensity at the minimum between (1 1 0) and (2 0 0)

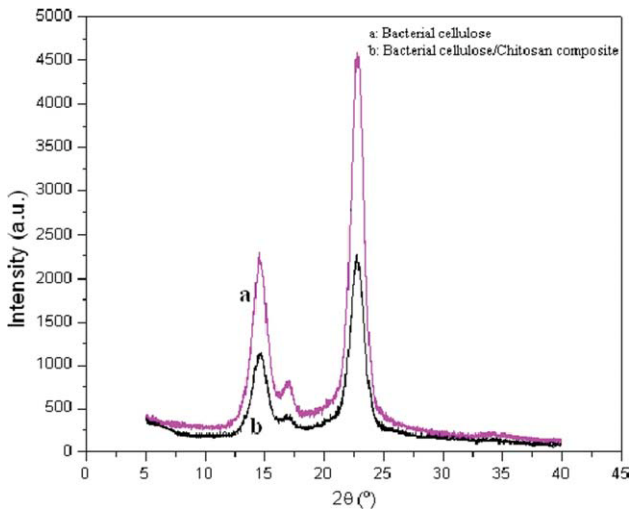

Figure 3 XRD patterns of BC and BC/Ch composite. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I
Diffraction Peaks, FWHM, Crystallite Size, and Crystallinity Index of Pure BC and BC/Ch Composite

	2θ (°)	FWHM (°)	*t* (nm)	CrI (%)
Bacterial cellulose	14.2	0.7897	10.02	87
	16.6	1.1596	6.85	
	22.4	0.8828	9.07	
Bacterial cellulose/ chitosan composite	14.2	0.8672	9.08	75
	16.6	1.7717	4.46	
	22.4	0.8974	8.92	

peak. Pure BC shows the highest degree of crystallinity (87%). The presence of amorphous chitosan in the BC/Ch composite accounts for the reduction of crystallinity values to 75%. Although it was an estimative of crystallinity based on the X-ray diffractograms, it is relevant to point out that the BC exhibits high crystallinity, which is associated with its high mechanical properties. The values for crystallite sizes for diffraction peaks, full-width at half-maximum (FWHM), and crystallinity index for BC and BC/Ch composite are listed in Table I.

Thermo-gravimetric analysis (TGA) is a continuous process, involving the measurement of sample weight in accordance with increasing temperature in the form of programmed heating. As TGA provides better understanding of thermal decomposition behavior, the thermal stability and thermal decomposition of BC, BC/Ch composite, and chitosan were investigated using TGA, and the results are given in Figure 4. For pure BC and chitosan, the thermal decomposition temperature (10% mass loss) measured by TGA is around 230°C and 260°C, respectively. For BC/Ch composite, the thermal decomposition temperature (10% mass loss) is about 235°C, which is between that of pure BC and chitosan and is more close to pure BC. However, the thermal decomposition temperature (50% mass loss) is about 263°C and 380°C for pure BC and chitosan, 380°C for BC/Ch composite which is more close to pure chitosan. The percentage weight loss at 300°C is 78.46% for BC and 37.49% for BC/Ch composite. All these results indicate that the thermal stability of BC in the composite has been improved by incorporating chitosan. As the thermal degradation temperature is affected by the structural parameters such as molecular weight, crystallinity, and orientation, as well as intermolecular interacting especially for composite. We believe that the increase in thermal stability for BC/Ch might be because of the strong intermolecular interacting by hydrogen bond formed by OH group and NH_2 group of BC and chitosan.

To investigate the mechanical characteristics of BC/Ch composites, the tensile tests were performed according to ASTM D-882-97 standard test method. Four samples were tested to calculate the average value and derivations. Figure 5 shows the stress-strain curve of the neat BC and BC/Ch composite sample and morphological changes after tensile test.

For both pure BC and BC/Ch composite, they exhibit almost linear elastic behavior from beginning until they break. No yielding point can be observed. These results indicate that the pure BC and BC/Ch composite are brittle materials and they have typical brittle properties. The tensile strength at break is about 460 MPa, 410 MPa, and 65 MPa for pure BC, BC/Ch composite, and pure chitosan, respectively. And the elongation at break is about 12%, 6.8%, and 23% for pure BC, BC/Ch composite, and pure chitosan, respectively. The Young's modulus found from the slope of the curve is 6.7 GPa for pure BC, 3.6 GPa for BC/Ch composite, and 0.3 GPa for pure chitosan. The BC/Ch composite displays 10% of decrease in tensile strength, 30% of decrease in elongation at break, and 45% of decrease in Young's modulus compared with pure BC. However, it has been reported that tensile properties of chitosan can be greatly improved by blend other polymers.[21,22] In our case, The BC/Ch composite displays 530% of improvement in tensile strength, 240% of decrease in elongation at break, and 4000% of increase in Young's modulus compared with pure chitosan. The reason might be because of the fact that inherently high modulus and strength of bacterial cellulose allow the mechanical properties of BC/Ch composite to improve.

After tensile test, we check the morphological changes for both surface and cross-section of fracture region. On the surface, we can see some BC nanofibrils assemble together and align to the pull direction. From cross-section, we can clearly see the alignment of nanofibrils. This alignment of nanofibrils can improve the strength of the sample. These mechanical properties of BC/Ch composite make it

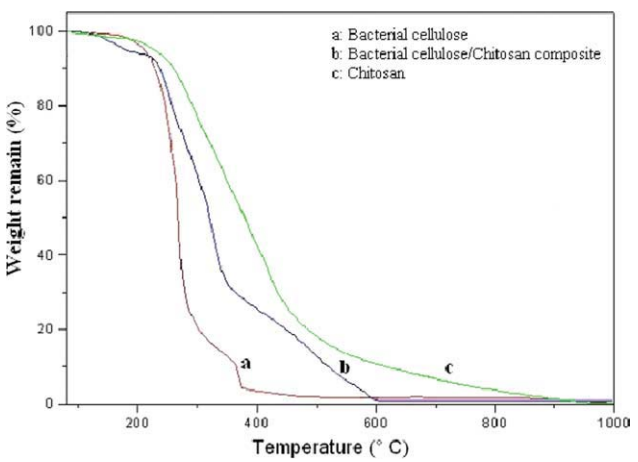

Figure 4 TGA spectra of pure BC, BC/Ch composite, and chitosan. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

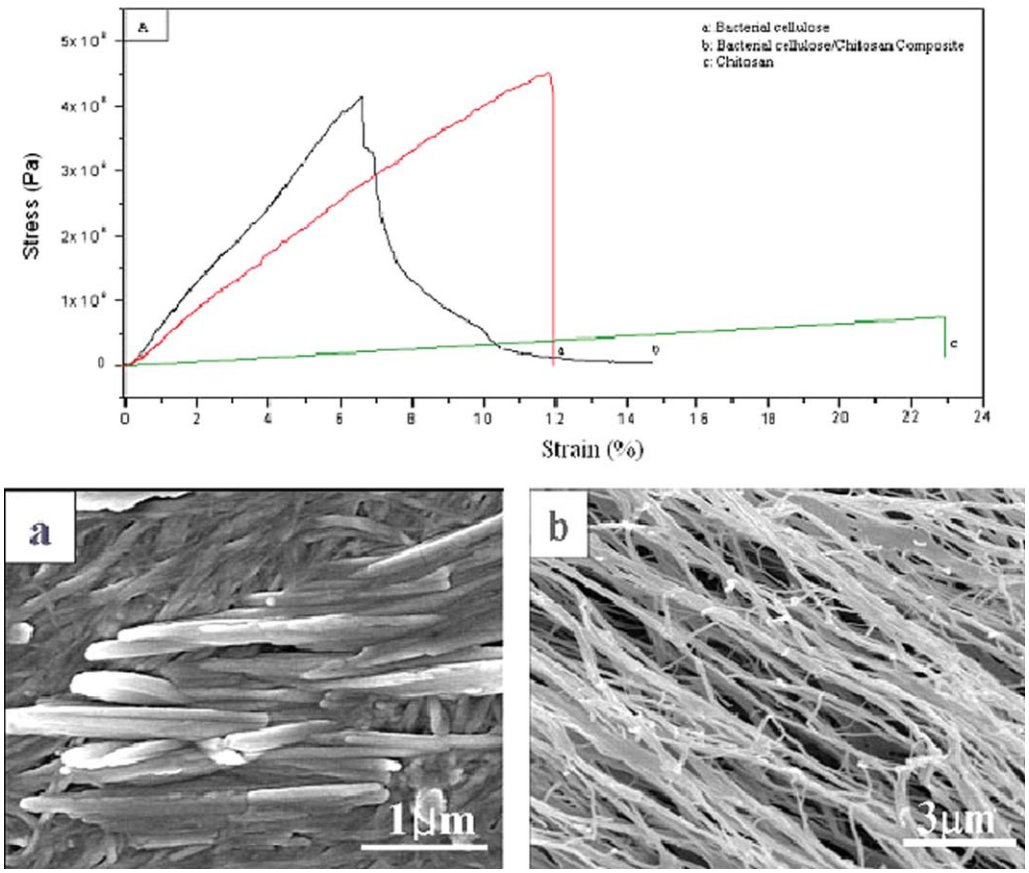

Figure 5 Stress-strain curves of tensile test (A) SEM images of fractured region on BC/Ch composite (B) (a: surface morphology; b: cross-sectional morphology). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

possible to be used as potential scaffold material in tissue engineering or other medical applications such as wound dressing, etc.

Figure 6 shows the cell attachment on pure BC and BC/Ch composite. Fibroblast cells (3T3) were seeded on cylinder scaffolds. After 48 h of incubation, there were many cells attached on pure BC scaffolds [Fig. 6(A)]. And most of the cells remained in round-shape. But for BC/Ch composite scaffolds, cells adhered and almost completely spread on the surface [Fig. 6(B)]. They had many pseudopodia and formed a layer on the surface. These results indicated that the cells stretched their morphology and were proliferating. This preliminary experiment suggests that BC/Ch composite has better biocompatibility compared with pure BC in terms of fibroblast cell culture. It would

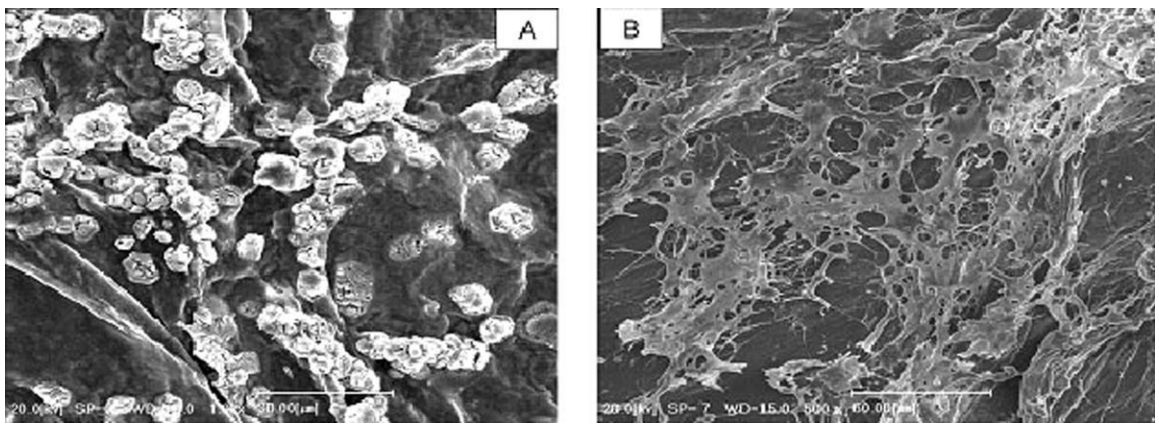

Figure 6 3T3 fibroblast cell attachments of pure BC (A) and BC/Ch composite scaffolds (B) of 48 h seeding the cells.

have potentials to be used as wound dressing materials or tissue regeneration scaffold *in vitro*. Further investigation such as cellular proliferation and differentiation assays are underway.

CONCLUSIONS

BC/Ch composite has been prepared by immersing wet BC pellicle in chitosan solution followed by freeze-drying. SEM images show that chitosan molecules can penetrate into BC forming multilayer structure of BC/Ch composite. The scaffold has very well interconnected porous network structure and large aspect surface. By incorporation of chitosan in BC, crystallinity tends to decrease while the thermal stability has certain improvement. At the same time, the BC/Ch composite displays 530% of improvement in tensile strength, 240% of decrease in elongation at break, and 4000% of increase in Young's modulus compared with pure chitosan. The reason might be because of the fact that inherently high modulus and strength of bacterial cellulose allow the mechanical properties of BC/Ch composite to improve. Cell culture test results indicate that BC/Ch composite has much better biocompatibility than pure BC. Thus, the prepared BC/Ch scaffolds are bioactive and may be suitable for cell adhesion/attachment suggesting that these scaffolds can be used for wound dressing or tissue-engineering scaffolds.

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