Bacterial Cellulose/Collagen Composite: Characterization and First Evaluation of Cytocompatibility

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ABSTRACT: The novel bacterial cellulose (BC)/collagen composites were prepared by immersing wet BC pellicle excreted by *Acetobacter xylinum* in collagen solution followed by freeze-drying process. The product looks like a foam structure. The morphology of BC/collagen composite was examined by scanning electron microscope (SEM) and compared with pristine BC. SEM images showed that collagen molecules was not only coated on the BC fibrils surface but also could penetrate inside BC and hydrogen bond interactions were formed between BC and collagen. The prepared BC/collagen composite was also characterized by Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), thermogravimetric analysis (TGA), and mechanical test. With the incorporation of collagen in the BC, no changes happened in the crystal structure but the thermal stability was improved. Tensile test results indicate that the Young's Modulus and tensile strength have a big increase while the elongation at break has a slight decrease. The cytocompatibility of composite was preliminarily evaluated by cell adhesion studies. The tests were carried out using 3T3 fibroblast cells. The cells incubated with BC/collagen scaffolds for 48 h were capable of forming cell adhesion and proliferation. It showed much better cytocompatibility than pure BC. So, the prepared BC/collagen 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. Therefore, these results suggest that these novel BC/collagen scaffolds may have the potential to be sued for some biomedical applications. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 120: 2938–2944, 2011

Key words: bacterial cellulose; collagen; characteristics; cytocompatibility

INTRODUCTION

Bacterial cellulose (BC) is a kind of extracellular polysaccharide presented the biofilm produced by several bacteria, notably by *Acetobacter xylinum*, as long nanofibers. BC is composed of glucose molecules joined by β(1→4)-glycosidic bonds forming branchless linear chains.[1] BC displays many unique properties including high water uptake capacity, high crystallinity, and an ultrafine nanofibril network structure.[2] Compared with other natural biodegradable polymers such as collagen, chitin, and gelatin, BC presents much higher mechanical properties, which are essential for scaffolds in tissue engineering.

BC is used in a wide range of applications, from the food industry to electroacoustic devices, such as phone diaphragms. Several applications for BC in medical fields have already been reported such as artificial skin for humans with extensive burns,[3] artificial blood vessels for microsurgery,[4] scaffolds for tissue engineering of cartilage,[5] and wound-dressing.[6] In medicine, nonporous cellulose membranes are used as stent coatings, for duramater substitution in tumor or trauma cases, or as skin protection in the cases of burn and deep wounds. In odontology, cellulose films are applied for periodontal tissue recovering.[7] To broaden the biomedical applications of BC, various attempts have been made to produce BC composites with high functionality.[8–12] Among them, BC based composite is one of candidates that have great potential applications for tissue engineering and drug delivery.

Collagens are natural biopolymer found in most tissues and organs and are widely employed in the construction of artificial skin substitutes used in the management of severe burns. In these applications, collagen not only plays the role of physical scaffold for the proliferating cells, but also enhances cell adhesion, proliferation and function.[13,14] Collagen creates a natural extracellular environment that is important for cell communication and layer formation.

The purpose of this study is to prepare a composite consist of BC and collagen by postmodification in an attempt to use the synergic beneficial aspects of both materials. It is expected that this new

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composite should have better potential for biomedical applications.

MATERIALS AND METHODS

Materials

Collagen (from porcine tendon, Type I) was purchased from Sigma. Other chemicals of the highest purity available were used and were purchased from Sigma-Aldrich.

Biosynthesis of bacterial cellulose pellicles

Gluconacetobacter xylinum was 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 14 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.

Preparation of bacterial cellulose/collagen composite scaffold

The wet BC pellicle was placed between two sheets of filter paper to remove free water. Then it was immersed in collagen-dissolved acetic acid solution for 30 min in room condition. After that, it was withdrawn from the vessel and the excess collagen solution was removed using filter paper. Finally, it was dried by freeze-dryer (IP3 Jouan, France) at −40°C for 3 days.

Characterization of BC/collagen composite

The prepared BC/collagen composite scaffold was characterized by scanning electron microscopy (SEM), Fourier transform-infrared spectroscopy (FTIR), X-ray diffraction (XRD), thermogravimetric analysis (TGA), and tensile test. SEM images (surface and cross section) of the samples were taken with a microscope (Hitachi *S*-4200, Japan) to study the morphological changes. FTIR spectra were obtained using a Perkin–Elmer System 2000 FTIR spectrophotometer for the evaluation of chemical structures using a KBr pellet. XRD pattern were recorded on an X-ray diffractometer (D/MAX-2500, Rigaku), by using Cu-Kα radiation at 40 kV and 30 mA. The diffraction angle ranged from 5 to 40°C. Crystallinity index obtained from the X-ray diffraction data was calculated according to the literature method.[15] This fast and easy method uses the intensity of (2 0 0) peak and the minimum intensity between (2 0 0) and (1 1 0) peaks, assuming that the intensity of (2 0 0) peak represents both crystalline and amorphous parts while the minimum intensity mentioned previously is for amorphous part only. According to this method, crystallinity index, Cr*I* can be written as

$$\mathrm{Cr}I = \left(I_{(200)} - I_{(\mathrm{am})}\right)/I_{(200)}$$

where $I_{(200)}$ is the intensity at (2 0 0) peak and $I_{(\mathrm{am})}$ is the minimum intensity between (1 1 0) and (2 0 0) peak. TGA test was carried out with a NETZSCH STA 409 PC/PG system. All analyses were performed with a 10-mg sample in an aluminum pan under dynamic nitrogen atmosphere between 30 and 1000°C. The experiments were performed at a scanning rate of 20 K/min. Tensile test was done on an in-house universal testing machine system in room condition with pulling speed of 2 mm/min. Load cell sensor (Daecell Korea, UU-K010) was used to measure the applied load. Linear scale sensor (Sony Japan, GB-BA/SR128-015) was used to measure moving distance. The test specimens were prepared by cutting the membranes to 10 mm wide and 50 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, a standard test method for tensile elastic properties of thin plastic sheeting. Five samples were tested to calculate the average value. Pulling speed of the testing machine was 2 mm/min. The test was performed in an ambient condition.

Cell attachment studies

The BC/collagen cylinder scaffolds (20 mm diameter × 4 mm height) were used for the cell attachment studies. Before 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's 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. After that, 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 nonadherent cells and the attached cells were fixed with 2.5% glutaraldehyde buffer solution (pH 7.4) at 4°C for 12 h.

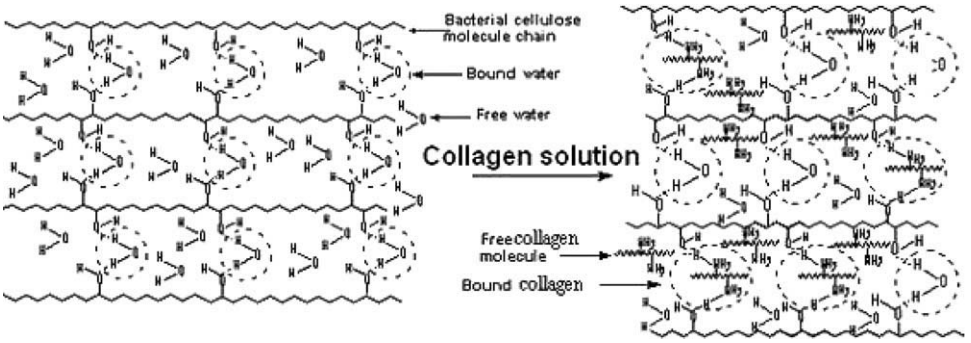

Figure 1 Schematic of BC/collagen interaction.

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

Statically cultured BC formed a pellicle. This pellicle was composed of a small amount of nanofibrils holding about 99% of water. The ultrafine, highly entangled nanofibrils, which have length in the range of 1–9 μm, form a dense reticulated structure, stabilized by extensive hydrogen bonds. In the humid state the pellicle is a homogenous, moldable, and handle-resistant gel. If this pellicle 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.[16] In the present study, the soft pellicle obtained by *Acetobacter xylinum* was treated with collagen acetic acid solution, instead of being dehydrated. Thus, we expect that, by immersing the BC pellicle in collagen solution, multiple layers of water surrounding polyglucosan chains would be displaced, inducing the formation of bonds between cellulose and collagen, with consequent marked structural modification (Fig. 1).

More detailed analysis by SEM revealed that the BC after drying is composed of long nanofibers. Figure 2 shows SEM images of freeze-dried BC and BC/collagen composite, highlighting details of the microstructure. As seen from Figure 2(a), a dense, flawless, and homogeneous cellulose matrix can be observed on the surface. The matrix is formed by an interlaced network of long cellulose microfibrils with high aspect ratio and mean diameter around 40 nm. This high aspect ratio may affect the mechanical properties of BC such as Young's modulus and tensile strength. From the cross-sectional images we can clearly see porous layer structure with high aspect ratio. The pore size is around 15 μm, which is connected each other. Evidently, a well-organized three-

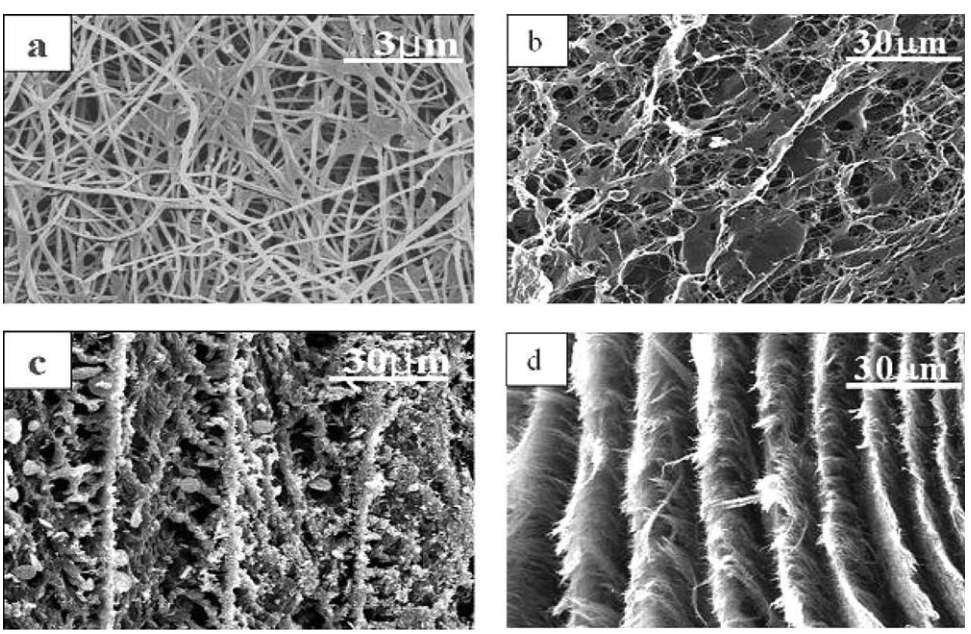

Figure 2 SEM images of the BC (a: surface; c: cross section) and BC/collagen composite (b: surface; d: cross section).

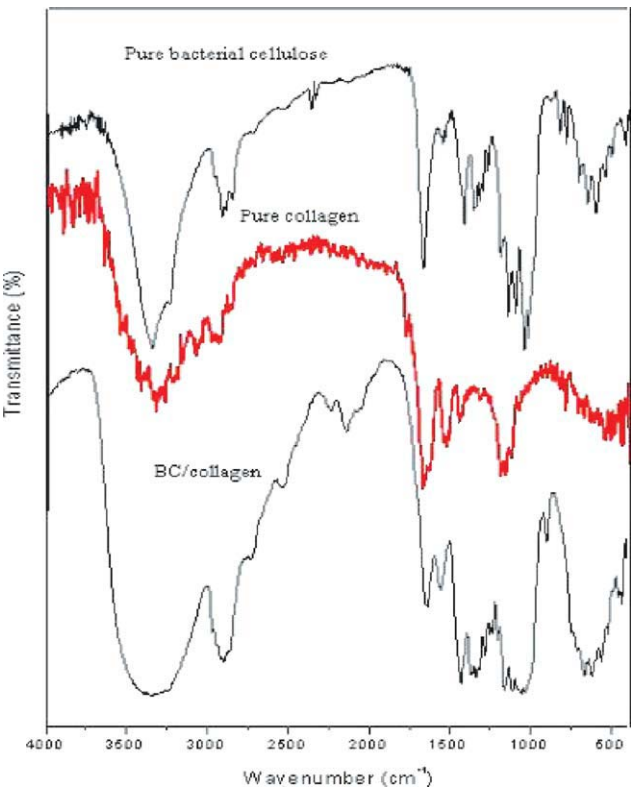

Figure 3 FTIR spectra of BC, collagen, and BC/collagen composite. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

dimensional porous network structure was observed. The BC sample has porous morphology, which has been investigated in the last decade.[17] 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.[18] The cellulose is crystallized outward the organisms, particularly in *Acetobacter 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 collagen solution, the morphology showed some changes. Figure 2(b,d) presents the surface and cross-sectional SEM images of freeze-dried BC/collagen composites. BC nanofibers still can be observed on the surface. However, a smooth layer was formed due to the coverage of BC by collagen in most part. From the cross-sectional image, we can see that collagen molecules penetrated into BC and formed layers of BC/collagen composite. BC nanofibers can be observed between layers and interconnected each other to form a network structure. Note that the present nanofibrous BC and BC/collagen composite have well interconnected porous network structure that has large surface area and is necessary for cellular attachment and vascularization in biomedical application. This BC/collagen composite can promote cellular ingrowth when it is used for tissue engineering scaffolds.

FTIR spectra obtained from pure the BC, collagen, and BC/collagen composite are shown in Figure 3. For the pure BC, a broad band at 3300 cm^{-1} is attributed to O-H stretching vibration. Band at 2820 cm^{-1} represents the aliphatic C-H stretching vibration. Absorbance peak at wave number 1730 cm^{-1}, is attributed to hydrogen-bonded carbonyl stretching vibration.

A sharp and steep band observed at 1080 cm^{-1} is due to the presence of C-O-C stretching vibrations. For collagen, the band at wave number 3300 cm^{-1} becomes much broad due to the combination of O-H and *N*-H stretching vibrations. The characteristic bands of collagen are located at wave numbers 1648, 1540, and 1239 cm^{-1}, which are attributed to amide I, II, and III bands. For the BC/collagen composite, all these bands can be observed with slight sift of their location. A weak new peak at wave numbers 1230 cm^{-1} is assigned to the acetate C-C-O stretching due to the acetylation of cellulose. Since we use acetic acid as solvent for collagen, cellulose can be easily acetylated during the treatment. Two new peaks around 2200 cm^{-1} might be due to the acetic acid residual which is not completely removed after immersing process. The FTIR observation indicates that the BC/collagen composite has been successfully made by using the postmodification method.

Regarding the crystalline structures of cellulose, they are classified into four crystallization types, *viz.* cellulose I, II, III, and IV, and their crystalline structures can be transformed from one type to another.[19] Figure 4 shows the X-ray diffraction pattern of the pure BC and BC/collagen composite. For the pure BC, three main peaks can be identified in the spectrum, which are assigned to the (1 $\bar{1}$ 0), (1 1 0), and (2 0 0) reflexions planes of cellulose I.[20] The location of these three peaks is 14.60°, 16.85°, and 22.70°. For the BC/collagen composite, the diffraction peaks are almost same as the pure BC except decrease in intensity. These results indicate that no changes in the crystalline structure of the BC/collagen composite were occurred during the incorporation of collagen.

The estimated degree of crystallinity index of the pure BC was 87 and 75% for the BC/collagen composite. With the introduction of collagen, the crystallinity index was decreased. This might be due to the fact that during the immersing process some water molecules, which were bonded with polyglucosan chains, could be displaced by collagen molecule chains, inducing bonding formation between cellulose and collagen. This incorporation of collagen could disturb the regular arrangement of BC molecule chains, which in turn can decrease the

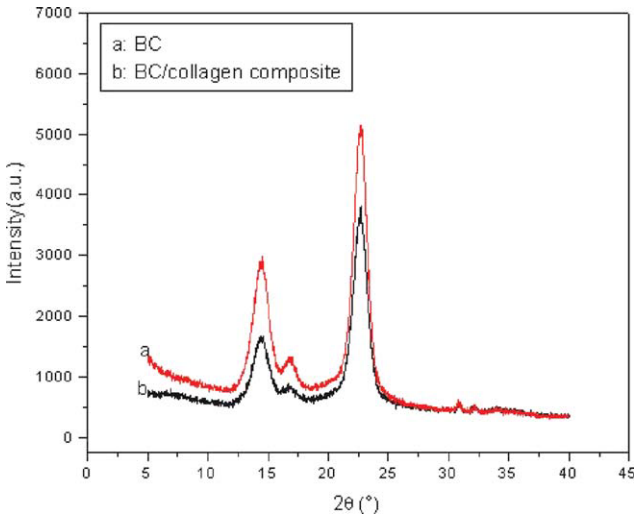

Figure 4 XRD patterns of the BC and BC/collagen composite. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

crystallinity index. Although it was an estimative of crystallinity based on the X-ray diffractograms, the BC exhibited high crystallinity, which might be associated with its high mechanical properties.

Thermogravimetric analysis (TGA) is a continuous process, involving the measurement of sample weight in accordance with increasing temperature in the form of programmed heating. Since TGA provides better understanding of thermal decomposition behavior, the thermal stability and thermal decomposition of the pure BC and BC/collagen composite were investigated using TGA. Figure 5 shows the TGA results. The TGA curves obtained by plotting percentage weight loss against temperature indicate that BC is stable up to 220°C. The percentile weight loss for the pure BC at 250°C was 29.66%, while for the BC/collagen composite it was 7.15%. In the case of BC, 50% weight loss was noticed at 262°C; meanwhile for the BC/collagen composite it was 352°C. With the introduction of collagen, the thermal stability of composite was improved and the thermal degradation temperature was increased. Another reason is due to the acetylation effect. Since we use acetic acid as solvent for collagen, cellulose can be easily acetylated during the treatment and this acetylation can also increase the thermal stability.

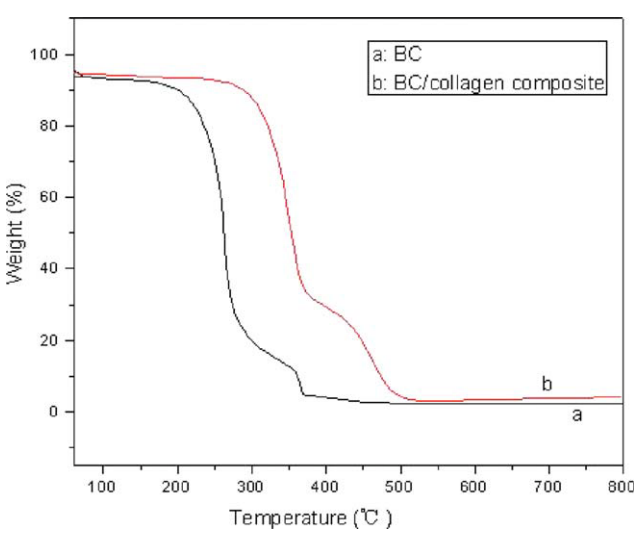

Figure 5 TGA spectra of the BC and BC/collagen composite. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Normally, when polymer pairs exist in two phases, the mechanical properties of the composite material are governed by the distribution of the respective polymers within the composite. In other words, the properties may be dominated by the higher volume polymer phase. Polymer-rich phase usually forms a continuous matrix, whereas the secondary phase plays the role of reinforcing the matrix by stress transfer between interfaces. Figure 6 shows typical stress-strain curves of BC and BC/collagen composite under tension. The stress-strain curves show quite different. For pure BC, rupture occurred at 6.5% strain, where the tensile strength was about 200 MPa. For BC/collagen composite, however, it exhibited almost linear elastic behavior. Elongation at the rupture was about 4% and the tensile strength was 275 MPa, which were much higher than that of pure BC. The Young's modulus found from the stress-strain curve was 4.5 GPa and 9.5 GPa for pure BC and BC/collagen composite, respectively. With the incorpoartion of collagen, the tensile strength and Young's modulus of composite were highly improved while the elongation at break tends to decrease.Tensile strength of untreated BC membranes is reported to higher than 150 MPa.[21] Its value depends on such parameter as culture time, post treatment, drying process, etc.

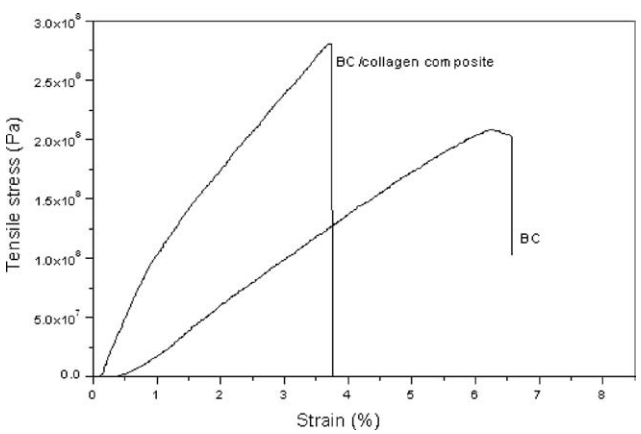

Figure 6 Stress-strain curves of the BC and BC/collagen composite.

TABLE I
Physical Properties of the BC and BC/Collagen Composite

Property	BC (other work)	References	BC (this work)	BC/collagen composite
Tensile strength (MPa)	150–240	21, 22	200 ± 18	275 ± 22
Young's modulus (GPa)	0.6–9.7	22, 23	4.6 ± 0.8	9.5 ± 1.6
Thermal degradation temperature (°C)	300	24	264	353
Crystallinity index (%)	60–80	25	87	75
Elongation at break (%)	2.6	22	6.5 ± 0. 5	3.75 ± 0.55

Table I summarizes the physical properties of the BC and BC/collagen composite obtained in this work in comparison with other literature data.

For the pure BC, the coiled and randomly arranged BC nanofibers were extended along the pulling direction under low pulling force. At this stage, the stress is small but the strain is big. After that, the aligned BC nanofibers can stand much big loads and shows an elastic behavior. This prealignment can greatly improve the tensile strength of the pure BC. However, for the BC/collagen composite, since collagen penetrated into BC and lots of BC nanofibers were covered by collagen to form layers of BC/collagen composite, the prealignment did not happen. As a result, it shows low elongation at the break point and almost linear elastic behavior.

Figure 7 shows the cell attachment on pure BC and BC/collagen composite. Fibroblast cells (3T3) were seeded on cylinder scaffolds. After 48 h of incubation, there were many cells attached on pure BC scaffolds [Fig. 7(a)]. Most of the cells remained in round-shape which means the cells have no tendency to proliferate. For BC/collagen scaffolds, cells adhered and completely spread on the surface [Fig. 7(b)]. They had many pseudopodia and formed a layer on the surface. These results indicated that the cells stretched their morphology and were proliferating. The reason may be due to the incorpoartion of collagen. As introduced before, collagen can enhance cell adhesion, proliferation. Collagen can create a natural extracellular environment that is important for cell communication and layer formation. This preliminary experiment suggests that BC/collagen composite has better cytocompatibility compared with pure BC in terms of fibroblast cell culture. It would have potentials to be used as wound dressing materials or artificial skin. Further investigation such as cellular proliferation and differentiation assays are underway.

CONCLUSIONS

Novel bacterial cellulose/collagen composite scaffolds were prepared by immersing wet BC pellicle in collagen solution followed by freeze-drying. We expected that during this immersing process hydrogen-bonded water surrounding polyglucosan chains would be displaced, inducing the formation of bonds between cellulose and collagen, with consequent marked structural modification. SEM images showed that collagen molecules could penetrate into BC forming multiplayer structure of BC/collagen composite. The scaffold had very well interconnected pore network structure and large surface area. With the incorporation of collagen in the BC, the crystallinity index tended to decrease while the thermal stability was improved. After incorporation of collagen, the tensile strength and Young' modulus was increased from 200 Mpa, 4.6 GPa to 275 Mpa, 9.5 GPa, while the elongation at break was decreased from 6.5% to 3.75%, respectively. Cell adhesion studies were carried out using 3T3 fibroblast cells. The cells incubated with BC/collagen scaffolds for 48 h were capable of forming cell adhesion and proliferation. So, the prepared BC/collagen scaffolds are bioactive and may be suitable for cell adhesion/attachment suggesting that these scaffolds can be used for wound dressing or tissue-engineering applications.

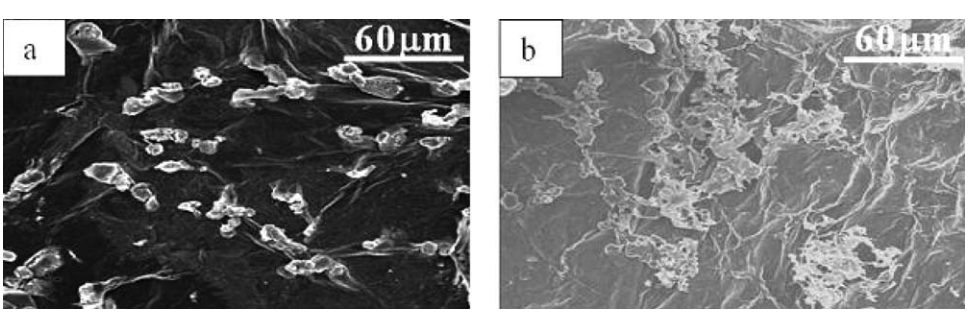

Figure 7 Fibroblast cell (3T3) attachments of pure BC (a) and BC/collagen scaffolds (b) of 48 h seeding the cells.

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