Skin tissue repair materials from bacterial cellulose by a multilayer fermentation method†

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Using an improved method, the multilayer fermentation method, bacterial cellulose (BC) was produced by Gluconacetobacter xylinus. The structure and morphology were analysed by an electronic microscope. The surface area and tensile strength were characterised. In vitro, the cytotoxicity of BC was determined by the proliferation, adhesion property, morphology, and viability of human adipose-derived stem cells (hASCs). Full-thickness skin wounds were made on the backs of 35 mice. The wounds were subsequently treated with two types of gauzes, two types of BC films, and three types of skin grafts using 5 mice per group, respectively. The improved method was reproducible and more efficient to control the thickness and homogeneity of BC. Low cytotoxicity of the BC film and good proliferation of hASCs on the BC film were observed. Histological examinations demonstrated significant fresh tissue regeneration and capillary formation in the wound area in the BC groups on day 7 compared with those in other groups. Pathological studies also showed a faster and better healing effect and less inflammatory response in the BC groups than those in other groups. These results indicate high clinical potential of the BC biosynthesized by our improved method.

1. Introduction

Currently, burn wounds, scald wounds, and chronic wounds represent a major and persistent burden in orthopedics and dermatology. Acute and chronic wounds need to be treated by artificial skin and/or wound dressings. Though various wound healing materials have been developed over time, the present commercial materials are far from ideal. For the majority of repair materials, the most important requirements are their ability to absorb exudate during the dressing process, in addition to their easy removal from the wound surface after recovery. Traditionally, skin tissue repair materials have been absorbent and permeable; for example, cotton gauze, a traditional dressing material. However, they can often adhere to the wound surface, resulting in secondary-injury when changing and removing dressings. In order to resolve these issues, many studies in the last 30 years have focused on tissue engineering in skin and wound healing, whilst additionally utilising new developments in cell biology and molecular biology.

As a biopolymer, cellulose is the most common biodegradable natural material and is present in a large amount of natural substances. Although the structural unit of BC is the same as that of cellulose from plants or algae, BC has many unique properties. BC is highly hydrophilic and has properties of good water retentiveness, high permeability, optimal gelatinization, and high mechanical strength. BC has been widely applied in medical and industrial fields, not just in medical science, but also in cosmetics and veterinary medicine. BC holds great potential to be used for medicinal purposes especially in the field of regenerative medicine. BC can be used as artificial skin, wound dressing, artificial vessels, and artificial cartilage. It is thus widely studied as an optimal substrate material for biomedical applications or tissue engineering. BC can also control the movement of microorganisms and undergo ordered controllable nano-scale material assembly. For most skin repair materials, an important characteristic is their ability to lock exudate during the dressing process, in addition to their ease of removal from a wound surface after recovery. Experimental results and clinical treatments have demonstrated the exceptional performance of...
BC-based wound healing materials. Some papers have reported BC applied as wound dressing or artificial skin resulted in a good healing effect on different wounds.9 But the difference of the wound healing effect between different pure BC films has not been reported yet. In addition, reproducible and production techniques will be essential for the ultimate approval for applications and greater acceptance of BC.10

Controlling the organisation of BC fibrils has been proven to be relatively successful. Three-dimensional (3D) nano-fibril network of BC with controlled micro-porosity can be obtained by placing paraffin wax and starch particles of various sizes in a growing culture medium.11 The structural diversity of BC is beneficial for applications in a variety of medical devices. The cell compatibility of BC was studied with cell-culture with adipose-derived stem cells (ADSC) as the seed cells. It was reported that the ADSC on the BC films retained the biological activity of stem cells. BC also has good biocompatibility.12 By using the skin defects model in C57BL/6 mice, the absence of toxicity in vivo supports the view that BC may be amenable for use as a tissue engineering biomaterial in the research of Jeong et al.13 In one example, a patient’s facial surface with severe deep second-degree burns was covered with a single sheet of BC in which holes for the eyes, nose, and mouth were made after placement. After 44 days, the wounded face was entirely healed with no need for skin grafting and no signs of extensive scarring.14 In the healing of skin tissue, transforming growth factor β1 (TGF-β1) and basic fibroblast growth factor (bFGF, also known as FGF2 or FGF-β) are two important factors, especially in the late reconstruction of tissues. The two cytokines perform many cellular functions, including the control of cell growth, cell proliferation, cell differentiation, and apoptosis.15,16

In the present study, the multilayer BC was biosynthesized by the improved multilayer method which also incorporated the static fermentation and shaken-flask fermentation methods at various intervals as described below. To study the relationship between the healing effect and different materials, the structures and basic properties of normal BC and multilayer BC were investigated by scanning electron microscopy (SEM) and tensile testing. The proliferation and adhesion of hASCs on BC were studied to evaluate the biocompatibility of the BC materials. The wound healing effects of BC were compared with five other materials: clinically widely used gauze; Vaseline gauze; three different skin grafts, including pig skin and rat skin as xenogeneic and mouse skin as allogeneic grafts. An enzyme linked immunosorbent assay (ELISA) study was performed to analyse the expression of the two cytokines mentioned above (TGF-β1 and bFGF). The relation of the two factors with cell proliferation and tissue regeneration was investigated.

2. Materials and methods

2.1 Preparation

BC was biosynthesized by Gluconacetobacter xylinus (G. xylinus, ATCC 53582 (American Type Culture Collection, Manassas, VA, USA)). G. xylinus was inoculated and cultured at 30 °C in a Hestrin and Schramm (HS) medium. The HS medium contained 2% (w/v) glucose, 0.5% (w/v) yeast extract powder, 0.5% (w/v) peptone, 0.27% (w/v) disodium phosphate (Na2HPO4), and 0.115% (w/v) citric acid.17 The yeast and peptone were purchased from Beijing Shuangxuan Microbe Culture Medium Products Factory, PR China, and the reagents were purchased from Sinopharm Chemical Reagent Beijing Co. Ltd., PR China.

An improved method was created by combining both static and shaken culture processes to control the thickness of the BC film. As soon as the BC pellicle formed in a culture flask, the flask was gently shaken and the first thin BC film sank below the surface of the liquid. A thin BC film was biosynthesized from a flask which was shaken every day. A thick BC film was biosynthesized from a flask which was shaken every 2 days. A multilayer BC film could then be produced at the surface. The second BC film then sank below the surface of the liquid. Repeating the procedure, several layers of BC films of a similar thickness were obtained during the whole period of BC growth. By regulating the length of fermentation time, the thickness of the BC film was relatively reproducible. The method described above is the “multilayer fermentation method.” The BC films were first purified by washing with distilled water for 2 days. They were then processed with boiling 1 wt% NaOH for 30–45 minutes to eliminate bacteria and proteins. The BC films were rinsed with distilled water until the pH reached 7.0. Afterwards, the BC films were washed several times with high-purity water and were subsequently stored in high-purity water at 4 °C.

2.2 Characterisation

The BC films were characterised by SEM in order to determine their surfaces and structures. The wet BC films were frozen in liquid nitrogen and freeze-dried under vacuum. The films were coated with carbon and gold in preparation for SEM of the surfaces and cross-sections in the usual way. SEM observations were performed with a scanning electron microscope (JSM 7401F, JEOL Ltd., Japan). Based on the dead volume measurement method, the pore size distribution and specific surface area of thin and thick BC films were measured at 77 K using a Belsorp-mini II system (BEL Japan Inc., Japan).

A tensile test was used to study the mechanical properties of the BC films. The testing was carried out at room temperature on a tensile tester (6P-TS 2000S, Shenzhen GaoPin Test Machine Co., Ltd., PR China) with a constant deformation rate of 5 mm min⁻¹. The humidity was controlled at 60%. Freeze-dried BC was prepared with a freeze drier machine (FD-1A-50, Beijing Boyikang Laboratory Instruments Co., Ltd., PR China). The dried BC films were compared with Vaseline gauze (Shaoxing Fuqing Medical Products Co., Ltd., PR China). The wet BC films were compared with 3 other dressings, pig skin, Vaseline gauze, and mouse skin, concurrently. The samples were cut into rectangular slices with a thickness of 0.1–2.5 mm and a width of 10 mm. The cut samples were immersed in 0.9% sodium chloride solution. For a single layer sample, the thickness was measured independently: length = 60 mm and width = 10 mm. The initial length of the sample between two gauges was always kept at 40 mm. A minimum of four specimens were tested on each tensile test. Measurements of tensile strength (σ), elongation at break (ε), and modulus of elasticity (E) were recorded.

2.3 Cell evaluation

The hACs were obtained and identified by the Institute of Microcirculation, Chinese Academy of Medical Sciences.
All the cells were cultured at 37 °C in an incubator with a humidified 5% CO2 atmosphere. The medium was changed every 3 days and the cells were transferred at least once a week. The cells were detached for 1–2 minutes by a 0.25% trypsin–0.02% EDTA solution in PBS. After trypsinization, the cells were collected by centrifugation at 1000 rpm for 5 minutes. The cell suspension was transferred to a 15 mL centrifuge tube. After the removal of trypsin, the remaining cell pellets were re-suspended in the medium supplemented with 10% FBS.

Cells of the second to third passage were used for all experiments. Sterilised materials were individually placed into single wells of a 24-well plate. Thereafter, the materials were washed with PBS and immersed in PBS for 24 h. Before seeding, the materials were washed with the medium and immersed in the medium for 24 h. The human adipose-derived stem cells were seeded in the cell plate in the control group, whilst the cells were seeded on the BC films that were placed in the cell plate in the experimental group. The cells were finally seeded in complete cell culture medium and incubated during days 1, 3, 5, 7, 9, and 11 at 37 °C in a humidified 5% CO2 atmosphere. The total number of cells initially deposited in each well for incubation was 8 × 10^4 per well. 500 μL medium was then added to each well.

In vitro cytotoxicity was studied in a 96-well plate. All materials were sterilised in high purity water under a sterilization instrument at high temperature and high pressure for 20 minutes. Following the cultivations, the cellular metabolism of hASCs on BC film was determined by a CCK-8 proliferation assay. Each test included 4 samples on days 1, 3, 5, 7, 9, and 11. First, both the well and control well were changed with 500 μL fresh medium and supplemented with 50 μL CCK-8 solution. The 24-well plate was incubated for 2 h and optical density (OD) values of absorbencies were measured at 450 nm. The OD values were obtained with a Microplate Reader (Sunrise, TECAN Trading AG, Switzerland). The results were then analysed with SigmaPlot software and a proliferation curve for BC was drawn.

The adhesion of hASCs was investigated by frozen sectioning/HE staining and SEM of the hASCs–BC film complex. Prior to the observation, the BC–hASCs complex was fixed with 2.5% polyoxymethylene (CH2O) for 12 h. The growth pattern and morphology of hASCs on BC were observed by invert phase contrast light microscopy (XDS-1B, Chongqing Optical Instruments Company) and SEM (S-3000N, Hitachi Ltd., Japan).

2.4 Animal surgery

Normal 6–8 week old male BALB/c mice and C57BL/6 (20–25 g) mice were used in the experiments and maintained under clean conditions. All the mice were purchased from Tongji Medical College of Huazhong University of Science and Technology (HUST), PR China. All procedures involving animal use conformed with the “Guide for the Management and Use of Laboratory Animals”, as published by Hubei Province, China (2005) or ISO 10993-1 (2003). The protocol was approved by the Institutional Animal Care and Use Committee at Tongji Medical College, HUST, PR China.

Full-thickness skin injuries were performed by removing a 10 × 10 mm2 section of dorsal flank skin in male BALB/c mice. Then, a piece of cover material of the same size was placed on the wound surface. Each mouse was kept in a single cage for observation. Fig. 1 shows the process of the full-thickness skin lesion and wound dressing in mice. The mice were divided into 7 groups according to the materials used for covering their skin wounds (Table 1).

Groups of mice were sacrificed on days 4, 7, 11, and 14. The wound tissues were then collected for histological study. The serum separators were used to allow samples to clot for 1 h at room temperature before centrifugation for 10 minutes at approximately 1500 rpm at 4 °C. The prepared serum was freshly stored in a refrigerator at −80 °C before testing. The light microscope and camera system was a BX51 instrument, Olympus Inc., Japan. The pathological sections of the wounds were investigated with hematoxylin and eosin (HE) staining. The serum was prepared by drawing blood from the eyeball on the 11th day. The ELISA kits (USCN Life Science Inc., PR China) utilised sandwich enzyme immunoassay for the in vitro quantitative measurement of TGF-β1 and bFGF in mice sera. The enzyme microplate reader was Synergy2 SL luminescence microplate reader, BioTek Instruments, Inc., USA.

![Fig. 1](image)

**Fig. 1** The establishment of the full-skin injury model in a BALB/c mouse: (A) shave off the hair of the mouse; (B) make a full-thickness skin lesion of 10 × 10 mm2 on the back; (C) transplant the BC film on the wound area of the mouse; (D) bandage treatment of the mouse. All the scale bars equal 10 mm.

**Table 1** Seven groups in the animal surgery

<table>
<thead>
<tr>
<th>Materials</th>
<th>Category</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterilised gauze</td>
<td>Commodity materials group</td>
<td>5</td>
</tr>
<tr>
<td>Vaseline gauze</td>
<td>Commodity materials group</td>
<td>5</td>
</tr>
<tr>
<td>Thin BC film</td>
<td>Experimental group</td>
<td>5</td>
</tr>
<tr>
<td>Thick BC film</td>
<td>Experimental group</td>
<td>5</td>
</tr>
<tr>
<td>Pig skin</td>
<td>Xeno-transplant control group</td>
<td>5</td>
</tr>
<tr>
<td>Rat skin</td>
<td>Xeno-transplant control group</td>
<td>5</td>
</tr>
<tr>
<td>Mouse skin</td>
<td>Allo-transplant control group</td>
<td>5</td>
</tr>
</tbody>
</table>
All collected tissues were fixed in 4% PBS-poloxymethylene solution (pH 7.4) for 72 h and embedded in paraffin after a series of dehydration in ethanol and xylene. Specimens were sectioned into 4 μm slices and subsequently stained with haematoxylin and eosin. For each specimen, 4 random slides were obtained and evaluated. The slides were observed and the photos were taken using a light microscope. The embedding machine was an automatic tissue embedder (TB-718, Hubei Taiva Medical Technology Co., Ltd., PR China). The manual rotary microtome was RM2235, Leica Microsystems GmbH (Germany). The rectangular water bath flattening table was HI1220, Leica Microsystems GmbH (Germany). The slide drier machine was TissueTek slide warmer PS-53, Sakura Finetek Japan Co. Ltd. Japan.

All kit components and samples were brought to room temperature before the ELISA assay. The concentration of TGF-β1 in mice from the naive animal, the thick BC group, and the mouse skin group were tested by an ELISA assay. Standard solution was obtained from the mixture of lyophilised TGF-β1 (recombinant protein in a buffer with preservatives) and standard diluent (2% bovine serum albumin (BSA), and 0.02% sodium azide in Tris–buffered saline (TBS) buffer). 100 μL of standard solution and 100 μL of serum samples were added to each well in a 96-well strip plate. The wells were incubated for 2 h at 37 °C. The liquid of each well was removed without washing. Detection reagent A (detection antibody, 50% glycerol) was diluted with diluent solution (2% BSA and 0.02% sodium azide). 100 μL of detection reagent A was then added to each well and incubated for 1 h at 37 °C after being covered with the plate sealer. The solution was aspirated. 350 μL of wash solution (TBS buffer) was added to each well 3 times. Detection reagent B (HRP-linked avidin, 50% glycerol) was diluted with diluent solution (2% BSA and 0.02% sodium azide). 100 μL of detection reagent B was then added to each well and incubated for 30 minutes at 37 °C after being covered with the plate sealer. The samples were aspirated and washed 5 times. The substrate solution (0.05% 3,3′,5,5′-tetramethylbenzidine) was diluted with diluent solution (2% BSA and 0.02% sodium azide). 90 μL of substrate solution was then added to each well and incubated for 15 minutes at 37 °C after being covered with the plate sealer. The liquid turned yellow by the addition of 50 μL per well stop solution (1 mol per L H₂SO₄). The OD value was measured immediately using the microplate reader at 450 nm. The concentration of bFGF was calculated based on the standard curve of bFGF.

2.5 Statistical analysis

Where indicated, the data are expressed as mean ± standard deviation and were analysed statistically by the paired Student’s test method.

3. Results

3.1 Characterisation

After 10 days of fermentation, the BC film was obtained as a whole layer in the flask, whilst multilayer BC films were obtained as several layers in the flasks. The normal BC was fermented for 10 days using a static culture method, resulting in a thickness of 10 mm. The film was shaken each day in the fermentation of thin BC films. The thickness of the BC film in one flask was controlled at around 1 mm, whilst that of thick BC films was controlled at 2 mm (see Fig. S1†). By the improved “multilayer fermentation method,” more pieces of the BC films were biosynthesized in one flask. Specifically, the thicknesses of BC films in any one flask were controlled at a similar thickness.

The water content value of the BC was very high. The results of tensile strength (δ), elongation at break (ε), and modulus of elasticity (E) are shown in Table 2. The dry BC film was more brittle than the wet BC film and as a result it broke earlier in the tensile test. Compared to wet gauze and dry gauze, the tensile strength of the dry BC film was significantly higher. This behaviour can be attributed to the fine 3D structure of BC nano fibres. More importantly, the BC fibres may act as reinforcing filler when compared with cotton fibres. In our case, the thickness of the mouse skin was usually close to 1 mm and its water content was about 50–70%. The water contents of BC films were about 98–99%. Neglecting the volume of the water, the thickness of the mouse skin and the thick BC film was similar. Furthermore, the wet BC film showed a similar elongation at break as with mouse skin.

Table 2 Tensile test of the different samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thickness (mm)</th>
<th>δ (MPa)</th>
<th>ε (%)</th>
<th>E (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet BC</td>
<td>2.10</td>
<td>1.96</td>
<td>23.00</td>
<td>17</td>
</tr>
<tr>
<td>Wet gauze</td>
<td>0.14</td>
<td>1.23</td>
<td>11.88</td>
<td>18</td>
</tr>
<tr>
<td>Pig skin</td>
<td>2.25</td>
<td>10.04</td>
<td>42.79</td>
<td>46</td>
</tr>
<tr>
<td>Mouse skin</td>
<td>0.88</td>
<td>1.13</td>
<td>33.29</td>
<td>6</td>
</tr>
<tr>
<td>Dry BC</td>
<td>0.55</td>
<td>10.32</td>
<td>9.00</td>
<td>131</td>
</tr>
<tr>
<td>Dry gauze</td>
<td>0.18</td>
<td>7.02</td>
<td>17.00</td>
<td>65</td>
</tr>
</tbody>
</table>
The SEM results (Fig. 2) illustrate a highly swollen 3D network with a distinct tunnel and porous structure of BC nanofibres. The sterilised gauze shows a smoother structure than the Vaseline gauze, whilst the Vaseline gauze shows a larger fleece than the gauze. The thin BC film shows the smallest porous network amongst the three types of BC films. The diameters \( D \) of different single fibres were measured by the images analysis. The data were recorded as 
\[
\begin{align*}
D_{\text{gauze}} &= 14.178.4 \pm 380.5 \text{ nm}, \\
D_{\text{Vaseline gauze}} &= 16.141 \pm 407.6 \text{ nm}, \\
D_{\text{thin BC}} &= 189.2 \pm 57.9 \text{ nm}, \\
D_{\text{thick BC}} &= 138.6 \pm 37.6 \text{ nm}, \\
D_{\text{normal BC}} &= 133.4 \pm 54.1 \text{ nm}.
\end{align*}
\]

The fibre of a thin BC is larger than that of thick BC and normal BC. The diameters of the fibres in the sterilised gauze and the Vaseline gauze are more than 70 times the diameters of BC films. The data show the dramatic difference in the fibre diameters of gauze, Vaseline gauze, and BC films. Though they have the same molecular formula, the BC films have fundamentally different nanofibre architecture. From the SEM images of thin BC, thick BC, and normal BC, differences in the stability of the hydrogels and in the network architecture are evident. The images of the two gauzes show larger pores while all the BC films have nano-scale pores. The pores of the thick BC film are the smallest among the BC films.

The results obtained demonstrated that the thin BC films had a higher surface area and greater total pore volume (total porosity) than thick BC films (Table S1↑). The average pore diameter for thick BC films was almost 3 times that of the thin BC films. In our synthesis, the surface area decreased with the total pore volume (TPV) of BC. The pore size of thin BC films was predominantly 1.88 nm (see Fig. S8↑). The average pore diameters of a thick BC film were larger than that of a thin BC film, but the thick BC film had had a higher surface area. The pore-size of thick BC films varied from 1.66–86.3 nm and had a much broader distribution.

3.2 Cell evaluation

The stem cell evaluation was performed by CCK-8 assay to evaluate cell growth after seeding and culturing hASCs on the BC film. Fig. 3 shows a continuous promotion of proliferation of hASCs on BC films, which indicates good biocompatibility of BC as well as good biocompatibility of the control cell plate. As Fig. 4 illustrates, the proliferations of hASCs were fast. After day 9, the proliferation showed a plateau phase. The hASCs were fibroblast-shaped on the BC film and formed a single layer by the 9th day (Fig. 4A). The frozen section/HE staining of hASCs–BC complex exhibits a continuous layer, which demonstrates a nice proliferation of the stem cells on the BC film. From Fig. 4B, it exhibits a continuous layer, which demonstrates the nice proliferation of the stem cells on the BC film. The adhesion of hASCs to the BC film was good, as demonstrated by both SEM and frozen section/HE staining examinations. Owing to the nano-scale ultrastructure of the BC film, the hASCs proliferated and adhered well to the surface of BC. These findings suggest good biocompatibility of BC. The above results also indicate a wide potential for BC in tissue engineering.
3.3 Animal surgery

The observations of the full-thickness skin lesion treated with various dressings in BALB/c mice are shown in Fig. 5. The inflammation in the wound area in BC groups was less than that in the rat skin group. The xeno-transplant groups (the pig skin group and the rat skin group) showed strong immunological rejections, which resulted in sclerosis of the skin around the wound on day 11 and dark necrotic grafts on day 14 (see Table S2†). The allo-skin transplant (the mouse skin group) showed moderate inflammatory response in the wound area. However, the allografts were rejected around day 11. Reduction in the size of the skin wound area in the thick BC group was faster than that in the thin BC group, thus indicating that the thickness of BC should be over 2 mm when acting as skin repair tissue material. The wound healing in thick BC groups was faster than that in the other groups, even faster than the allo-skin transplant group. Both the photos and the HE pathological sections of the thick BC group showed the best wound healing among the seven groups. The wound size changes of the best two groups (one from the mouse skin group and the other from the thick BC group) have been quantified. The original wound size was divided by reduction rate of the wound size (equal to the wound size change), from which we gained the healing rate of both groups. The reduction rate of the wound size of the thick BC group was faster than that of the mouse skin group. The results demonstrated that the thick BC group showed a faster wound healing rate than the mouse skin group (Fig. S10†).

Due to the large holes and short drying time of sterilised gauze, it was difficult to remove from the regenerated tissue. Removing gauze resulted in pain and injury to the wound. The gauze could be separated from the surface until day 14, at which point the wound was almost fully recovered. Both the thin BC and the thick BC groups demonstrated good attachment of the BC film to the wound tissue. The transparency of BC was good enough to allow observation of the wound surface. The water content of BC was the highest amongst all the dressing materials. The plasticity of BC was as highly effective as in sterilised gauze and Vaseline gauze. The results demonstrated that the suitable mechanical properties and high biocompatibility of the BC film ensured good protection of the wound and a suitable environment for cell proliferation and tissue regeneration in the wound healing procedure.

As a natural biomaterial, BC causes less irritating stimulation to the wound tissues. The tissue regeneration and cell proliferation in the thick BC group were the best among all the groups (Fig. 6). In the skin materials control groups, severe inflammation spread from the outer skin to the inner skin, and even to the muscular tissue. The inflammatory cells were dominated by lymphocytes, macrophages, and neutrophilic granulocytes, also called polymorph nuclear neutrophils or neutrophils, on day 7. In the thick BC group, there were more fibroblasts and extensive regeneration of tissue. The inflammatory cells were mainly neutrophils and showed few lymphocytes and macrophages. In addition, there was heavier inflammation in the wound tissue in xeno-transplant groups than that in the BC or gauze groups on day 14. Unlike the groups which showed more inflammatory response, the cells of the thick BC group were mainly fibroblasts and few macrophages.

Compared to the other groups, the thick BC group showed a better healing effect, even better than the mouse skin group. We also investigated the effect of TGF-β1 and bFGF in the wound healing period. We used a naive BALB/c mouse as the control. The levels of TGF-β1 and bFGF were determined by ELISA. The results are shown in Fig. 7. The concentration of TGF-β1 in blood serum samples of naive mice was 133.9 pg mL⁻¹ while the concentration of bFGF was 1.58 pg mL⁻¹. On day 11, the thick BC group showed a good healing result. The concentration of TGF-β1 and bFGF recovered nearly to a normal level. The wounds in the thick BC group were cicatrized. A normal concentration of bFGF in the thick BC group was correlated with an active proliferation of fibroblast. In the mouse skin

![Fig. 5](image_url) Fig. 5 The macro-observations of the full-thickness skin lesion and dressing experiments in BALB/c mice (one of three same cases in each group was shown). Photographic findings of wounds covered with BC films, and controls. All skin wound windows were defects of 10 × 10 mm². Amongst all the groups, the thick BC group showed the best healing effect and fastest healing rate. All the scale bars equal 5 mm.

![Fig. 6](image_url) Fig. 6 Light microscope images of the pathological sections in BALB/c mice surgeries. HE histological examination of wounds covered with BC films, and controls. Each image was chosen from 4 random slides after the HE staining. Among all the groups, the thick BC group shows the best healing effect and the fastest healing rate. All the scale bars equal 100 μm. Arrows indicate specific cell types in the histological section; ←: fibroblast, ←: neutrophil, ←: lymphocyte, ←: macrophage.
group, a higher expression of bFGF was observed. Besides, the average time for wound healing was shorter in the thick BC group than that in the mouse skin group. For example, the wound healing in the thick BC group was 3 days faster (Fig. S10†). Overall, the thick BC group was the first condition to reach a normal level of cytokines when compared with the allograft group on day 11.

4. Discussion

The most important characteristic for most repair materials is their ability to absorb exudate in the dressing process and during removal from a wound surface. BC has demonstrated vast potential as a novel wound healing dressing and skin tissue repair material. Due to the unique nanostructure of the wet BC film, BC has high mechanical strength and remarkable physical properties. Following the purification, BC contains no impurities and no functional elements other than hydroxy groups. Due to its special structure, BC can be a natural scaffold material for the regeneration of a wide variety of tissues. As a result of its excellent biocompatibility, its application in tissue engineering scaffolds is very valuable. The properties of BC, its clinical performance, as well as the progress in the commercialization of BC for wound care fields are reported.4

It is reported that normal twisting ribbons of BC could be prepared when the incubation temperature is 28 °C.9 Since BC only grows at the interface between its culture medium and air,20 the management of the micro-architecture of the bioengineered scaffolds for artificial tissues allowed material and cell-interaction properties to be designed to mimic native materials.21 BC consists of ribbon-shaped nano-fibres structured in a refined network. BC can be synthesized by two different methods: static and shaken cultures.22 In this study, by using the improved “multilayer fermentation method,” uniform BC films were biosynthesized by G. xylinus. Some papers provided pictures of BC films with an obviously rough surface.23 But we show that under the right condition, we can do something to control the growth of G. xylinus, and obtain smooth films. Of course, the surface of BC was non-uniform owing to the bundling and aggregation of thin layers or filaments in the case of no shaking after inoculation. The surface area and TPV for BC films were lower than for mesoporous BC (mesopore diameter of about 10 nm) with a specific surface area of 200 m² g⁻¹ and a pore volume about 0.5 cm³ g⁻¹.24 The mesoporous BC was prepared by the method comprised of thorough washing and sterilization of the aquogel, quantitative solvent exchange, and subsequent drying with supercritical carbon dioxide at 40 °C and 100 bar. Despite this, the shape of the pore size distribution of thick BC films was found to be similar for all the BC samples with maximum distribution of pore size (1.88 nm) as shown in Fig. S8†. This is surprising, since the average pore diameter and surface area show significant differences. This might be related to the activity and the movements of bacteria being influenced by shaken frequency. The impact of the multilayer fermentation method on the behavior of bacteria is worthy of further study.

As a skin tissue dressing, the mechanical properties must be investigated to discover whether a similarity in elongation is needed. The tensile test showed a similar elongation at break of the BC film with that of the BALB/c mouse. The network structures of BC and the tensile test results showed similarities to that of a collagen network; the BC might therefore be able to share in the extensive application of collagen. Instead of using high temperature, the magnetic Ag nanocomposite has also been demonstrated by Suresh Kumar et al. for the ability of stabilizing bacterial culture medium.25 Some heat-sensitive biopolymers might be used in the in situ modification of BC, e.g., collagen, silk protein, or β-glucan, hyaluronic acid, and so on.

BC was found to be very attractive for applications in cell immobilization, cell migration, and the production of extracellular matrices. The fact that neither the implant of BC, fibrosis, capsule formation, nor giant cells were detected around the implants and connective tissue was also very nicely integrated with the BC structures demonstrated that BC did not elicit any foreign-body reaction. Cytotoxicity is a critical factor to assess the biocompatibility of biomedical materials.26 Though the structures on the two sides of the BC film were slightly different, there was no significant difference in the healing effect between them. Thus in the experiments, there was no distinction to either side of the BC film and therefore no factor to dictate a choice of either side as dressing. The metabolism of the cell contacting any materials could vary depending on the surface properties of the material to which the cell attached. It was important to consider the chemistry and topography on the surface of the material.

In the device design, the ideal materials should facilitate cell growth, organisation, and differentiation. There is a great amount of research on the skin tissue engineering devices, which use different types of materials in association with different cells.27 Chiaopakobkij et al.28 reported that pure BC supported the proliferation of human keratinocytes and gingival fibroblasts.28 The SEM morphologies of hASCs on BC film are similar to that of an asymmetric film with one more compact side and another more porous side. In the microscopy images, the cells showed a migration along the nanofibril network of BC. On the basis of our previous cytotoxicity result of BC as the skin tissue repair materials, animal surgery was used to further evaluate the wound healing ability of BC.

Since regular stem cells were not suitable for the present study, whilst the use of embryo stem cells face many ethical challenges, and there is but a limited supply of mesenchymal stem cells,
adipose-derived stem cells became a favourite for use in tissue engineering. hASCs can be safely and readily isolated from adult humans in large quantities without extended time for expansion. They are also easy to maintain in culture. Bodin et al. reported that microporous BC scaffolds were seeded with urine-derived stem cells (USC), which were then induced to differentiate into urothelial and smooth muscle cells (SMC). 29 Mendes et al. reported that in vitro evaluation of the interaction between cells and BC was performed through viability staining analysis of the cells over the biomaterial, which showed that 95% of the mesenchymal stem cells that migrated into the cellulose film were alive and that 5% were dead. The scanning electron microscope observed mesenchymal stem cells with normal morphology attached to the cellulose film surface. 30 Further studies of the differentiation fate of hASCs on these skin repair materials need to be performed.

Naturally derived materials have attracted growing interest as promising tools in cell transplantation and tissue engineering applications. 31 We have compared the healing effect on full thickness skin wounds with seven different materials in order to find the best skin tissue repair material and improve the functional outcome of BC. After transplanted on the back of BALB/c mice, the skins from pig and rat were immunorejected by the BALB/c mice but BC was not, indicating that BC is compatible with the BALB/c mice. Good biocompatibility of BC with the pig and rat models has been demonstrated in the literature. 32,33,34 The biocompatibility of BC has been investigated and demonstrated successfully in the past, in applications such as artificial skin, artificial blood vessels, artificial cornea, artificial heart valve prosthesis, artificial urethra, artificial bone, artificial cartilage, artificial knee menisci, 35 and deliveries of drug, hormone, and protein. 36 Schumann et al. implanted BC scaffolds to replace the carotid arteries of pigs and found no macroscopic signs of inflammation around the implants. 37 Helenius et al. implanted BC subcutaneously into rats and found no macroscopic signs of inflammation around the implants. 38

When the full-thickness skin tissue is damaged, it does not have the inherent potential to regenerate; the skin regeneration is guaranteed by the skin tissue around the wound area. Moreover, the regeneration of follicular and hair in the wound area is faster than that of other non-wound skin areas. 39 Encouragingly, we also have found that a BC film with no active cells showed a similar effect on the growth of follicles and compared hair with the tail skin of the allo-transplant group. This finding is most likely due to the fact that regeneration of follicular and hair does fatefuly depend on the skin transplant materials. In the present case, the reason for the slight inflammations in the BC groups might be related to the nano superstructure of the BC. By contrast, the rehabilitation of the wound surface was fastest in the BC group. There were multiple fibroblasts and extensive tissue regeneration. The BC skin tissue repair material evidently has a regenerative effect in promoting the healing of epithelial tissue and reducing inflammation.

The absence of toxicity in vitro and in vivo supports the view that BC may be amenable for use as tissue engineering biomaterial. 40 In terms of the tissue compatibility in vivo and the healing effect on mice, generally interactions between BC materials with cells in the wound tissues lead to slight inflammatory and non-immune responses. The underlying processes in BC induced tissue regeneration are complicated and are not yet fully understood. 35,36 Owing to the good biocompatibility and the physical barrier of BC, the results in animal surgery show that the BC can protect the wound tissue and create suitable conditions for wound healing and tissue regeneration. Compared with the two gauzes, the nano-structure and nano-fibres of BC might provide better biocompatibility. Healing in the thick BC group was faster than that in the thin BC group, which may be due to the differences in thickness and physical barriers. Both mass transfer and the nano-structure effects play the main roles on wound healing.

TGF-b1 is a polypeptide member of the transforming growth factor beta superfamily of cytokines. While TGF-b1 inhibits epithelial, endothelial, and hematopoietic cells, it stimulates the growth of some mesenchymal cells, such as fibroblasts. 41 As a member of the fibroblast growth factor family, bFGF shows mitogenic effects on fibroblasts, endothelial cells, smooth muscle cells, and osteoblasts. It could mediate the formation of new blood vessels: a process known as angiogenesis. 42,43 From Fig. 7, the direct impacts on the expression of TGF-b1 and bFGF were not observed between the thick BC group and the mouse skin group. It seems apparent that the stage of the wound was related to the expression of the factors. The classic model of wound healing is divided into three sequential phases: (1) inflammatory phase, (2) proliferative phase, and (3) remodeling phase. 44 The ELISA assay demonstrated that the groups treated with thick BC films had the fastest healing process, especially during the remodeling phase. The healing effect of BC can be attributed to multiple factors. Further studies are needed to explore the molecular mechanism of skin tissue repair materials from BC.

At a nanoscale network, the biocompatibility of BC is reported to be perfect for tissue engineering scaffolds and medicine delivery. 45,46 BC especially fulfilled several criteria for an optimal skin tissue repair material with characteristics such as moderate strength and elastic properties, support for attachment, proliferation, cell migration, and obvious improvement in healing speed. 47 The spatial arrangement of BC nanofibrils provides it with the potential of smart material design, 48 which might induce the growth and differentiation of stem cells, such as hASCs. Confirming this, different BC products have been successfully applied as skin tissue repair and wound dressing materials. In addition, BC could have other applications in wound healing and regenerative medicine, such as guided tissue regeneration, periodontal treatments, or as a replacement for dura mater (the film surrounding brain tissue). 49

5. Conclusions

The skin tissue repair materials based on BC have been biosynthesized by G. xylinus. Multilayer BC has a similar nanostructured network as normal BC. However, the multilayer fermentation method is more efficient to control the reproducible thickness and homogeneity of BC. Until today, gauze is a major clinical wound dressing. Manufacturing and marketing BC based wound dressings and/or skin tissue repair materials require reproducible biosynthesis and application. We feel certain that the multilayer fermentation method will soon bring a reliable bioreactor design. In vitro and in vivo studies indicate better biocompatibility and histocompatibility of BC. The BC film can promote the proliferation and adhesion of hASCs. Histological examinations demonstrated significant fresh tissue regeneration.
and capillary formation in the wound area in the BC groups on day 7 in comparison with those in other groups. Pathological studies also showed a faster and better healing effect and less inflammatory response in the BC groups than those in other groups on day 14. Furthermore, the thick BC film group showed a better and faster healing effect than the allo-skin transplant group. These results indicate the high clinical potential of the BC biosynthesized by the multilayer fermentation method. Thus, the skin tissue repair materials from BC exhibit better integrated properties that are promising for use in the skin tissue regeneration field and wound dressing field.

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