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Bacterial cellulose and bacterial cellulose-vaccarin membranes for wound healing

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ABSTRACT

Bacterial cellulose (BC) and bacterial cellulose-vaccarin (BC-Vac) membranes were successfully produced in large scale. BC was synthesized by *Gluconacetobacter xylinum*. BC-Vac membranes were prepared by immersing BC in vaccarin solution. The surface morphologies of BC and BC-Vac membranes were examined by a scanning electron microscope (SEM) and an atomic force microscopy (AFM). The images showed that BC-Vac exhibited the characteristic 3D nanofibrillar network of BC matrix but there was adhesion between fibers. The mechanical properties of BC and BC-Vac membranes were evaluated and the results indicated that the adding of drug vaccarin into the BC membranes increased the malleability indicated by the increment in elongation at break compared with BC. Fourier transform infrared spectroscopy (FTIR) analysis was conducted to confirm the incorporation of vaccarin in BC-Vac and investigate the hydroxyl interactions between BC and drug vaccarin. Cell viability and cell attachment studies demonstrated that BC and BC-Vac membranes had no cytotoxicity and could be a good carrier for cell growth. The wound healing performance was examined in vivo by rat skin models. Histological observations revealed that wounds treated with BC-Vac epithelialized and regenerated faster than treated with BC. Therefore, BC-Vac was considered as a potential candidate for wound dressing materials.

1. Introduction

Cellulose, a water-insoluble polysaccharide, is well known as the most abundant macromolecule in nature [1,2]. It can be produced by vascular plants and many microorganisms, such as the genera *Gluconacetobacter*, *Rhizobium*, *Agrobacterium*, *Rhodobacter* and *Sarcina* [3,4]. Bacterial cellulose (BC) and plant cellulose possess similar chemical structure, but the self-assembling nanofibrous structure of BC is different from that of plant cellulose [5]. Moreover, BC presents high purity and contaminants such as lignin, pectin, and hemicellulose, which makes it be able to retain a greater degree of polymerization [1]. Since the first report of BC production, there have been plenty of studies about the biosynthesis, production and applications of BC [6–8]. BC is produced in the form of a swollen membrane (~99%) in the interface air/culture medium and is prospective natural cellulose.

With a fibrous structure of three-dimensional non-woven network and highly hydrophilic nature, BC exhibits unique physic-mechanical properties such as high water absorption capacity, tensile strength, good permeability, crystallinity and biocompatibility, which makes BC useful in a wide range of applications in various fields, especially in the aspect of wound dressings and tissue engineering. The ideal wound dressing materials could accelerate the healing process, prevent infection, and restore the structure and function of the skin [9]. Due to the nanometer-size distribution and random alignment within the BC fibers, BC would be able to mimic the structure of the naturalextracellular matrix (ECM), which could encourage the proliferation of epithelial cells and the formation of new tissue [10]. Moreover, the inherent properties make BC be an excellent wound healing system with higher sorption of liquids for cleaning wound exudates, permitting cell respiration, high-gas permeation and easy and painless dressing change without damage to the newly formed epithelial lining skin [11–13]. Various polymers and biopolymers have been incorporated as bacterial cellulose-based materials and widely used in the field of wound dressing. Almeida et al. [14] investigated the BC-glycerin membranes and first used for human skin irritation evaluation under occlusion. UI-Islam et al. [13] studied the BC-MMT nanoreinforced composites facilitating the development of BC sheets as wound dressings and regeneration materials for therapeutic applications without any side effects. Lin et al. [5] prepared BC-chitosan membranes and assessed their cytocompatibility and antibacterial activities. The effects of the membranes on wound healing were also examined in rat skin models.

Vaccarin, a major flavonoid glycoside in Vaccariae semen, can attenuate endothelial cell oxidative stress injury in association with neovascularization in vitro.

and in vivo. Angiogenesis is a multistep complicated process that involves numerous steps, which can be conceptually visualized as initial

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activation phase of endothelial cells (ECs) that often results in release of proteases leading to degradation of the basement membrane. Then, ECs migrate to newly formed interstitial space followed by cell proliferation phase forming new vessels. Finally, blood flow initiates and new vasculature attains stability. [15,16]. A research showed that vaccarin promotes endothelial cell proliferation in association with neovascularization, which is considered one of the major active constituents and has gained ever increasing research attention [17].

In this study, BC membranes were produced using *Gluconacetobacter xylinum*. BC-vaccarin (BC-Vac) membranes were first prepared by treating BC membranes with vaccarin solution. The surface morphology, tensile strength and chemical properties of BC and BC-Vac membranes were evaluated. Cytocompatibility including cell viability and cell attachment was assessed in vitro. For the prepared BC and BC-Vac membranes, their wound healing performance was examined in vivo by using rat skin models and histological observation. The composite BC-Vac membranes were developed in the purpose of accelerating the wound healing process.

2. Experimental

2.1. Materials

G. xylinum was kindly provided by Donghua University (China). Glucose, bacto-peptone, and citric acid were purchased from Sinopharm Chemical Reagent Co., Ltd. Disodium hydrogen phosphate, potassium phosphate monobasic, acetyl fluorescein, hematoxylin and eosin were received from Sinopharm Chemical Reagent Co. Ltd. (China). Vaccarin was purchased from Shanghai Shifeng Technology Co., Ltd. (Shanghai, China). Fetal Bovine Serum (FBS) was purchased from CLARK Bioscience (USA). The mouse skin fibroblast cells (L929) were obtained from Shanghai Institutes for Biological Sciences. ICR male mice were gained from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China). Penicillin, streptomycin, Dulbecco's Modified Eagle's Medium and 24-well and 96-well cell culture plates were purchased from Key GEN Bio TECH Ltd. (Nanjing, Jiangsu Province, China). All the chemicals were of analytical grade. Deionized water was used for the preparation of all solutions.

All animal experiments were conducted according to the Guides for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), and were approved by the animal ethics committees of Jiangnan University (JN NO 20130327-0702).

2.2. BC production and purification

BC membranes were produced using *G. xylinum*. The bacterium was cultured in Hestrin and Schramm (HS) liquid medium in static condition. The medium was composed of 5% (w/v) glucose, 1.6% (w/v) bacto-peptone, 0.2% (w/v) citric acid, 0.2% (w/v) disodium hydrogen phosphate, 0.3% (w/v) potassium phosphate monobasic [18]. The liquid medium was firstly sterilized at 120 °C in autoclave for 2 h. Cells pre-cultured in a test tube containing a small cellulose membrane on the surface of the liquid medium were inoculated into 100 mL Erlenmeyer flask containing 10 mL of HS medium [19]. The flasks were kept at 30 °C for 7 days in the bacteriological incubator. After the incubation period, the BC membranes were withdrawn from the culture medium and treated with 1% (w/v) NaOH at 80 °C for 2 h. This procedure was repeated three times in order to eliminate remove components of the culture liquid. Then the membranes were rinsed 3 days in deionized water until its whitening and reaching pH 7.0.

2.3. Preparation of BC and BC-vaccarin (BC-Vac) membranes

BC disks (7 cm diameter) were prepared from 2 mm thick wet membranes (~47 mg dry weight). BC disks for testing were placed in a Petri dish and dried at 50 °C in a ventilated oven for 24 h, BC water content was carefully lowered to 50% by hand pressing between two polytef plates at room temperature [20]. BC-Vac membranes were prepared by soaking BC disks in 10 mL of aqueous solution of 2 mg vaccarin for 24 h in order to assure complete absorption of vaccarin. After the total absorption of the solution, BC-Vac membranes were placed over a petri dish and dried as described above. The dried BC and BC-Vac membranes were kept in a desiccator until their use.

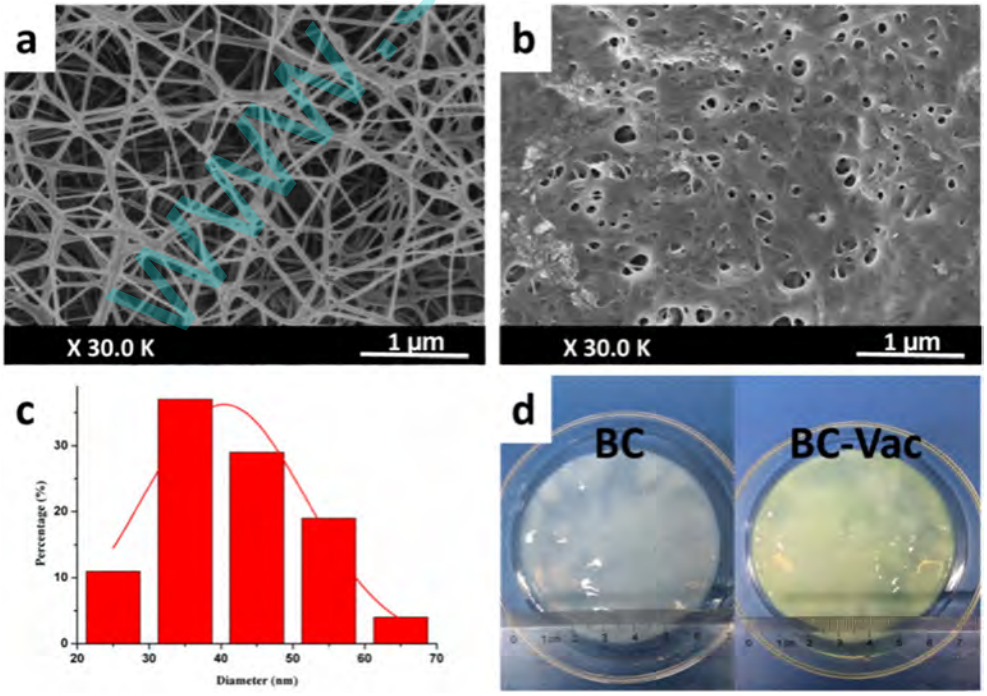

Fig. 1. SEM images of BC (a) and BC-Vac (b) membranes, diameter distribution of BC membrane (c), digital photos of BC and BC-Vac membranes (d).

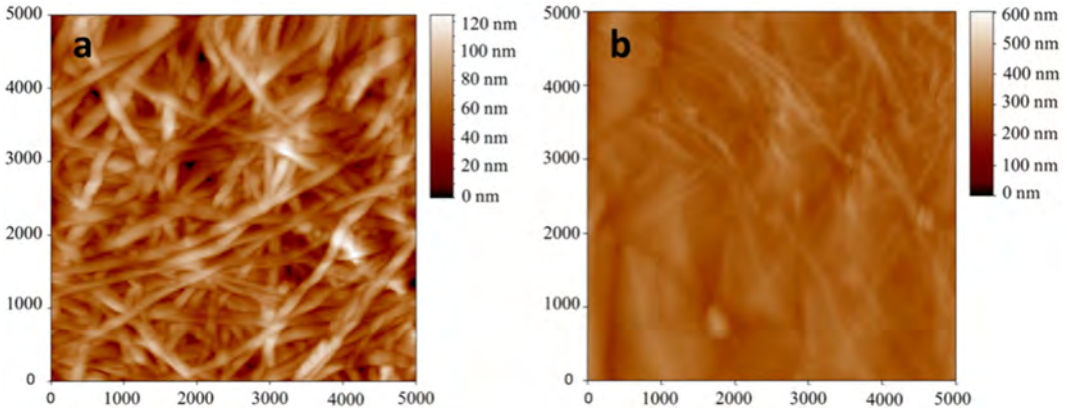

Fig. 2. AFM images of BC (a) and BC-Vac (b) membranes.

2.4. Characterization

2.4.1. Morphology of BC and BC-vaccarin (BC-Vac) membranes

Field emission scanning electron microscopy (FESEM, S-4800, Hitachi Zosen Corporation, Japan) and atomic force microscopy (AFM, Benyuan CSPM 4000) with tapping mode were used to investigate the surface morphologies of dried BC and BC-Vac membranes. The samples were sputter coated with a thin layer of gold before the SEM imaging.

2.4.2. Mechanical properties

Mechanical properties of the dried BC and BC-Vac membranes were tested using a uniaxial testing machine (INSTRON1185) at a crosshead speed of 10 mm/min and gauge length of 5 cm. The samples were cut in rectangular form of 6 cm in length, 1 cm in width. The thicknesses of the samples were measured using a DUALSCOPE MPO digital micrometer having a precision of 1 μm. The results were an average based on at least 5 tests for each material.

2.4.3. Chemical analysis

The chemical functional groups in the range of 4000–500 cm^{-1} of BC and BC-Vac membranes were investigated by Fourier transform infrared spectroscopy (FTIR, Nicolet Nexus, Thermo Electron Corporation) using ATR reflection. The spectra were recorded with 16 scans at a resolution of 4 cm^{-1}.

2.5. In vitro cytotoxicity studies

Cell line L929 (mouse skin fibroblast cells) were used for the in vitro cytotoxicity studies. Cells were initially maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA), supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin and 100 μg/mL streptomycin. The dishes were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and the culture medium was changed once every 1–2 days. Changes in cell viability caused by the contact with membranes were evaluated by indirect cytotoxicity tests of the MTT assay [21]. The attachment of cells on the membranes was evaluated by direct cytotoxicity tests of the MTT assay.

2.5.1. Cell viability study

The indirect method was carried out by seeding L929 cells into 96-well plates followed by incubation overnight. BC and BC-Vac membranes were cut into the samples with the area of approximately 3 cm^2 and sterilized by ethanol evaporation for 24 h and UV irradiation for 2 h. A certain amount of DMEM were used to incubate the sterilized membranes according to the Chinese standard ISO10993. After incubation, 100 μL of the media extract was transferred into each well. Cells from the indirect tests were incubated for 24 h and 72 h and tested with the MTT assay. The randomly chosen fields of cells were photographed at 100× amplification under a microscope video system (Olympus, IX70, Japan). The optical densities were measured at 570 nm with a 630 nm reference wavelength using a microplate spectrophotometer (EPOCH, Bio Tek Instruments, Inc. Highland Park). At each time point, six samples were used to measure the number of the viable cells. The percentage of viable cells was calculated using the following formula (1):

$$\%\text{cell viability} = A_{membrane}/A_{control} \times 100. \quad (1)$$

Where $A_{membrane}$ is the absorbance at 570 nm of the cells with membranes, and $A_{control}$ is the absorbance at 570 nm of the control cells.

2.5.2. Cell attachment studies

For direct cytotoxicity tests, 1.5 cm diameter membranes were incubated with 1 mL culture medium in 24-well plates for 24 h and L929 cells were seeded onto the membranes at a density of 10.0×10^4 cells/well.

The growth of L929 cells on the BC and BC-Vac membranes was qualitatively analyzed using SEM. In order to observe cell attachment manner on the membranes, chemical fixation of cells was carried out in each sample. After 48 h of incubation, the membranes were rinsed twice with PBS and subsequently fixed in 2.5% glutaraldehyde for 1 h. After that the samples were rinsed with deionized water and then dehydrated with ethanol. Finally, the samples were kept in a vacuum oven and then sputter coated with gold for the cell morphology observation by using SEM. For the fluorescent observation, cells were seeded on the surface of matrixes plated in 24-well cell culture plates at a density of 10×10^4 cell per well and incubated for 24 h. After that the culture media was removed and the culture plates were washed with PBS for three times. 0.5 mL acetyl fluorescein (FDA, 0.05 mg/mL in PBS) was added into the cells and the plates were stained for 30 min in dark. Then the dye liquor was removed and washed with PBS to remove excessive dye. Cells were observed under a fluorescent inverted microscope (Ti–U, Nikon, Japan).

Table 1
Thickness, tensile strength and elongation at break of dried BC and BC-Vac membranes, obtained from mechanical tensile assays.

Sample	Thickness (mm)	Tensile strength (MPa)	Elongation at break (%)
BC	0.796 ± 0.051	400.60 ± 51.19	9.56 ± 5.32
BC-Vac	0.851 ± 0.032	459.73 ± 48.21	19.36 ± 10.45

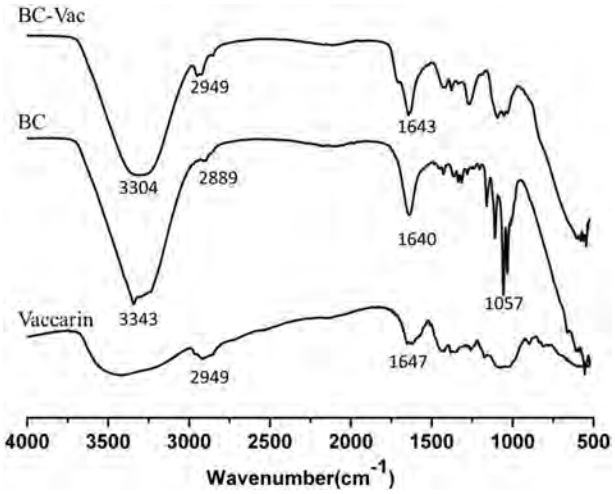

Fig. 3. ATR–FTIR spectra of Vaccarin, BC and BC-Vac membranes.

2.6. Wound healing assay and histological examination

The in vivo wound healing was evaluated using ICR male mice with an average body weight of 25–30 g. After anesthesia and hair removal, one 10 mm × 10 mm wound was prepared on each of the rat's back. Animals were divided into two groups. One is the controlled group, in which each wound was covered with the commercial dressing. Petrolatum gauze and nano silver dressing with a size of 10 mm × 10 mm were applied on each wound, respectively. In the second group, each wound was covered with the same size of BC and BC-Vac membranes, respectively. After the wound dressing materials were applied on each wound, they were fixed with the self-adhering elastic bandage. No dressings were replaced during the whole healing process. 8 mice were used in each group which was approved by the animal ethics committees of Jiangnan University (JN NO 20130327-0702).The mice had access to a standard diet and water ad libitum. All mice were housed in individual cages and maintained in a controlled environment with a 12-h light/12-h dark cycle at 24 ± 2 °C and 60 ± 5% relative humidity. After various postoperative days, standardized digital photographs of the wounds were taken and the wounds size were measured. For the histological examination, the wound dressings were removed from the wounds and the tissues samples were obtained from the wound and the surrounding skin. One sample was used to evaluate from each animal. The pieces of tissues were fixed in a 4% formaldehyde solution for 24 h and embedded in paraffin. The specimens were sectioned into 5-μm thick slices in perpendicular to the surface of wound. The histological sections were stained with hematoxylin and eosin (H&E) and examined under an optical microscope (BX53, Olympus, Japan). The pathologic picture was chosen randomly.

3. Results and discussion

3.1. Membrane characterization

The morphologies of the BC and BC-Vac membranes are shown in Fig. 1a and b. Fig. 1a displays the SEM image of pure BC membrane, which can be seen that BC had a three-dimensional (3D) network structure with an average diameter of 39.1 ± 9.3 nm, as indicated in Fig. 1c. After the soaking of drug vaccarin into the BC membrane, the surface morphology of BC-Vac membrane changed which is shown in Fig. 1b. The characteristic 3D nanofibrillar network of BC matrix could still be observed, but there was adhesion between fibers. It was due to the intermolecular attraction forces between BC and drug vaccarin. Fig. 1d shows the digital photos of BC and BC-Vac membranes. BC membrane was translucent while BC-Vac membrane appeared a light yellow color and quite homogeneous, which clearly indicates a good dispersion of vaccarin inside the BC nanofibrillar network surely due to the second-order interactions, such as electrostatic interactions, hydrogen bonding.

AFM images were obtained to visualize the shapes and surface morphologies of BC and BC-Vac membranes, as presented in Fig. 2. The pure BC membrane (Fig. 2a), it shows clear nanofibrillar network structure while BC-Vac membrane (Fig. 2b) does not exhibit very clear fibrous network. The AFM images were consistent with the SEM results and both of them could confirm the incorporation of drug vaccarin into the BC membranes.

In terms of mechanical properties, BC membrane has an asymmetric structure composed of a fine network of nanofibrils which makes it have high tensile strength [2]. The tensile strength of BC was about 400 MPa, which was higher than that of previous studies [5]. The addition of drug vaccarin into the BC membranes increased the tensile strength and malleability as indicated by the increment in elongation at break compared with BC (Table 1). In addition, BC-Vac membranes were thicker and had greater elasticity. This feature is quite significant for the clinical application of BC membranes since bendable type materials are easier to be fixed and removed [20].

Compatibility among the components is essential for producing high-quality and stable of drug-loaded membranes. Fourier transform infrared spectroscopy analysis (Fig. 3) was conducted to investigate the interactions between BC and drug vaccarin. BC and vaccarin molecules possess free hydroxyl (acting as potential proton donors for hydrogen bonding) and carbonyl (potential proton receptors) groups. Vaccarin shows the absorption band assigned to the hydroxyl group and hydrogen bond at 3200–3600 cm^{-1} and the characteristic peak at 1647 cm^{-1} was associated with the stretching vibrations on carbonyl groups. The band at 2949 cm^{-1} appears to be an indicative of the

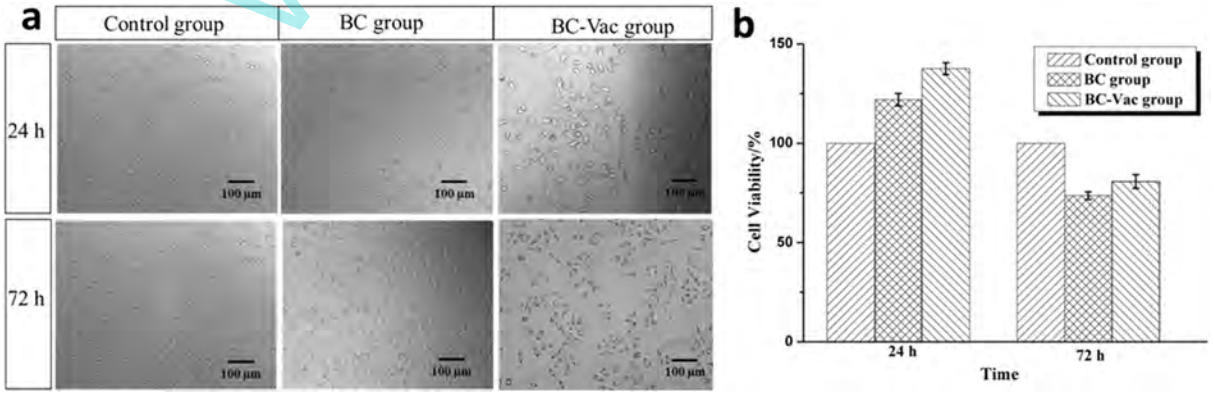

Fig. 4. Cytotoxicity (a) and cell viability (b) of BC and BC-Vac membranes towards L929 cells with the indirect method. The viability of control cells was set at 100%. Values are mean ± SD.

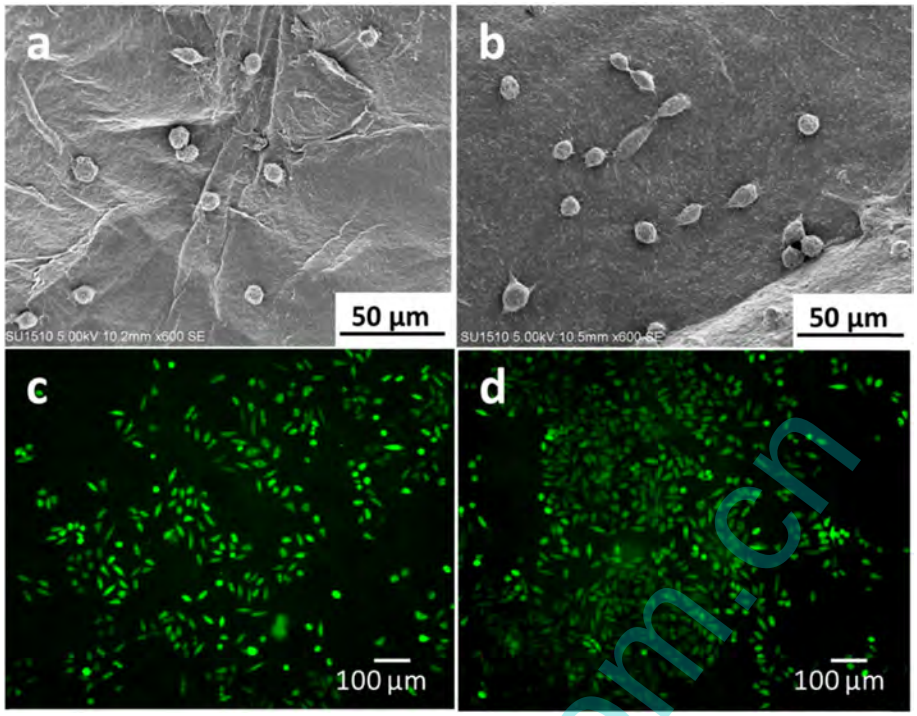

Fig. 5. SEM images and fluorescent dye figures of cell attachment on the BC (a, c) and BC-Vac (b, d) membranes.

vaccarin presence. BC membranes, presented the typical FTIR spectra of cellulosic substrates with strong bands at around 3343, 2889, 1640 and 1057 cm^{-1}. The strong band at 3343 cm^{-1} arose from the stretching of hydroxyl groups. The bands at 2889 and 1640 cm^{-1} originated from the C–H stretching and the H–O–H bending of the absorbed water. A strong band at 1057 cm^{-1} was due to the C–O–C pyranose ring skeletal vibration [22]. Compared with the spectra of pure BC, it can be seen that the absorption peaks at 3343 cm^{-1} and 1640 cm^{-1} in the spectrum of BC were shifted to 3304 cm^{-1} and 1643 cm^{-1} in the spectrum of BC-Vac membranes, respectively. This indicated that there was an interaction between the hydroxyl groups of BC and drug vaccarin.

3.2. Cell viability and cell attachment

A primary requirement for wound healing materials is that they must be biocompatible, which is the necessity of materials performing with an appropriate host response in a specific situation [23].The biocompatibility of the BC and BC-Vac membranes was examined by measuring cytotoxicity after indirect and direct contact, and the results are shown in Fig. 4. As shown in Fig. 4a, both BC and BC-Vac membranes showed non-toxic impact on L929 cells and the cells proliferated normally. After 24 h incubation with extracts of the BC and BC-Vac membranes, cell viability was 121.9% and 137.5% in the condition of low concentration, respectively. This was probably due to the fact that vaccarin could promote the cell proliferation [17]. After 72 h incubation, cell viability was 73.6% and 80.7%, respectively. The cell relative growth rate (RGR) for both BC and BC-Vac membranes was above 74%, which met the requirements of biomedical materials [5]. BC-Vac membrane had lower toxicity and better biocompatibility than BC membrane.

The attachment of L929 cells on the BC and BC-Vac membranes was analyzed using SEM and fluorescent inverted microscope. Fig. 5a and b showed the attached cells on the surface of BC and BC-Vac membranes.

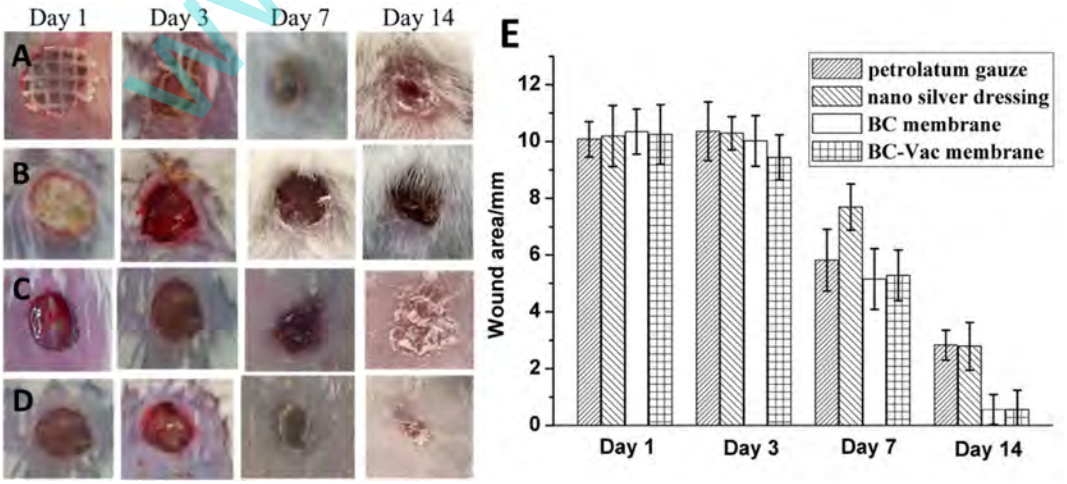

Fig. 6. Photographic images on day 1, 3, 7, 14 of wounds treated with petrolatum gauze (A), nano silver dressing (B), BC (C) and BC-Vac (D) membranes.

The images revealed that the L929 cells maintained a fusiform morphology on the membranes and the BC-Vac membranes showed better live cell morphology. Fig. 5c and d exhibited the fluorescent dye figures of cell attachment on the BC and BC-Vac membranes. It could be seen that the number of cells attached on the BC-Vac membrane was much more than that on BC membranes. These results demonstrated that the BC-Vac membranes may be biocompatible and safe for using as wound healing materials.

3.3. Wound healing experiments

Photographic images of skin wound healing after treated with BC, BC-Vac membranes and other commercially materials are shown in Fig. 6. Each wound was observed in 1, 3, 7, 14 days. As seen, BC and BC-Vac membranes exhibited faster and better wound healing than petrolatum gauze and nano silver dressing, which is also confirmed by Fig. 6E. After the treatment for 7 days, the wound area treated with BC and BC-Vac membranes remained less than that treated with the commercial dressings. After the treatment for 14 days, the BC and BC-Vac treated wound area was about 0.56 and 0.5 mm, respectively. Moreover, the wound covered with BC and BC-Vac membranes showed better fluid retention compared to the other two groups. It was also found that the soft and flexible BC and BC-Vac membranes could be removed from the wounds easily without causing any interference with the healing process while gauze adhered to desiccated wound surfaces and induced trauma on removal. The wounds covered with BC-Vac membranes showed better self-healing performance than BC. It may be due to the effects of drug vaccarin which can promote the endothelial tissue proliferation, which was confirmed by histological examination.

3.4. Histological examination

Histological images of skin wounds treated in different groups are shown in Fig. 7. The granulation tissue in the dermis was observed at 200× amplification in each group. Microscopic observations on day 3 showed little difference between groups. On day 7, an organized layer of necrotic tissue had covered the wound surface which was observed in the group of petrolatum gauze with numbers of inflammatory cells while in the group of nano silver dressing, a great amount of inflammatory cells were observed [23]. BC and BC-Vac membranes treated group exhibited more new neovascularization and fibroblasts and less inflammatory cells on day 7 and 14. The epithelialization process was activated at the surface of the wounds treated with BC and BC-Vac membranes [5]. On day 14, there were granulation tissue companied by inflammatory cells on the petrolatum gauze treated wounds and there was only granulation tissue on the wounds treated with nano silver dressing. On the other hand, there appeared stratified squamous epithelium, dense newborn subcutaneous tissue, collagen fiber and hyperplasticfibrous connective tissue appeared on the wounds covered with BC and BC-Vac membranes. These results confirmed the effectiveness of wound healing by BC and BC-Vac membranes. Moreover, the wound area covered with BC-Vac membranes was observed to have more active fibroblasts which could contribute to epithelialization and tissue reconstruction.

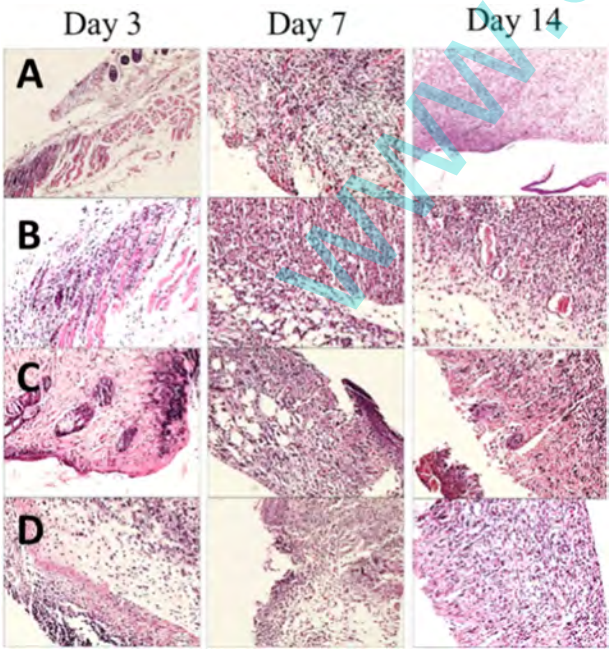

Fig. 7. Histological images on day 3, 7, 14 of wounds treated with petrolatum gauze (A), nano silver dressing (B), BC (C) and BC-Vac (D) membranes.

4. Conclusions

In the present study, BC and BC-Vac membranes were successfully prepared as wound healing materials. BC-Vac membranes showed better physical and mechanical properties than BC membranes which were probably more suitable for this purpose. Cell viability and cell attachment in vitro showed nontoxicity of BC and BC-Vac membranes. In addition, BC-Vac membranes exhibited improved biocompatibility due to that drug vaccarin can promote the endothelial tissue proliferation. Animal studies showed that BC-Vac-covered wounds healed more rapidly than wounds covered with BC and other commercial dressing materials. Histological examinations demonstrated that more active fibroblasts and epithelialization were observed in the wounds treated by BC-Vac membranes than others. Considering the properties of BC and the nature of drug vaccarin as well as their clinical performance, the commercialization of BC-Vac membranes for wound dressing materials seems very promising.

Acknowledgments

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