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Bacterial cellulose/hyaluronan nanocomposite biomaterials as wound dressings for severe skin injury repair

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Bacterial cellulose (BC), a network of pure cellulose nanofibers with fine crystallinity, high mechanical strength and wet capability, and good biocompatibility, is a good material candidate for wound dressing. Hyaluronan (HA) has obvious curative properties, promoting the healing of wound skin tissue and reducing scar formation. This study explored an "orifice plate" culture method to obtain BC samples of different sizes but consistent thicknesses. Novel BC/HA nanocomposites with a 3-D network structure were obtained through a solution impregnation method. The total surface area and pore volume of the BC/HA composite films gradually decreased with the increase of HA content. The elongation of BC/HA composite films at break point gradually increased as the HA content increased while the tensile strength of the BC/HA composite films decreased during the same process. The BC/HA composite films had a better water uptake capability than pure BC, and water vapor transmission rate (WVTR) measurements showed that the BC/HA composite films can satisfy breathing requirements of injured skin. The BC/HA composite films facilitated the growth of primary human fibroblast cells, showing their low toxicity, and the BC/HA with 0.1% HA lead to higher levels of cell viability than the pure BC. In vivo experiments indicated that the BC/HA with 0.1% HA had the shortest wound healing time while BC/HA with 0.05% HA yielded best tissue repair results. The BC/HA composite films are expected to be useful as novel wound dressing materials for clinical skin repair.

1. Introduction

Every year, more than 6 million patients suffer from severe burns and over 0.3 million person ultimately die from these injuries worldwide.[1, 2] The treatment of acute and chronic or large surface area wounds has relied upon dressings or artificial skin to cover them. Wound healing is one of major importance for the survival and positive clinical outcome of burn patients.[3] Injured fetal tissues can be completely recreated without fibrosis during early gestation, but the wound repair process commonly leads to scars in human adults.[4] Scarring and fibrosis, often observed in surgical recovery and wound healing, also cause reduced functional restoration through the formation of a non-functional mass of fibrotic tissue.[5] Many studies have been devoted to finding good bioactive materials for use as wound dressings to optimize the rate of healing and the aesthetic repair of wounds. Various wound dressing materials have been developed to prevent infection, absorb wound exudates and allow the diffusion of atmospheric oxygen to the wound.[6,7] However, wet environment was needed in wound healing and lack of water in wound can affect cell metabolism and lead to wound drying. The wet environment of wound dressing not only benefits tissue regeneration but also effectively alleviate the pain.[8] Bacterial cellulose (BC) has advantages as skin tissue repair material in terms of high mechanical strength, water uptake ability and excellent biocompatibility. Wet BC feels very soft, so its shape is variable and controllable, these properties favor the intimate contact of BC with the skin. For example, when BC was used to cover severe second-degree facial burns for 44 days, complete healing was observed.[9] Wound healing is a biological process which involves hemostasis, inflammation, migration, proliferation and maturation. Keratinocytes, fibroblasts and vascular endothelial cells are the first cell types activated by the trauma, and these cells play important roles in the skin wound healing process. Maturation consists in tissue remodeling during healing and in this stage, fibroblasts cover the surface of the wound as a new layer of skin.[6] Chiaoprakobkij et al. performed senescence analysis on cultured human keratinocytes and fibroblasts. Their findings demonstrated that BC films only supported the growth, spreading, and migration of human keratinocytes.[10] Hyaluronan (HA) is present in abundance in the body, especially in the skin, articular joints, vitreous humor of the eye, and the vasculature.[11] Exogenously, HA has good biocompatibility, biodegradable and gel-forming properties. It is useful to promote cell adhesion, migration and differentiation, and is involved in a host of cellular processes including

morphogenesis, regeneration and wound healing.[11, 12, 13] HA has obvious curative effects in promoting the healing of wound skin tissue and reducing scar formation.[14] It also has viscoelastic, lubricating and hydrating properties useful in medical and cosmetology applications including optical surgery, adhesion prevention, cosmetic formulations and cosmetic surgery.[15] HA can be produced on a large scale by biotechnology but its poor mechanical properties, rapid degradation and clearance in vivo have limited its wide spread application.

In our previous work, we used a solution impregnation method to obtain novel BC/HA nanocomposites.[16] The composites displayed enhanced properties in terms of elongation at break and thermal stability as compared to pure BC, which are important factors for materials applications in the biomedical field. In the present study, we used a "multilayer" fermentation method to obtain homogeneous BC films, and used an "orifice plate" culture method in 24- or 96-well plates to obtain BC films of different sizes. BC films cultured in flasks require a secondary sizing operation to satisfy the need of in vitro experiments, usually by cutting into square or circular samples. In the current case, the BC films were formed into round-shaped samples by growing them directly in 24- or 96-well culture plates. In this way, potential damage or structure changes on the edge of the BC membranes, leading to water retention variations causing large errors in the in vitro evaluation processes, may be avoided. The small round BC films cultured in well plates are suitable for toxicity and biological safety testing, as well as for many characterization methods for which the accumulation of statistical data requires a lot of small but uniform samples.

The mechanical strength, water uptake ability and WVTR of wound dressing, the three important parameters in skin repair, were also assayed in this work. The in vitro cytotoxicity of BC and BC/HA films were evaluated by combining two different assays: MTT with either Calcein AM/PI staining or Dead/Live cells staining. The adhesion and morphology of fibroblasts was investigated by FE-SEM. Additionally, the biocompatibility and effect of BC and BC/HA nanocomposites on open full-thickness wound healing on Wistar rats were also evaluated.

2. Materials and methods

2.1 Preparation of BC and BC-HA films

BC was biosynthesized by *Gluconacetobacter xylinum* (*G. xylinus*) (ATCC53582), by growing the bacteria on a Hestrin and Schramm (HS) medium.[17] Before incubation the medium was sterilized for 20 minutes at 121°C, and then cooled to room temperature. The bacteria were inoculated into a flask at an inoculum concentration of 10% (v/v), and transferred to the well plates. The HS medium volume in the flask, the 24-well plates and 96-well plates were 150 mL, 2.5 mL and 300 μL, respectively. We obtained multilayer and homogenized BC films by the "multilayer" fermentation method. After incubation at 30°C and a controlled humidity level of 40% in the 250 mL flask for 14 days, or in the well plates for 3 or 4 days, the BC films were dipped into sterilized water. The bacteria and proteins of BC were eliminated in three steps. The BC films were first washed for 3 days with sterilized water, then boiled with 1 wt% NaOH for 40 minutes, and finally washed with sterilized water several times every day until the pH reached 7.0. The BC films were sterilized for 20 minutes at 121°C and stored in sterilized water at either room temperature or 4°C.

Hyaluronan (HA; Mw 400-1000 kDa, purity > 98%) was purchased from the Amresco company. The BC/HA composite films were prepared by the solution impregnation method. BC/HA composite films used for performance testing were prepared by the following method: The BC films were sterilized and then loss water 3 hours on an aseptic operating table at room temperature in order to allow more HA to impregnate into BC. The BC films were then dipped into 20 mL of hyaluronan solution at a concentration of either 0.05%, 0.1%, 0.2%, or 0.5% (wt/v) for 24 h at room temperature. Freeze-dried films were obtained in three steps, namely by storing the BC/HA composite films at -20°C for 2 days, at -80°C for another 2 days, and by freeze-drying at -50°C for 24 h. The BC/HA composite films used for cell culture were prepared from steriled HA solutions obtained by filtration through a 0.22 μm membrane filter. 1 mL or 200 μL HA solution can combine with the BC films in the 24- or 96-well plates, respectively, within 24 h. The preparation processes used to obtain wet, dry, cell culture and animal experiment BC/HA composite films are described in Fig. 1.

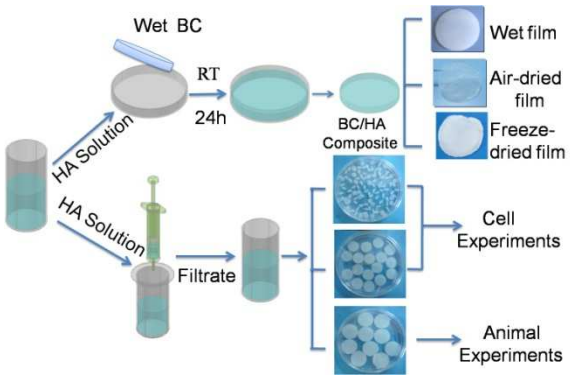

Fig.1 Preparation process for wet, air-dried and freeze-dried, cell culture, animal experiment BC/HA films.

2.2 Characterization

The surface morphology and the structure of BC, HA, BC/HA composite films and gauze were characterized by field emission scanning electron microscopy (FE-SEM, S4800) with an accelerating potential of 5.0 kV, the samples were sputter-coated with gold for 30 s.

The tensile strength of the wet BC and BC/HA composite films was measured with an universal mechanical analyzer (BTC-EXMULTI-PAC2). The films were cut into dumbbell-shaped specimens and the distance between the two clamps was 40 mm. All the samples were tested at a stretching speed of 5 mm/min.

The water uptake ability of the BC films was conducted as following steps. The BC, BC/HA composite films and gauze samples were cut into round shape with a diameter of 20 mm and then immersed in PBS solution for 10 min, 30 min, 1 h, 2 h, 4 h and 12 h at room temperature (25°C), at a controlled humidity level of 30%. The wet BC, BC/HA composite films, and gauze were quickly shaken twice to remove excess water, and then weighed. The water uptake by the films was determined using the equation $w = (w_t - w_0)/w_0$, where w_0 is the weight of the dry film

and w_t is the wet weight at different times.[10]

The moisture permeability experiments were carried out in 15 mL centrifuge tubes, with 15 mm inside and 17 mm outside diameters. Water (10 mL) was added to each tube, the BC films were placed and fixed at the opening of the tubes for the experiment group, while opened tubes were used for the control group. A precision balance (with mg resolution) was used to measure the weight of water in each tube at 0 h. The tubes were stored at 37 ± 1°C, at a controlled humidity level of 30%, and then weighed again after 24 h to determine the mass difference G. The WVTR was calculated according to the formula WVTR ($g \cdot m^{-2} \cdot d^{-1}$) = G/A·t where G is weight change of the water (g), A is the area of the test sample (m^2) and t is the duration of the test (d), respectively.[18] The effective area of the centrifuge tubes measured in this work was equal to 176.63 mm^2 (πR^2).

2.3 Cell evaluation

Primary human fibroblasts were isolated carefully from human foreskin samples, which were obtained from donors (18-25 years old) undergoing circumcision and had given their informed consent. All the procedures were approved by the ethics committee of the Wuhan Union Hospital (Wuhan, China). The foreskins were washed several times with sterile phosphate-buffered saline (PBS) (Thermo Scientific HyClone, Rockford, IL, USA) and digested as described by Häkkinen et al. for the isolation of human fibroblasts. The cells were cultured in Dulbecco-modified Eagle medium (DMEM) (HyClone, USA) with 10% fetal bovine serum (FBS) (Gibco Invitrogen, USA) and 1% penicillin-streptomycin (PS) (MP Biomedicals, USA).[19] The cell cultures were incubated at 37°C in humidified atmosphere containing 5% of CO_2. The medium was replenished every 2-3 days and the cells were transferred after 3 or 4 days. Cells obtained after 3 to 7 cycles were used for all the experiments. The total number of cells initially deposited in the 24- and 96-well plates for incubation was 8×10^3 and 4×10^3 cells/mL, respectively.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation and cytotoxicity assay was carried out as follows: Primary human fibroblasts were cultured in 96-well plates (4×10^3 cells/mL) on BC, BC/HA composite films and tissue culture plates for 1 day, 3 days, 5 days and 7 days. The medium in each 96-well plate was then removed completely and 120 μL of MTT (Beyotime, China) mixed solution was added to each culture well, which consisted of 100 μL of culture medium and 20 μL of MTT solution (0.5 mg/mL). After incubation at 37°C and in 5% CO_2 atmosphere for 4 h, 100 μL of formazan solution (Beyotime, China) was added to each well before incubation at 37°C in 5% CO_2 atmosphere for another 4 h. Finally, solution (200 μL) from each sample was transferred to a 96-well plate, OD was measured by enzyme mark instrument (Tecan, infinite F50, Switzerland), at optical absorbance of 570 nm. The cell viability index was calculated using the following equation: Cell viability (%) = ($OD_{experiment} - OD_{blank}$) / ($OD_{control} - OD_{blank}$) × 100%.[20] Five replicate measurements were obtained for each sample.

Primary human fibroblasts were cultured in 24-well plates (8×10^3 cells/mL) on BC, BC/HA composite films and tissue culture plates for 1 day, 7 days and 14 days. The culture medium was then removed and PBS (1mL) was added into each well of the plate to dilute the serum-containing esterase and to wash the cells, and the dual fluorescence Calcein AM/PI assay reagents (500 μL) (Dojindo, Japan) were added into each well before incubation for 20 min at 37°C in 5% CO_2. The fluorescent reagents were then removed and 500 μL of PBS was added to each well. A fluorescence microscope (Olympus Dp71, Th4-200, Japan) was used to record fluorescence images. Six fields were randomly selected and the number of primary human fibroblasts attached on the surface in a defined area was determined, and the primary human fibroblasts were observed at 200 × magnification.

The BC and BC/HA composite films with cells were fixed with 3% glutaraldehyde solution for 24 h at 4°C after the cell-materials were washed twice with PBS. The glutaraldehyde solution was then removed and the samples were washed twice with PBS. The cell samples were stored at -20°C for 2 days, -80°C for another 2 days, and freeze-dried at -50°C for 24 h. The samples were characterized by FE-SEM (JSM-6700F, Japan) with an accelerating potential of 5.0 kV and magnifications of 200-1000 after sputter-coating with gold for 30 s.

2.4 Animal wound model and histological analysis

Normal 8-10 week old male Wistar rats with an average weight of 400 g were used for the in vivo experiments. All the Wistar rats were purchased from the Beijing Vital River Laboratory Animal Technology Co., PR China. All the procedures involving animal use were approved by the Institutional Animal Ethical Committee (IAEC) at the Tsinghua Laboratory Animal Research Center, PR China.

The Wistar rats were randomly divided into 2 groups on the basis of the materials to be used for covering their skin wounds, which included BC, BC/HA composite films (0.05, 0.1, 0.2 and 0.5%) and gauze. Every group had 5 parallel samples. The rats were anesthetized by injection of chloral hydrate 3.5% (10 mL/kg) in the abdominal cavity. Full-thickness skin injuries were prepared by removing a round 20 mm diameter section of dorsal flank skin from the anaesthetized rats. A piece of cover material of the same size was then placed on the dorsal wound surface and fixed by gauze and skin stapler (Manipler AZ MANI, Japan). The rats were kept in a single cage for observation after the operation and recovery from the anesthesia. Photographs of the wound region were taken on day 0, 7 and 14 to visualize changes in wound size over specific time. The percentage of wound closure (wound healing rate) was calculated according to the following formula: wound closure (%) = $(A_t - A_0) / A_0 \times 100\%$.[21] where A_0 is the original wound area and A_t is the wound area at the specific time of observation.

Wound tissues were collected post-operation from each group of rats on days 7 and days 14. The wound tissues were immersed in 4% paraformaldehyde solution to fix the samples. Pathological sections from each wound tissue were analyzed by hematoxylin and eosin (H&E) and by Masson trichrome stain. Histological images were recorded on an inverted microscope (Leica DM4000M, Germany).

2.5 Statistical analysis

The values reported are expressed as mean ± standard deviation (SD). The Origin 8 software was used for graph plotting. A value of $P < 0.05$ was considered significant. Each experiment included

at least three replicates.

3. Results

3.1 Characterization

The SEM images display the surface morphology and structure of BC, HA, BC/HA composite films and gauze (Fig. 2). BC was biosynthesized by *G. xylinus* which is a kind of aerobic bacterium, and BC was more easily to form at the air/liquid interface than in the medium. We have already conformed that BC has two sides, the top side of BC appears more compact and denser fibrils than the bottom. The structure differences influence the cell growth and wound healing.[22] The BC film was composed of intercourse nanofibers. Comparatively, the HA film appeared to have a relative smooth surface. The morphology of the BC/HA composite films was very different from that of pure BC or HA, and nanofibers of the BC/HA composite films became more compact with the increase of HA concentrations. The pore size of the BC/HA composite films gradually became smaller with the increase of HA concentration as shown in Fig. 2. Fu et al. reported that the diameter of the gauze fibre is more than 70 times the diameter of BC film.[7] The dramatic difference in the fibre diameters of BC, BC/HA composite films and gauze is shown in Fig 2. The bottom side of BC and BC/HA composite films appeared looser and larger pore size than the top side of BC and BC/HA composite films. The structure of the BC, BC/HA composite films and gauze may influence their performance as wound dressing.

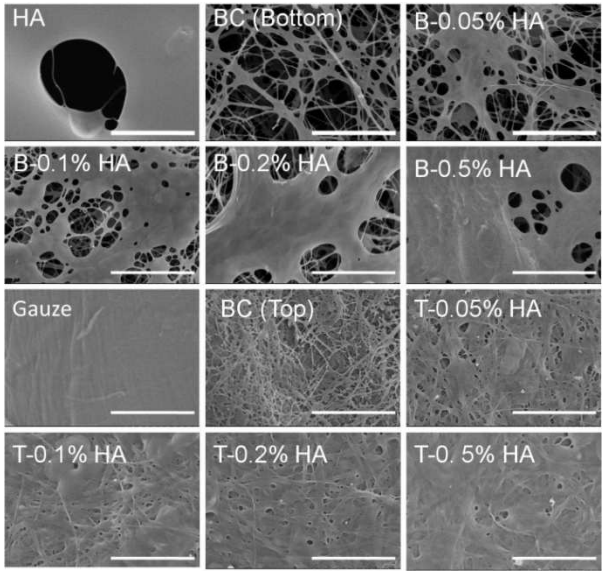

Fig.2 FE-SEM micrographs showing the morphology and surface structure of HA, BC-Bottom, the bottom side of BC/HA composite films (B-HA), gauze, BC-Top and the top side of BC/HA composite films (T-HA). The scale bar is 3 μm.

Wound dressing materials need good strength, flexibility, bending and sustainability properties.[23] In this study, the stress-strain behavior of the wet BC and BC/HA composite films was investigated at the room temperature (25°C). Evaluation of the strength and elasticity of the hydrogel films was carried out by tensile strength and elongation tests.[24] The Young's modulus of the wound dressings was determined by the value of the secant modulus at 5% strain. Young's modulus of 0.05% HA, 0.1% HA, 0.2% HA, 0.5% HA and BC were 0.61 ± 0.04, 0.64 ± 0.11, 0.55 ± 0.02, 0.47 ± 0.06 and 0.76 ± 0.13 MPa, respectively, as summarized in Table 1. This result may be related to the disruption of intermolecular hydrogen bonding in cellulose, due to the formation of cellulose-hyaluronan hydrogen bonds. The ultimate tensile of the BC/HA composite films was decreased with the increase of HA concentration. This behavior can be attributed to the intermolecular interactions between BC and HA that might reduce the crystallinity and mechanical strength of the composite materials. The elongation at break point (failure strain) of the BC/HA composite films was higher than that of pure BC, showing an increasing trend with increasing HA concentrations (Table 1). FE-SEM results showed that the density of the BC/HA composite films became more and more compact with the increase of HA concentration (Fig. 2). The elongation at break point correlates well with the amount of HA added. Li et al. reported that BC could be used as potential wound dressing while Czaja et al. showed that BC had been used as skin repair materials, all of these applications were due to its excellent mechanical strength.[25, 26] Though the ultimate tensile strength of BC/HA composite films was lower than that of pure BC, the elongation at break point of the BC/HA composite films was higher than that of the pure BC. Therefore, we expect that BC/HA composite films should be superior as wound dressing to pure BC and have the capability to be used as artificial skin.

Table 1. Quasi-static tensile mechanical properties of the BC/HA composite films and pure BC measured at room temperature. (n = 5)

Sample	Young's modulus (MPa)	Ultimate tensile strength (MPa)	Failure strain (%)
0.05% HA	0.61 ± 0.04	1.03 ± 0.02	38.39 ± 1.23
0.1% HA	0.64 ± 0.11	0.96 ± 0.04	40.11 ± 2.31
0.2% HA	0.55 ± 0.02	0.94 ± 0.02	45.57 ± 1.56
0.5% HA	0.47 ± 0.06	0.91 ± 0.01	48.14 ± 1.86
BC	0.76 ± 0.13	1.06 ± 0.03	34.52 ± 3.01

The water uptake ability of wound dressing materials plays an important role in their biomedical applications. It is a gravimetric test for the determination of the maximum amount of fluid absorption and retention on the wound for a dressing material.[27] If the wound dressings have a poor water uptake ability, this can result in the accumulation of wound exudate, and the wound will have hydroncus reducing the speed of healing. Porous wound dressing materials can help with the drainage of wound exudates and allow the permeation of atmospheric oxygen to the wound. BC has a 3D structure with nano- or micro-pores, while HA has a high capacity for lubrication, water sorption and retention. These factors can affect the water uptake ability of materials serving as wound dressings.

The water uptake ability of the BC/HA composite films, the BC and gauze was compared in Fig. 4. The water uptake ability of 0.05% HA, 0.1% HA, 0.2% HA, 0.5% HA, BC and gauze was about 46, 45, 28, 32, 44 and 8.0 times of their weight after immersion in PBS 10 min (Fig. 3). With the increase of immersing time, the water uptake ability of the dressings increased gradually and reached 57, 58, 59, 61, 52 and 9 times of their original weight for 0.05% HA, 0.1% HA, 0.2% HA and 0.5% HA, BC and gauze, respectively after 720 min (Fig. 3). The

higher values of BC and the BC/HA composite films compared to gauze maybe due to the denser nanofiber networks of the BC which present larger surface to volume ratio than that of gauze. BC/HA composite films had higher water uptake ability than pure BC, that may due to the water sorption and retention properties of HA. The HA composites with BC have slightly higher water uptake ability than pure BC, this means that BC/HA composite films may absorb more exudes and keep the wound moist for a longer time than BC-only in the wound. So, BC/HA composite films have more advantage over gauze and BC as wound dressing.

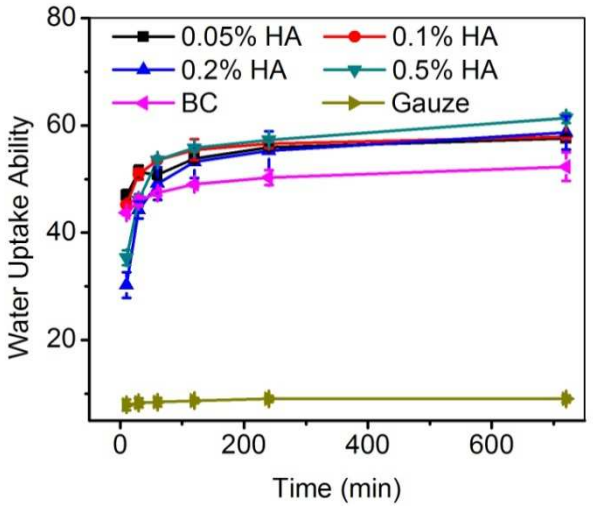

Fig.3 Water uptake ability of the BC/HA composite films, BC and gauze at room temperature (25ºC), at a relative humidity of 30%. Five replicates were analyzed and averaged for each sample.

The amount of water vapor lost through a dressing to the atmosphere from the wound bed over defined time periods was measured by WVTR test method. Wound dressings should have proper water vapor transmittance parameters. If this parameter is too high, it is difficult to keep the wound in a moist environment; if it is too low, it can affect the normal metabolism of the tissue.

In previous report, Lamke et al. determined that the evaporative water loss rate for normal skin is about 204 ± 12 $g \cdot m^{-2} \cdot d^{-1}$ at a skin temperature of 35.8 ± 0.2°C. The evaporative water loss rate from first degree and granulating wounds was 278.4 ± 1.1 and 5138 ± 202 $g \cdot m^{-2} \cdot d^{-1}$, respectively. Lamke et al. also found that the evaporative water loss could be reduced by covering with a wound dressing, such as the biological dressings can reduce the water loss by 90%, while the artificial dressings can reduce it by 73%.[28] In this study, the WVTR for one layer of gauze (G1) was about 1250 ± 130 $g \cdot m^{-2} \cdot d^{-1}$, and 1190 ± 120 $g \cdot m^{-2} \cdot d^{-1}$ for two layers of gauze (G2) (Table 2). However, the WVTR for BC was 700 ± 70 $g \cdot m^{-2} \cdot d^{-1}$, which was dramatically lower than that of the gauze. With the modification of the HA, the WVTR could be further lower, for example, 0.2% HA can decrease the WVTR of the BC to 670 ± 60 $g \cdot m^{-2} \cdot d^{-1}$, displaying the advantage of BC and HA in decreasing water loss of the wound. In clinical practice, to ensure the tight bound of the gauze to wound to prevent the infection of the wounds, several layers of gauze are often used. Gauze has a higher evaporative water loss rate than BC and the BC/HA composite films. This may be due to the large fiber and pores present in gauze, while BC and the BC/HA composite films have nano- and micro-sized pores which can reduce water evaporation from the materials. The WVTR decreased with the addition of HA. This may be due to the variation of the materials that the surface of BC/HA composite films became smoother and denser with the increased HA concentrations. Therefore, our experiment confirmed that BC and the BC/HA composite films are useful as wound dressings that can keep the wounds in a moist environment and fulfil normal skin breathing.

Table 2. Water vapor transmission rate (WVTR) of BC/HA composite films, BC and gauze investigated over 24 h at 37 ± 1°C, at a relative humidity of 30%. The test was repeated five times and the mean value of G/A/ t was calculated.

Materials	Thickness (mm)	Water vapor transmission rate(g·m-2·d-1)
Control		1860± 190
0.05% HA	0.11 ± 0.01	690 ± 60
0.1% HA	0.11 ± 0.02	690 ± 70
0.2% HA	0.11 ± 0.01	670 ± 60
0.5% H	0.12± 0.01	660 ± 70
BC (no HA)	0.10 ± 0.02	700 ± 70
G1 (one layer)	0.03 ± 0.01	1250 ± 130
G2 (two layer)	0.06 ± 0.01	1190 ± 120

3.2 Cell evaluation

BC has a higher biocompatibility, faster and better healing effect and lower inflammatory response than that in the control group (no wound dressing).[29] In this study, the morphology of primary human fibroblast cells grown on BC and the BC/HA composite films after 7 days we compared in Fig. 4. The fibroblast cells growing on BC and the BC/HA composite films displayed normal cell morphology with spindle shapes, and distributed in a radial or swirl pattern spreading to cover the material surface. Some cells still showed round shape on all the films, but for the BC films this was most obvious. The extent of cell growth on the BC/HA composite films looks better than for BC (Fig. 4 upper). FE-SEM results showed that the primary human fibroblast cells adhered more strongly to the BC and 0.05% HA films, while more cells proliferated on the surfaces of 0.1% HA, 0.2% HA, and 0.5% HA (Fig. 4 bottom). The cells need to adhere to the surface of the materials to grow and proliferate.[30] Altmann et al. confirmed that rough surfaces are more useful for cell adhesion, while smooth surfaces are better for cell proliferation.[31] Our previous study demonstrated that the surface of BC becames smoother as their HA content increased.[15] Chiaoprakobkij et al. reported that BC cannot support fibroblasts growth. [10] Laurent et al. reported that HA is synthesized in the plasma membrane of fibroblasts, its production increases in proliferating cells.[32] Therefore, the morphology of fibroblasts appeared growth better on BC/HA composite films than on pure BC.

MTT tests can be used to evaluate the activity of enzymes in the mitochondria and the integrity of the cell membrane. It can also respond to the degree of cell proliferation and activity. In this study, primary human fibroblast cells were used to evaluate their adhesion and proliferation on BC and the BC/HA composite films. Cell viability was tested after seeding the cells on the 96-well plates on BC and the BC/HA composite films after 1, 3, 5, and 7 days. The same number of cells (4×10^3 cells /mL) was added to each well. The result of MTT assay was presented in Fig. 5. BC/HA composite films supported the attachment and proliferation of the cells, and their viability were higher than that

of BC. The fact that BC has no cytotoxic effects on a variety of cells was confirmed in previous studies.[33, 34] Sanchavanakit et al. reported that BC films supported the growth, spreading, and migration of human fibroblast while not using well-based cell culture techniques.[35] Therefore, the modification of BC with HA can not only maintain the excellent biocompatibility of the BC, but also enhance the proliferation of the cells.

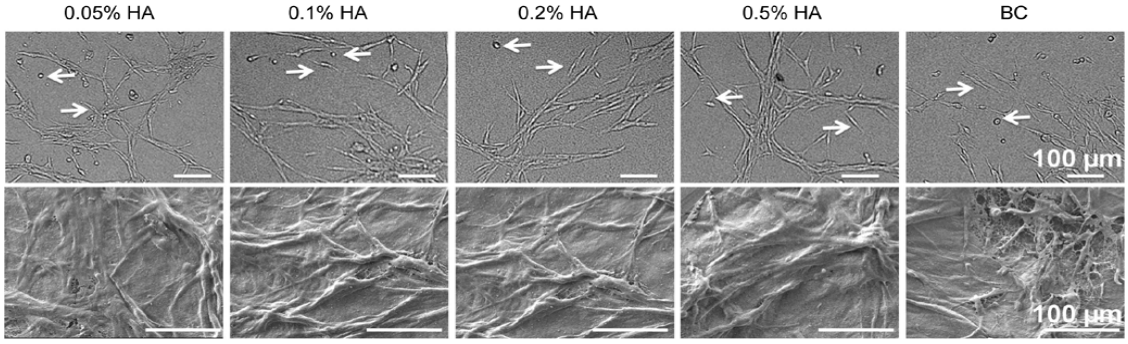

Fig.4 Light microscope images (upper) and FE-SEM images (bottom) showing the growth and morphology of primary human fibroblast cells attached to the scaffold. Round cells are indicated by an arrow (←) while spindle shape cells are indicated by an arrow (→). The light microscope images and FE-SEM images were recorded at 200 × magnifications (scale bar is 100 μm, upper) and 1000 ×magnifications (scale bar is 100 μm, bottom), respectively.

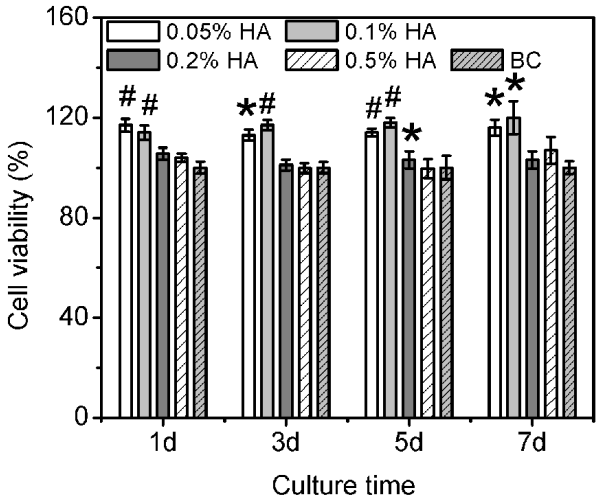

Fig.5 MTT measurement of primary human fibroblast cells viability after co-culture with the BC/HA composite films and BC on 1, 3, 5 and 7 days (n = 5). The data show significant statistical differences between the BC/HA composite films groups and BC group (*P < 0.05, # P < 0.01).

We also evaluated the viability of the primary human fibroblast cells on BC and the BC/HA composite films by Calcein AM/PI kit. Seldom dead cells appeared from day 1 to 14 on all the dressing materials. The average number of the cells attached to the surface of membranes gradually increased with time. The total number of the cells attached to the 0.05% HA, 0.1% HA films was significantly higher than the other samples on day 1, 7 or 14 (Fig. 6). The increase of the HA content apparently influence the increase of the cell number, being consistent to the previous reports. Thus, appropriate concentration of HA appeared to be more efficient in promoting cell proliferation with high viability.

3.3 Animal evaluation

Gauze is the traditional wound dressing, which is manufactured as bandages, sponges, tubular bandages and stocking. The gauze can stick to the wounds and its cost is lower than other wound dressings. Also, gauze is constituted of fiber, which structure is similar to BC. Therefore, we compared the wound healing rate between the traditional wound dressing gauze and novel hydrogel wound dressing in vivo.

In this study, the BC films, BC/HA composite films and gauze were applied as dressings on the model wounds of Wistar rat. Fig. 7A illustrates the process of establishing a full-skin injury model in the dorsal skin of the rat. The full-thickness skin injury diameter was 20 mm in the back, and the diameter of the films was also 20 mm to exactly cover the wounds. Fig. 7B shows the macroscopic appearance of excisional wounds covered with the BC and BC/HA composite films at day 0, 7 and 14. The sizes of all the wounds decreased on 7 days after the operation, and decreased more apparently after 14 days (Fig. 7B). Fig. 7B shows clear differences between BC/HA composites- and BC-treated wounds after 7 and 14 days. The size of wound treated by 0.1% BC/HA reduced the fastest after 7 and 14 days of treating.

The extent of wound skin repair was quantified by using the Image- pro plus software and plotted with Origin pro 8.0, the results are shown on Fig. 7C. The extent of wound healing was calculated by comparing wound size on day 7 and 14 with the original wound size on day 0, respectively (Fig. 7C). The wound covered with 0.1% HA had the smallest wound size on days 7 (34 ± 4%) and 14 (5 ± 1%). The wound treated with 0.05% HA also had a smaller wound size (52 ± 13% on day 7, and 14 ± 3% on day 14) than the other BC/HA composite films, BC and gauze (Fig. 7C). The wounds covered with 0.2% HA and 0.5% HA weren't significantly different from the control wounds treated with BC on days 7 and 14. The rate of wound healing was evaluated by calculation of the ratio between already repaired wound area and original wound area as a function of time, as shown in Fig. 7D. On day 7 and 14, 0.1% HA showed a highest wound healing rate than other groups did. This result was supported by macroscopic observations. This results may due to the different structure and mechanical properties between BC/HA composite films and BC films. FE-SEM results showed that both the bottom and top surface of the BC/HA composite films became denser with the increase of HA concentration (Fig. 2). The decreased pore size may influence the wound breath. Peppas et al. reported that the increase of crosslinking can enhance the

stronger of the hydrogel, but the hydrogel will become more brittle and less elastic.[36] Tensile mechanical properties of the

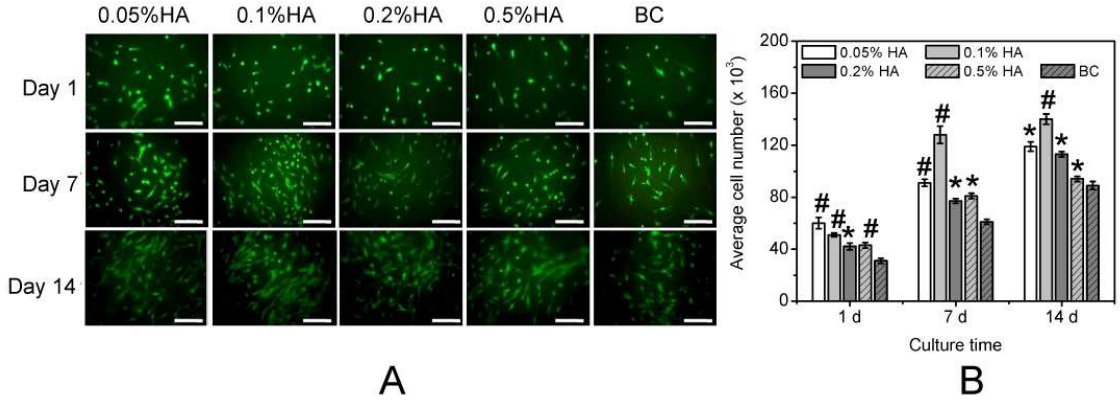

Fig.6 The photos of Calcein AM/PI staining after the primary human fibroblast cells co-cultured with BC/HA composite films and BC after 1 day, 7 days and 14 days (left). The live cells were stained with green colour while the dead cells were stained by red colour. The number of average cell was counted after the Calcein AM/PI staining (right). *The data shows significant statistical differences between the two groups ($P < 0.05$). #The data shows significant statistical differences between the two groups ($P < 0.01$). The scale bars are equal to 100 μm.

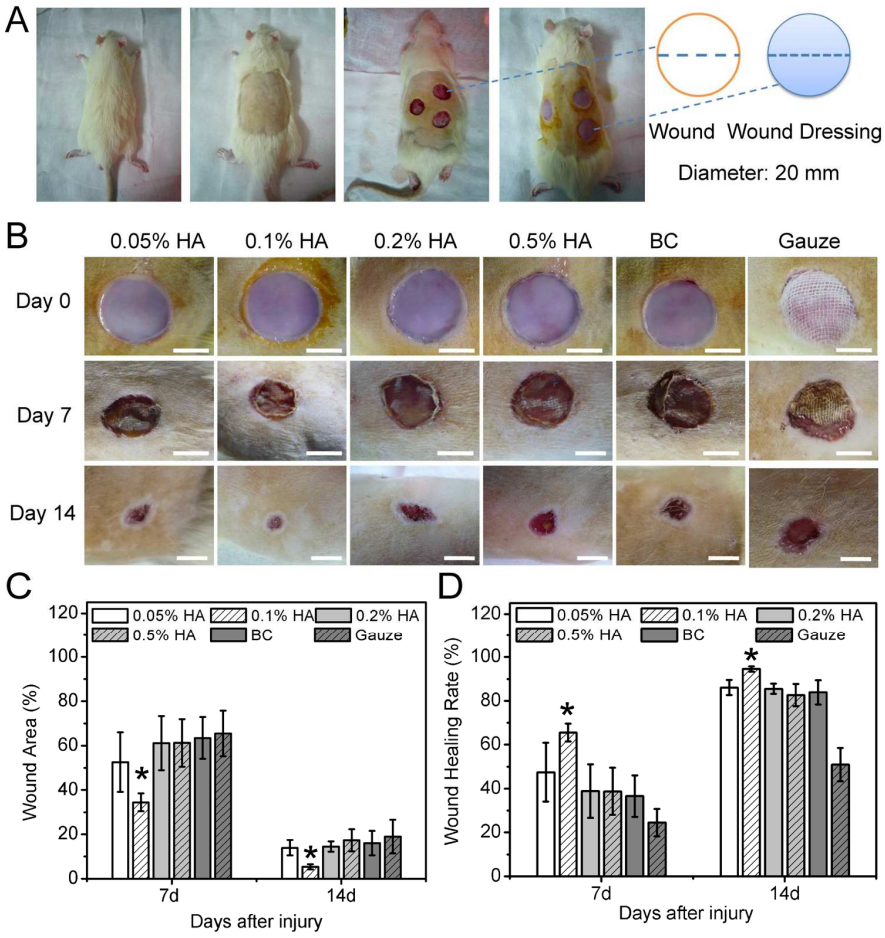

Fig.7 Skin wound healing accelerated by BC and the BC/HA composite films. A) Establishment of a full-skin injury model in a Wistar rat; B) Macroscopic observation of excisional BC- and BC/HA-treated wounds after 0, 7 and 14 days; C) Proportion of the wound area left 7 and 14 days after the injury (n = 6). D) Wound healing rate of the different wounds which were covered by BC-, BC/HA and gauze at 7 and 14 days after the skin injured. The data shows significant statistical differences between the two groups (*$P < 0.05$), the BC as control. All the scale bars are 10 mm.

BC/HA composite films and pure BC results showed that with the increase of HA concentration, the tensile strength of the composites decreased or they requires less force to break. Boateng et al. informed that greater tensile strength should produce a soft, elastic hydrogel film that follows skin movements.[37] Therefore, the high concentration of HA composite with BC may reduce the wound healing time. The surface area, pore size, fiber structure, mechanical strength, and moist environment of the 0.1% HA composite film are beneficial to wound healing.

Wound healing is a complex process requiring the collaborative efforts of many different tissues and cell lineages.[38] Tissue regeneration, granulation, tissue hyperplasia and cicatrization form a complex combination and the cooperation of these processes plays a critical role in wound healing. Histological analysis can give a microscopic view of the wound healing process over different days.

Hematoxylin and eosin (H&E) and Masson staining were carried out for evaluating wound healing of Wistar rat 7 and 14 days after the operation. The wound tissue covered with BC and the BC/HA composite films showed many newly formed blood vessels, especially the wound covered with 0.1% HA had the largest blood vessel areas which surrounded by continuous rings of vascular cells (Fig. 8A). The acquired images were utilized to quantify the blood vessel-to-tissue area ratio using Image-pro plus software, and column graphs were plotted with Origin pro 8.0. Anilkumar et al. found that HA not only provide a moist environment but increase cell migration and tissue remodeling.[39] Choi et al. reported that the pore size of the scaffolds influence the formation of vascular network.[40] Proper pore size of the 3D scaffolds can support the migration of cells and transport of nutrients, wastes and oxygen.[41, 42, 43, 44]. It should be pointed out that, the blood vessel-to-tissue area ratio of the gauze treated wound did not experience noticeable changes (Fig. 8B). That may be due to that the BC and BC/HA composite films had 3D structures, which are useful for cell migration, and keep the wound in a moist environment. The wound covered with the 0.1% HA had higher blood vessel-to-tissue area ratio than other groups (Fig. 8B). Therefore, in this study, HA concentration, surface morphology and mechanical strength of the BC/HA composite films influenced the cell migration and blood vessel formation.

The formation of granulating tissues and re-epithelialization play important roles in the wound healing process.[45] Granulating tissue is formed by new capillaries and fibroblasts, and is accompanied by inflammatory cell infiltration. Fibroblasts participate in the whole process of wound healing and influence the formation of collagen. Collagen is an important factor that affects wound reconstruction and ban scar formation.[46] After 7 days the HE data showed that fibroblasts appeared in all the groups, but more fibroblasts appeared in the wound covered with 0.1% HA than in the other groups, and many lymphocytes appeared in all the groups before gradually decreasing with time (Fig. 9). The results also revealed that the wounds covered with the BC/HA composite films already grew mature squamous epithelial cells after 7 days (Fig. 9). Masson staining of the wounds on day 14 showed that the collagen regenerated in the 0.05% HA- and 0.1% HA-treated wounds were denser than for the other BC/HA composite- and BC-covered wounds (Fig. 9). The migration and proliferation of epidermal and fibroblasts is essential to regenerate skin appendages such as hair follicles.[45] After 14 days, squamous epithelial appeared in all the wound tissues. Many hair follicles had grown in the wound covered with 0.1% HA, while the wound covered by 0.05% HA also appeared hair follicles, indicating that 0.1% HA and 0.05% HA can repair the wound with higher level of integrity than the other groups (Fig. 9). This is in good agreement with the MTT results which showed that the 0.1% HA and 0.05% HA composites can promote primary human fibroblast cells proliferation.

Conclusively, the 0.1% HA group showed the best wound healing performance among the six groups. This result may due to the fact that HA is one kind of ECM molecules in tissues, so, the

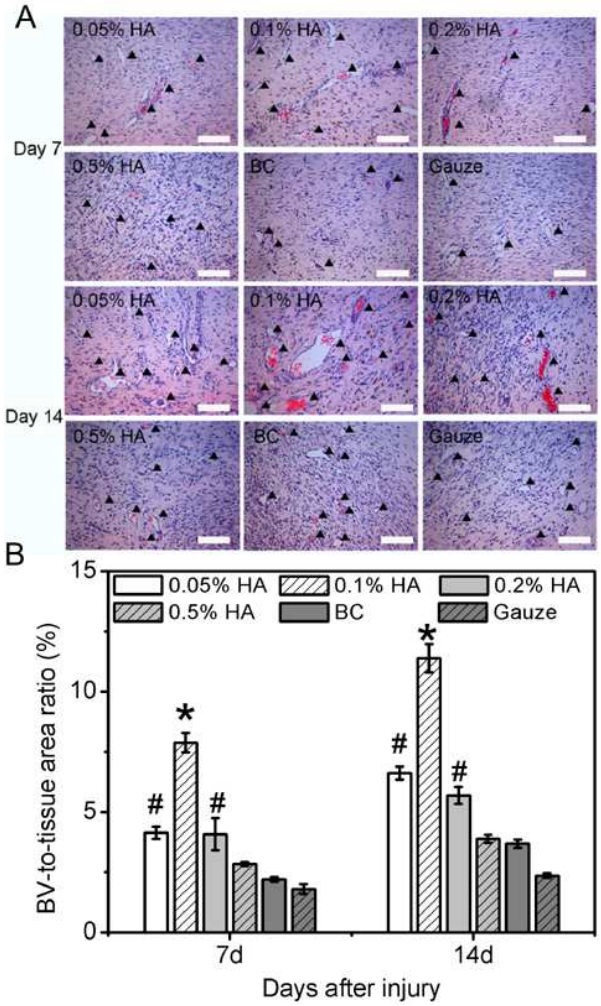

Fig.8 A) Representative HE stained tissue sections of subcutaneously implanted wound dressings with BC/HA composite films, BC and gauze, respectively, on 7 days and 14 days after implantation. Blood vessels are indicated by black arrowheads. All scale bars equal 100 μm. B) Plots of blood vessel-to-tissue area ratio of the wounds treated by BC/HA composite films, BC and gauze, respectively. The data were obtained from measurement of HE stained tissue sections. The data shows

significant statistical differences between the BC/HA groups (*P < 0.05, #P < 0.01) and BC (control, n=6).

modification of HA is undoubtedly beneficial for the growth of cells on the BC film. However, the HA modification can lead to the decrease of the pore size on the BC film, which may influence the wound breath. 0.1% HA composite film had the highest fibroblast cell viability (Fig. 5) and the most number of the cells (Fig. 6). So, certain concentration of HA (0.1% HA composite film) should be suitable for optimizing the biocompatibility of the BC.

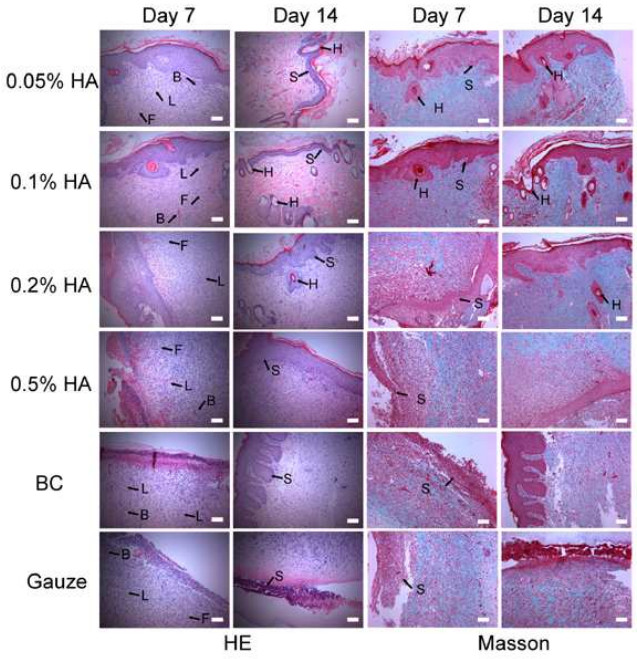

Fig.9 Light microscopy images for pathological sections of wounds on the Wistar rats after 7 and 14 days. The letters indicate specific cell types in the histological sections; F: fibroblast; L: lymphocyte; B: blood vessel; H: hair follicle; S: squamous epithelial. All scale bars equal 100 μm.

4. Conclusions

Patients with serve wounds or large areas of injury often endure not only pain from the body, but also economic pressure. BC has been found to be an excellent and cheap material for wound dressing and has been widely used in the biomedical field.[47] In this study, the comparison between BC and BC/HA composite films were carried out systematically. Clinical therapy requires a specific range of WVTR for wound dressing materials, which can be met by the BC/HA composite films. The BC/HA composite films have a better water uptake ability than pure BC, leading to better wound drainage. The structure of wound dressing can influence mechanical strength of the dressing and thus the wound cell growth and wound breath. Also, the relatively high strength of dressing could protect a wound from external rubbing or collision mechanical damage.[48] In this study, the BC/HA composite films had higher failure strain than the BC films did, however, the tensile strength of the composites decrease with the increase of HA concentration. So, the BC/HA composite film with certain concentration of HA may be the best dressing materials for wound healing. In vitro experiments on primary human fibroblast cells indicated that low concentration of HA in the BC/HA composite films enhance the proliferation and adhesion of the cells compared to pure BC. In the in vivo experiments, the BC/HA composite films with low HA concentrations 0.05% HA and 0.1% HA evidently promoted wound healing. In conclusion, appropriate concentration of HA can evidently improve the physical property and biocompatibility of the BC film, making BC/HA composite films an excellent material for rapid repairing of wound in vitro and in vivo. The BC/HA composite films, therefore, is an ideal candidate as wound dressing for skin repair, which may benefit to patients by reducing the pain and shorten their hospital stay.

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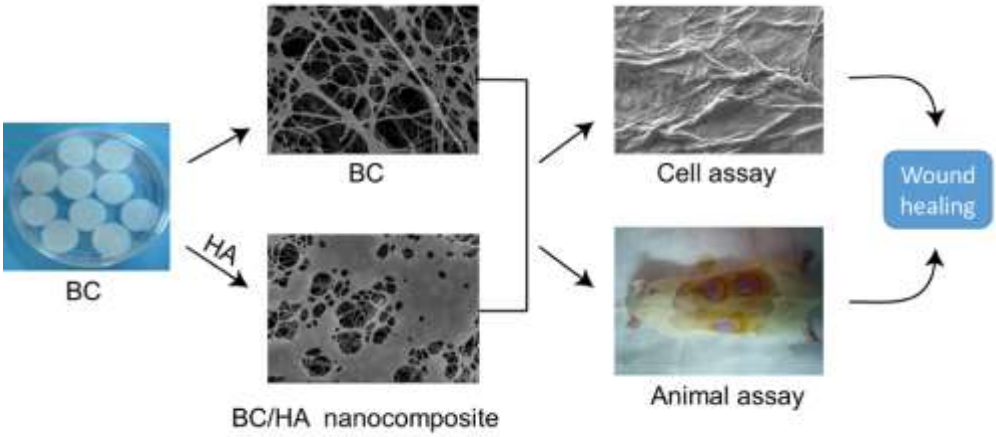

BC/HA composite dressings have better performance in wound healing

A table of contents entry
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