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# Bioactive Bacterial Cellulose Wound Dressings for Burns with Collagen *In-situ* and Chitosan *Ex-situ* Impregnation

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## Abstract

Bacterial cellulose (BC) is a biopolymer that commonly used for wound dressings regarding to its high *in-vitro* and *in-vivo* biocompatibility. Moreover, the three-dimensional fibers in BC become an advantageous for bioactive wound dressing application as they serve as templates for impregnation other supportive materials. Chitosan and collagen are two of the materials that can be impregnated to optimize the BC properties for serve as wound dressing material. Collagen can help skin cells grow on the wound sites, where chitosan has anti-bacterial properties and can bind red blood cells. BC-based wound dressings were made by impregnating collagen via *in-situ* method followed by immersing chitosan via *ex-situ* method into BC fibers for 24 hours. The intermolecular interactions of amine groups in the wound dressing were confirmed by FTIR. The XRD diffractogram showed wider peaks at 14.2°, 16.6°, and 22.4° due to the presence of collagen and chitosan molecules in the BC fibers. SEM images confirmed that chitosan and collagen could penetrate BC fibers well. Other tests, such as water content, porosity, antibacterial properties, and haemocompatibility, indicated that the wound dressing was non-hemolytic. *In-vivo* test indicated that BC/collagen/chitosan wound dressing supported the wound healing process on second degree burn.

**Keywords:** BC/collagen/chitosan; wound dressing; second degree burn; haemocompatibility; *in-vivo* test

## Introduction

The skin is the largest organ that is found on the outermost part of the body which serves as a cover to almost the entire surface of the human body. However, because it is located on the outermost part of the body and is directly exposed to the outside environment, the skin is very vulnerable to wounds trauma, such as burn. Burns are injuries to the skin that typically involve significant and complex skin tissue damage. Furthermore, the burn surface is known to be susceptible to rapid bacterial colonization with the potential for invasive infection. Partial thickness burns also usually produce a moderate to high amount of exudate that needs to be managed with the use of an appropriate dressing material [1], [2]. According to the American Burn Association, by 2021, there are more than 400,000 patients that received burn treatment annually.

Currently, the burn dressings type that most widely used clinically are tulle and gauze, which does not actively play an important role in promotes wound healing process. As the result, currently, extensive study related to the development of burn dressings that can play an optimal role in the wound healing process, not only serve as a wound cover, has been widely carried out. The characteristics of wound dressings that need to be developed include the capability to serve as a barrier to contaminants, prevent bacterial infection, maintain wound moisture, stop bleeding, absorb wound exudate, hasten wound healing, and minimize the possibility of scar tissue formation. In addition, the produced wound dressings must be easy to apply, sterile, non-toxic, non-allergenic, can facilitate air exchange and degraded easily in order to prevent the medical waste accumulation. Produced wound dressings also have to maintain a healthy tissue temperature to promote blood flow to the wound bed, enhance the function of cells like macrophages, neutrophils, and fibroblasts, and promotes epidermal migration, angiogenesis, and connective tissue production [3]–[5]. This type of wound dressing is generally produced from biopolymers which contain bioactive compounds such as alginate, collagen, chitosan hydrofibre, and hydrocolloid [6]–[8].

Bacterial cellulose (BC) is an exopolysaccharide polymer that produced by the *Gluconacetobacter* bacteria. In contrast with plant cellulose, which still needs to be purified from lignin, pectin, and hemicellulose, this type of cellulose is acquired in its pure form [9]–[11]. Its high purity makes BC as one of the biomaterials that highly recommended to be used for wound dressing application. In addition to purity, the unique multilayer 3D structural arrangement of cellulose fibers with a large surface area and high porosity is also an advantage for this applications [12], [13]. Additionally, BC is permeable and biocompatible, which enables it to keep wounds hydrated and directly applied to the wound. It is also biodegradable and natively produced as a hydrogel by bacteria, which makes it an ideal substrate for treating dry wounds in which appropriate moisture is needed to accelerate the healing process [14]–[16]. Numerous research have examined the efficacy of the BC utilization as a main material for wound dressing and it is found that BC efficiently accelerated wound re-epithelialization and showed remarkable therapeutic effects in promoting wound healing [17], [18]. However, BC polymer was known

does not possess antibacterial properties [19]. Therefore it is necessary to develop a modification strategy to optimize the use of BC as a wound dressing.

Currently, the most widely technique used in BC modification is the *ex-situ* solution impregnation technique. This method involves physically absorption, where a substance or other material is impregnated into pure BC fibers without any BC modifications. In this process, the presence of hydroxyl groups from the cellulose chain will result in strong hydrogen bonds between the BC molecules with the adsorbed molecules [20]. This type of technique is the simplest and most flexible method in BC modification strategy [21]. Another technique is known as *in-situ* modification, in this technique, the substance is included in the media culture from the beginning as the result the incorporated process was occurs during the BC fibril network synthesise. Both types of modification techniques can be used to modify the physical, chemical, mechanical, and morphological characteristics of the resulting BC composite. They can also be employed as a method for incorporating additional components, such as active substances and/or bioactive substances, which can be used to improve the antioxidant, antimicrobial, and anti-inflammatory properties of BC [22], [23]. In this study, considering some of wound dressing material properties that need to be addressed, these two methods were adapted for collagen and chitosan impregnation into the BC fibers.

Collagen is a natural polymer whose main structural component consists of the extracellular dermal matrix with many favorable properties to be utilized in wound healing processes, such as hemostasis, biodegradability, hydrophilicity, biocompatibility, and the ability to promote cell proliferation [24], [25]. Therefore, collagen is widely used as a biomedical material and occupies a superior position among the other polymers for skin tissue engineering applications. In addition, collagen dressings are interested because they have the ability to inhibit or inactivate MMP, increase production and absorption of fibroblasts, assist the absorption and bioavailability of fibronectin, assist the preservation of leukocytes, macrophages, fibroblasts, and epithelial cells, as well as preservation of wound tissue. Previous studies reported that the *in-situ* impregnation of collagen into BC fibers was shown to successfully facilitate collagen immobilization in BC nanofibers and was demonstrated to enhance the biological activity of the BC scaffold [26]. Hence, the *in-situ* impregnation method for BC/collagen production was adapted in this study. However, BC/collagen is known to not have the antibacterial properties that needed for wound healing treatment. Therefore, in this study, chitosan was added to achieve the overall wound healing treatment requirements.

Chitosan, as previously reported, possess many excellent properties to be used as active wound dressing, including biocompatibility, biodegradability, hemostatic properties, anti-inflammatory effects, and the ability to accelerate wound closure [27], [28]. This natural polymer is also known to have great potential to be used as an antimicrobial agent in medical devices. Chitosan can also serve as a non-protein matrix for 3D tissue growth which also plays a role in macrophage activation. In healing process, chitosan is able to accelerate tissue regeneration in wound contraction by stimulating inflammatory cells, macrophages and fibroblasts. Furthermore, chitosan has hemostatic properties which help blood clotting and reduce pain by blocking nerve

terminal. The hemostatic properties of chitosan can be attributed to its positive charge which allows chitosan macromolecules to interact electrostatically with the negative charge of red blood cells. Previous studies shown, that the *ex-situ* addition of chitosan into BC fibers for wound dressing production, significantly inhibited the development of *Staphylococcus aureus* and *Escherichia coli* bacteria. Furthermore, BC/Chitosan was reported to promote faster cell rejuvenation and regeneration compared to Tegaderm. In accordance with all the data and information that has been explained, in this study, collagen is impregnated *in-situ* while chitosan is impregnated *ex-situ*, not the opposite, is in order to produce a wound dressing with antimicrobial activity which also supports the wound healing process. This procedure is considered to be the most effective method for allowing collagen to enter and immobilize properly in the BC fiber and facilitating the chitosan to be physically bound to the outermost portion of composite, thus chitosan will serve as the first material to release when dressings are applied to the burn wound in order to inhibit the development of bacteria. In this work, the efficacy of BC-based wound dressings with *in-situ* collagen impregnation, followed by *ex-situ* chitosan impregnation as a dressing for second-degree burns was evaluated for its material properties and its *in-vitro* and *in-vivo* biocompatibility.

## **Materials and Methods**

### **Materials**

*Gluconoacetobacter xylinus* (American Type Culture Collection (ATCC) 23770) was obtained from the University of Wolverhampton Culture Collection. Yeast extract, peptone, mannitol, and agar were purchased from Lab M (Bury, UK). Tryptone soy agar (TSA) was purchased from Sigma-Aldrich (Irvine, UK). Bacteriological peptone, yeast extract and dextrose for Hestrin and Schramm (HS) culture media were purchased from Lab M, UK. Tryptone soya broth (TBC), disodium phosphate and citric acid were purchased from Sigma-Aldrich, (Irvine, UK). HeLa cells were purchased from ATCC, UK. Collagen was purchased from Sigma-Aldrich, UK. Chitosan (DDA 73.72%, MW 331.131 kDa) was purchased from Acros Organics, UK. Defibrinated horse whole blood was purchased from TCS Biosciences Ltd, UK.

### **BC/collagen Production**

BC/collagen was prepared by the *in-situ* addition of 0.1 g collagen into 250 mL Hestrin and Schramm media and sterilized in an autoclave at 120 °C. Next, 20 mL *Gluconoacetobacter xylinus* ATCC® 23770 was added into the culture medium and incubated under static conditions at 30 °C for 14 days. BC/collagen pellicle formed was harvested and purified with 1% (w/v) NaOH heated at 80 °C, followed by purification in ~100 °C hot distilled water for one hour.

### **BC/collagen/chitosan Production**

The purified BC/collagen gel was dried by pressure drying. The dry sample was put into 100 mL chitosan solution (0.02 g/mL of powder in 1% acetic acid solution) for 24 hours with 100 rpm stirring conditions at room temperature to form BC/collagen/chitosan. The BC/collagen/chitosan

gel formed was dried for 72 hours using a freeze dryer. The prepared sample was labeled as BC/chitosan/collagen and stored in a desiccator for further use.

### **FTIR Analysis**

The functional groups present in BC, BC/collagen, and BC/collagen/chitosan samples were recorded using an FTIR Spectrophotometer (Bruker, Alpha, Platinum-ATR, Germany). The scans were performed in the range of 400–4000  $\text{cm}^{-1}$  with 16 scan settings for each sample.

### **XRD Analysis**

To determine the crystallinity of the sample, X-ray diffraction (XRD) was performed using a Shimadzu XRD-6100 diffractometer at the Universitas Negeri Medan (UNIMED), Indonesia. In this study, the crystallinity index was calculated using equation (1), which was based on the equation proposed by Osorio-Madrado et al [29].

$$\text{Crystallinity Index (\%)} = \frac{\text{Crystalline Area}}{\text{Total Domain Area}} \times 100\% \quad (1)$$

### **Morphological Analysis**

BC, BC/collagen and BC/collagen/chitosan solid samples were plated with gold by using SC500 fine coating (Emscope, Kent, UK). The shape and morphology of the samples were observed by using a Zeiss Evo 50 EP, SEM (Carl Zeiss AG, Oberkochen, Germany).

### **Thermal Analysis**

Thermal properties of BC, BC/collagen and BC/collagen/chitosan samples were observed by using EXTAR 7300 Series at Universitas Lampung. The sample was heated at 30-610  $^{\circ}\text{C}$  with a heating rate of 10  $^{\circ}\text{C}/\text{min}$  and constant nitrogen gas flow (60 mL per minute). Thermal thermogravimetric differential analysis (DTGA) curves were also produced as the first derivative of the TGA curves.

### **Moisture Content**

The moisture content of all samples was tested by measuring the weight of the pure sample and the weight of the sample after drying in a freeze dryer. The ability of the sample to bind water was calculated with equation (2).

$$\text{Moisture Content (\%)} = \frac{\text{Weight of Wet Sample} - \text{Weight of Lyophilized Sample}}{\text{Weight of Wet Sample}} \times 100\% \quad (2)$$

### **Porosity Percentage Calculation**

The porosity of samples was analyzed by using equation (3), where samples were immersed in 96% ethanol for 10 minutes.

$$\text{Porosity (\%)} = \frac{V_{e0}-V_{e2}}{V_{e2}-V_{e1}} \times 100\% \quad (3)$$

$V_{e0}$  = Initial volume of ethanol

$V_{e1}$  = Volume of ethanol when the sample is immersed

$V_{e2}$  = The volume of ethanol after the sample is removed

### **Mechanical Test**

Tensile properties were determined using an Instron universal testing machine equipped, US, with a 1 kN static load cell, in accordance with active standard test method D638. The grips were made specifically for testing fibers. Each sample was tested three times with strain rate speeds of 10 mm min<sup>-1</sup> at 20 mm gauge lengths.

### **Haemocompatibility Test**

Haemocompatibility test was carried out by firstly washing the anticoagulant in defibrinated horse blood and 0.9% saline solution in centrifugal force at 3000 rpm for 10 minutes. Next, disk-shaped sample with a diameter of 8 mm was immersed in a horse blood cell suspension at 37 °C for 2 hours and agitated. A spectrophotometer was used to measure the absorbance of the sample at 540 nm, where distilled water was used as a positive control, while the negative control was a normal saline solution. The percentage of haemocompatibility was calculated by equation (4).

$$\text{Haemocompatibility (\%)} = \frac{A_s - A_{k(-)}}{A_{k(+)} - A_{k(-)}} \times 100\% \quad (4)$$

$A_s$  = Sample absorbance

$A_{k(-)}$  = Negative control absorbance

$A_{k(+)}$  = Positive control absorbance

### **Cytotoxicity Test**

25 cells/ $\mu$ L HeLa cells in RPMI-1640 media were added to each well of a 24-well plate and incubated for 24 h. After the incubation, the cells were washed twice with phosphate-buffered saline (PBS), then RPMI-1640 media were added into the well. The disk-shaped sample with a diameter of 8 mm was added into the well and incubated for 24 hours with three repetitions ( $n = 3$ ). After the incubation process was completed, MTT (0.5 mg/mL PBS) was added to each well and the cells were incubated again at 37 °C for 3 hours. The solubility of formazan crystals was achieved by mixing DMSO (100 L/well) with a gentle stirring for 10 min. The absorbance was read at 570 nm using a microplate scanning spectrophotometer (SPECTROstar® Nano, BMG Labtech, Germany). Equation (4) and (5) were used to calculate the level of toxicity and viability respectively.

$$\text{Cytotoxicity (\%)} = 1 - \frac{\text{Sample Absorbance}}{\text{Negative Control Absorbance}} \times 100 \quad (4)$$

$$\text{Viability (\%)} = 100\% - \text{Cytotoxicity (\%)} \quad (5)$$

### ***In-vivo* Test (Second Degree Burn)**

To test the efficacy of the samples in accelerating the healing process of burns, an *in-vivo* test was conducted on 28 male and female rats (*Rattus norvegicus*) aged 3 months old weighing 150–180 g. The animal testing procedure carried out in this study was approved by the Animal Research Ethics Committee (AREC) No.0734/KEPH-FMIPA/2020, Universitas Sumatera Utara. The entire research process was carried out at the Animal Research Laboratory Universitas Sumatera Utara, complying to university guidelines. The back area of the rats, where treatment would be carried out, was firstly prepared by shaving the skin. After that, the rats were anesthetized using xylazine (5.0 mg/kg) and ketamine (35.0 mg/kg). Next, the shaved area was cleaned with chlorhexidine, povidone iodine and alcohol (70%). A second degree burn was made on that area by placing a 20 x 20 mm piece of hot metal perpendicular to the back of the rat for one second, precisely at the position between the last thoracic vertebra and the first vertebral column. The rats were randomly divided into 4 groups of seven, as follow: control (-), BC, BC/collagen and BC/collagen/chitosan groups. The control group (-) rats did not receive any treatment. In this test, BC, BC/collagen and BC/collagen/chitosan wound dressings used to treat the burn wounds on the rats were not dried to minimize the pain and stimulate the healing process.

### **Wound Closure Test**

Burns were observed every three days for 21 days. The most significant changes in the wound areas were observed on day 7, 14 and 18. The wounds were measured by using a caliper and recorded with a camera. After that, the percentage of wound closure was calculated by using equation (6).

$$\text{Wound Closure Area (\%)} = \frac{AH_0 - AH_r}{AH_0} \times 100\% \quad (6)$$

$AH_0$  = Day-0 Wound Area

$AH_r$  = Relative Day Wound Area

### **Anti-microbiological Test**

The rats in the positive control group were given wound dressing accordingly (BC, BC/collagen, and BC/collagen/chitosan) and bioplacenton. Whereas the rats in the negative control group were not given any treatment. All the research samples were swabbed on day 4, 8, 12 and 16 for bacterial analysis in the microbiology laboratory. Each wound dressing was immersed in 2 mL of

normal saline and vortexed in a 10-fold serial dilution. The dilution volume of 1000  $\mu\text{L}$  of the sample was spread evenly on the surface of tryptic soybean agar (TSA). The cultures were left for 24 hours in an incubator at 37 °C prior to the calculation of bacterial colonies.

### **Histological Observation**

After 21 days, histopathological observation was carried out to all treated wound tissue areas. Samples were set in 10% buffered formalin and coated with paraffin. The visualization of paraffin-embedded specimens was observed under microscopic light after cutting and tagging with Masson's trichrome (MT) stain.

### **Statistical analysis**

One-way Anova was used to calculate the statistical data, and Dunnett's post-hoc test was then performed. Normally,  $p < 0.05$  is used to indicate the significant finding.

### **Results**

#### **FTIR Analysis**

The FTIR of BC spectra in Figure 1 show peaks at 3450-3300  $\text{cm}^{-1}$  indicating the presence of OH stretching and at 2895  $\text{cm}^{-1}$  as C-H stretching peak. The buckling peak of H-O-H water molecule was observed at 1640  $\text{cm}^{-1}$ ,  $\text{CH}_2$  was deformed at 1435  $\text{cm}^{-1}$ ,  $\text{CH}_3$  was deformed at 1370  $\text{cm}^{-1}$ , OH was deformed at 1336  $\text{cm}^{-1}$ , and C-O was deformed at 1320-1030  $\text{cm}^{-1}$ . Collagen showed a specific peak at 1659  $\text{cm}^{-1}$  indicating C=O stretching peak coupled with N-H buckling peak in amide I. The peak at 1659  $\text{cm}^{-1}$  indicated N-H buckling peak coupled with C-N stretching peak in amide II. The deformation of N-H peak and C-N stretching peak in amide III. This indicates that the addition of collagen to cellulose fibers can create the interconnections between one and the other fiber in the three-dimensional (3-D) structures of the cellulose fiber, in accordance with previous study that indicate that the presence of the hydroxyl groups from cellulose chains will produce strong hydrogen bonds between BC and absorbed molecules [20]. Moreover, in this study, the impregnation process was carried out via *in-situ* method, which means that the collagen loaded process was take place simultaneously with the BC synthesis process. As the result, this condition facilitate the N-H and C-N groups in collagen to produce a hydrogen bond with cellulose fibers and serve as a link to connect the one BC fiber to the another. The presence of chitosan was detected in the peak between 2880 and 2920  $\text{cm}^{-1}$  which was associated with the C-H groups in chitosan. Amide I band was shifted from 1624 to 1600  $\text{cm}^{-1}$  in BC/collagen/chitosan sample. This occurred because of the interaction between BC and chitosan in the composite. Previous research also showed a similar report [30]. The success of chitosan and collagen impregnation in BC was indicated by the appearance of peaks at 1585  $\text{cm}^{-1}$  and 1645  $\text{cm}^{-1}$  indicating C=O stretching as shown in Figure 1.

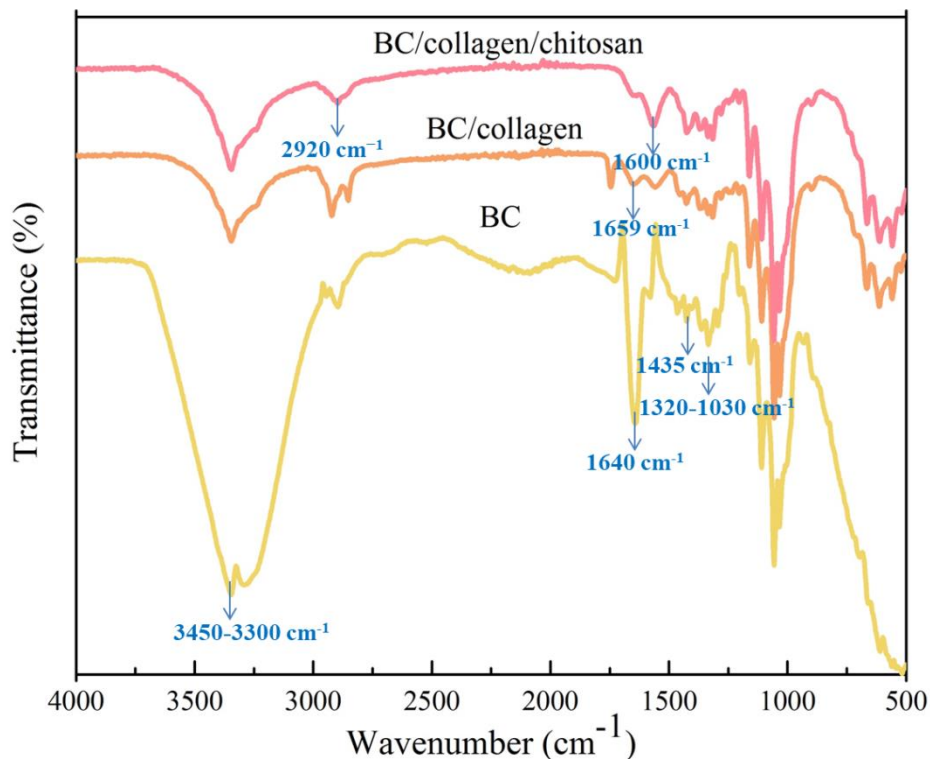


Figure 1. FTIR curve of BC, BC/collagen and BC/collagen/chitosan samples

### XRD analysis

The XRD diffractogram of BC sample (Figure 2) showed two specific peaks indicating  $1\alpha$  and  $1\beta$  cellulose, specifically at  $15^\circ$  and  $22.5^\circ$  crystal planes (110) and (002). BC was not completely crystalline, so broad diffraction peaks could still be observed on the BC curve [30]. The widening peaks of BC/collagen and BC/collagen/chitosan samples on the XRD graph showed the amorphous nature of collagen and chitosan. They reduced the crystallinity degree of BC, which was in accordance with a number of past studies [12]. In other words, the decrease in the intensity of XRD peaks in BC/collagen/chitosan sample occurred due to the impregnation of amorphous collagen and chitosan, thereby reducing the degree of crystallinity of BC [31].

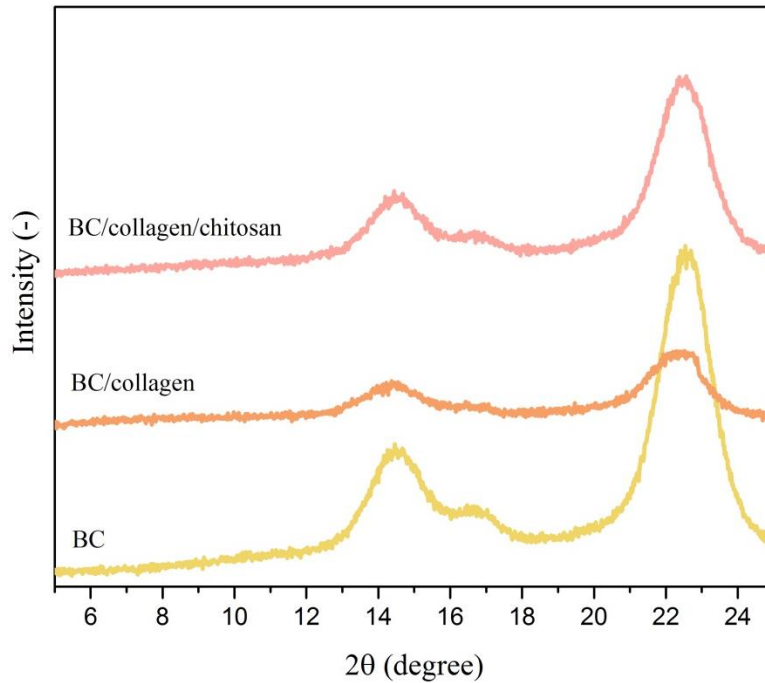


Figure 2. XRD curves of BC, BC/collagen and BC/collagen/chitosan wound dressings

### Morphological Analysis

Surface analysis carried out to wound dressing samples (Figure 3) using a scanning electron microscope (SEM) showed woven bacterial cellulose fiber structures. The *in-situ* addition of collagen in the media resulted in morphological changes in the cellulose fibers (Figure 3b). There was a widening of the pores in the cellulose fibers due to the attachment of collagen onto the cellulose fibers during the synthesis stage involving *Gluconacetobacter xylinus* bacteria. The morphological appearance of cellulose fibers was similar to previous studies, which showed that the addition of collagen in cellulose fibers created interconnections between one fiber and the other in cellulose fibers 3D structures that supported the growth of fibroblast cells [31]. In addition, as demonstrated in Figure 3c, the surface appearance of the BC sheet seems more compact and dense after chitosan has been impregnated into the BC fiber. This is due to a strong hydrogen interaction between the chitosan and BC molecules as a result of the chitosan solution being trapped between the BC fibers [32].

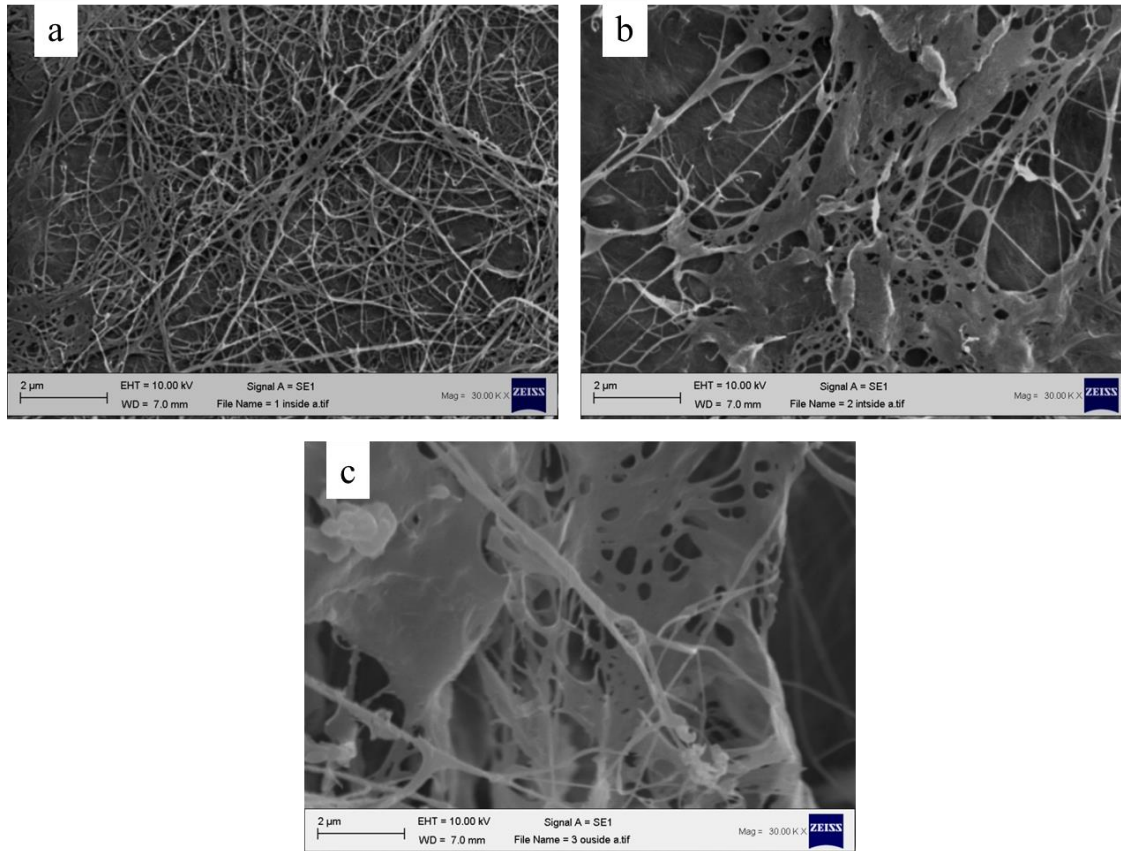


Figure 3. SEM morphology of (a) BC, (b) BC/collagen and (c) BC/collagen/chitosan wound dressings

## Thermal Analysis

Thermal analytical techniques such as TGA as shown in the curve in Figure 4 (a) are widely used in the pre-formulation of biomaterials for medical applications. Thermokinetic data can be used to understand thermal decomposition reactions and help determining suitable storage conditions for the biomaterials. Comparing collagen to chitosan TGA curve is useful to show the effect of collagen and chitosan impregnation on the thermal stability of BC matrix. The thermogravimetric data showed that the thermal degradation of BC began at about 255 °C and lost about 50% mass between 325-326 °C. The TGA curve showed that the impregnation of collagen and chitosan into the BC matrix caused thermal degradation to occur at a lower temperature, at around 180 °C, which was about 70 °C lower than the degradation temperature of pure BC. Nevertheless, the TGA data showed that BC/collagen/chitosan biomaterials wound dressings were safe for use because they were stable at room temperature and stably sterilized in an autoclave before their applications onto wounds – considering that the sterilization temperature in the autoclave was only around 110-120 °C [33].

TGA curve analysis for BC/collagen/chitosan showed 6 – 7% mass loss at ambient temperature at 90-110 °C, which was associated with water loss [30]. While the molecular decomposition occurred at around 255-303 °C. The maximum mass loss occurred at 299 °C temperature range. This phenomenon more clearly shown in the DTGA curve in Figure 4 (b). DTGA curve analysis revealed that the maximum mass loss occurred in BC/collagen/chitosan sample was lower than BC and BC/collagen samples (Table 1). This result was explainable because of collagen and chitosan presence in the sample, as well as considering that collagen and chitosan had lower stability than BC. Moreover, the highest residual mass was observed in BC/collagen/chitosan, reaching 33.98%, compared to the residual mass of BC and BC/collagen which was 9.13% and 9.17% respectively. This finding could be related to the impregnation of chitosan into BC that has increased the residual mass, as reported in previous studies [12].

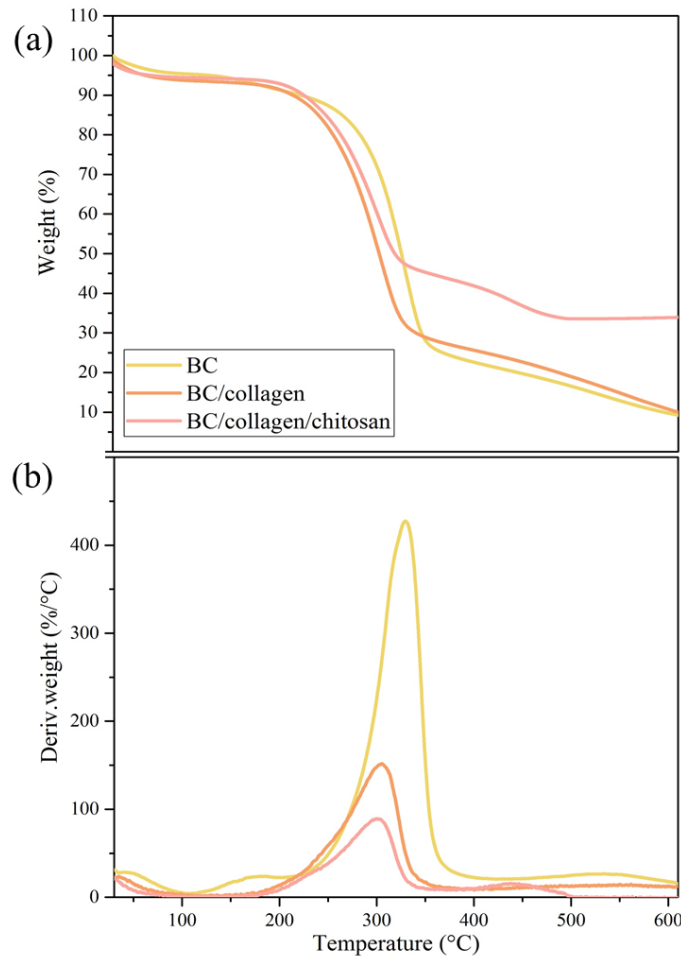


Figure 4. (a) TGA and (b) DTGA curves of BC, BC/collagen and BC/collagen/chitosan wound dressings

Table 1.  $T_{max}$  and Residual Mass of BC, BC/collagen and BC/collagen/chitosan wound dressings

Sample	BC	BC/collagen	BC/collagen/chitosan
$T_{max}$ (°C)	329	305	299

<b>Residual Mass (%)</b>	9.13	9.17	33.98
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### Moisture content and Porosity

The high capability of BC to bind water is one of the advantages, that made BC as an appropriate main material in the active wound dressings development, especially for burn wound dressing which is known to produce high amount of wound exudate. However, as indicated in Table 2, the *in-situ* addition of collagen is known to reduce the water content by 2%, and the subsequent addition of chitosan caused a decrease in water content of up to 5% compare to pure BC. The decrease in water content in this study was recognized to still meet the criteria for the BC/collagen/chitosan employed as a wound dressing.

One of the properties that also crucial to evaluate before applying a material as a wound dressing is porosity, because it is expected that materials for wound dressings to have pores that can promote oxygen exchange but in appropriate size hence they still able to act as a barrier for contaminants. In this study, collagen *in-situ* addition is known to reduce the porosity by around 1%, and the further addition with chitosan via *ex-situ* method was observer reduce the porosity value by around 2% as shown in Table 2. The porosity reduction is the result of the impregnation process of collagen and chitosan, where these materials trapped in the BC pores. However, it is well recognized that the porosity of BC/collagen/chitosan still appropriate for its application as a wound dressing material.

### Haemocompatibility Test

Materials requiring direct blood contact is known to cause hemolysis, which is the rupture of the red blood cells themselves. Therefore, the assessment of hemolytic properties becomes important, especially for biomedical materials with applications as wound dressings. According to ASTM F756 standard, the hemolytic index of the tested samples can be classified into: (a) hemolytic materials with hemolysis values > 5%, (b) slightly hemolytic materials with hemolysis values between 2 and 5% and (c) non-hemolytic materials with hemolysis values below 2% [33], [34]. *In-vitro* testing of BC, BC/collagen, BC/collagen/chitosan wound dressings for blood compatibility testing revealed that all samples were haemocompatible, with hemolysis values < 2 % (n=4). The haemocompatibility evaluation shown that the BC/collagen/chitosan wound dressing has a hemolysis values of  $1.51 \pm 0.01$  % (Table 2 ). This value was lower than previous studies which showed that BC/collagen/chitosan produced by the *ex-situ* method had higher haemolytic properties, namely  $1.65 \pm 0.08$ %, although both of the values indicated that the material was non-haemolytic and can be applied as wound dressing [12].

Table 2. Moisture content (%), porosity (%) and haemocompatibility (%) data of BC, BC/collagen and BC/collagen/chitosan (n = 4).

Sample	Moisture Content (%)	Porosity (%)	Haemocompatibility (%)
BC	98.7 ± 0.12	78.5 ± 0.11	1.50 ± 0.03
BC/collagen	96.9 ± 0.10	77.2 ± 0.05	1.58 ± 0.01
BC/collagen/chitosan	95.5 ± 0.10	76.8 ± 0.07	1.51 ± 0.01

## Tensile Properties

Tensile strength was tested on lyophilized samples of pure BC, BC/collagen, and BC/collagen/chitosan wound dressings. Mechanical strength is one of the most important properties of the materials used in wound dressing applications. Figure 5 shows the tensile strength properties of all samples. Due to the interconnected nano-network structure formed by strong hydrogen bonds, BC-based wound dressings had a high tensile strength. Pure BC samples were tougher and more brittle than both BC/collagen and BC/collagen/chitosan. The mechanical properties of BC/collagen/chitosan samples were observed to be softer, more ductile, and flexible. The addition of collagen and chitosan and the presence of carbonyl bonds have reduced the crystallinity. All tested samples, however, demonstrated good mechanical properties, making them promising flexible wound dressing materials.

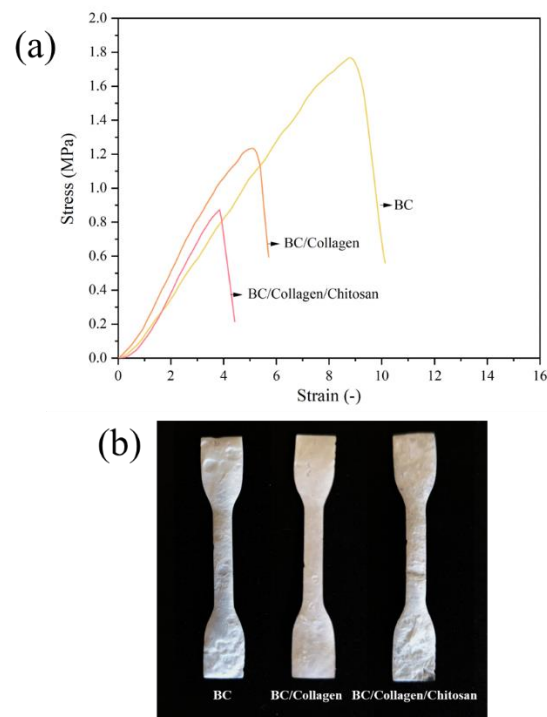


Figure 5. (a) Tensile test analysis and (b) photographs of wound dressing sample.

## Microbiological Examination

The wounds created on the rat samples were treated differently according to the control group. All wounds were swabbed periodically for microbiological examination. BC/collagen/chitosan wound dressings showed the least amounts of bacteria on the wounds. The chitosan content in the sample had antibacterial properties that played a role in preventing bacteria growth in wounds.

Chitosan acted as an antibacterial agent with electrostatic interactions between positively charged chitosan molecules and negatively charged cell membranes. The antimicrobial activity of chitosan is based on the presence of amino groups in the polymer chain. This amino group can be protonated, giving chitosan a positive charge and resulting in cell permeability changes and cell membrane lysis [35].

Despite the advanced development of antiseptics, wound infection remains a major problem. The emergence of multidrug-resistant pathogens has led to a constant need for more efficient topical antimicrobial products. The main drawback is that most of the antimicrobials are highly cytotoxic and thus affect the tissue healing process [36]. Therefore, chitosan becomes the highlight, in which it is biocompatible and non-toxic to living cells and tissues in addition to its antimicrobial and antifungal properties. Chitosan biocompatibility has been tested *in vitro* using various cell types such as fibroblasts, keratinocytes, and hepatocytes, as well as myocardial and endothelial cells [37].

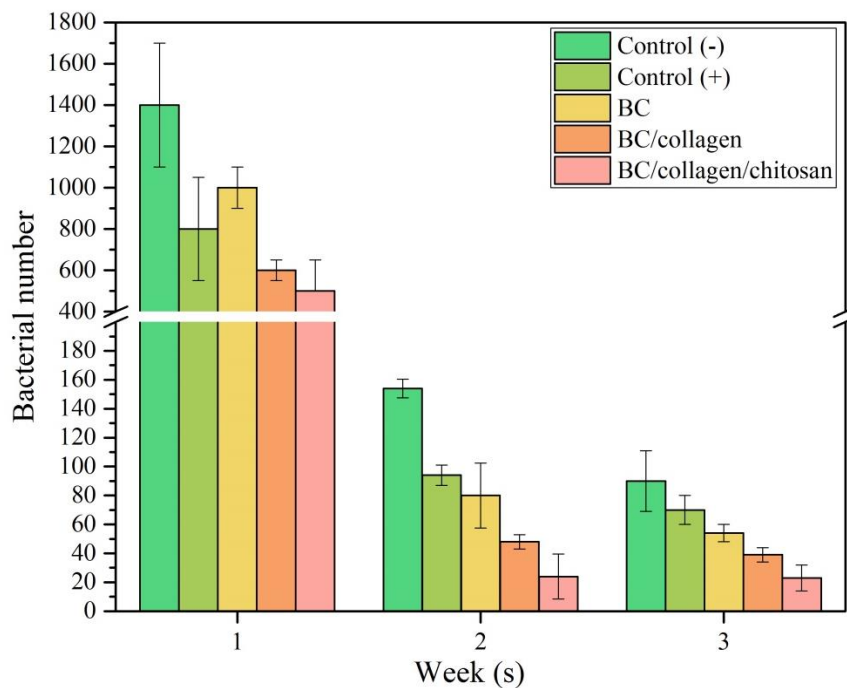


Figure 6. Number of bacteria contained in burns receiving different treatment for 3 weeks.

### Toxicity test

A number of studies have reported that BC was cytocompatible so it could be used for biomedical applications [31], [38]. Cytocompatibility is one of the many properties of BC that

makes this material to have the potential for wound dressing fabrications. Need to note that the surface of wounds is a good growing media for bacteria. Therefore, modifications have been carried out to make BC-based material with antibacterial properties. However, antibacterial properties to accelerate wound healing are mostly having harmful effects on host cells [38], [39]. In the wound healing process, the materials in wound dressings should be able to support the growth of new cells useful to close the wounds [40]. Therefore, the benefit-to-harm ratio of antimicrobial selections in wound dressings needs to be evaluated. The use of BC-based wound dressings impregnated with silver nanoparticles to treat chronic wounds is one of the examples in which the evaluation results showed that the benefits of silver nanoparticles as an antimicrobial agent outweighed the risks to the cytotoxic effect from the silver [33], [34], [40]. The objective of the toxicity test was also to determine how collagen and chitosan released from the BC matrix affected the survival of mammalian cells. Cytotoxicity test carried out by MTT assay in Figure 7(a) showed very good 24-hour cell survival rate and cell exposure that reached 90% as shown in Figure 7(b). This result far exceeded the IC50 allowable threshold, where 50% mortality was defined as highly toxic to cells [33]. The results in this study guarantee that BC, BC/collagen and BC/collagen/chitosan prepared as wound dressings would not be toxic to vital organs.

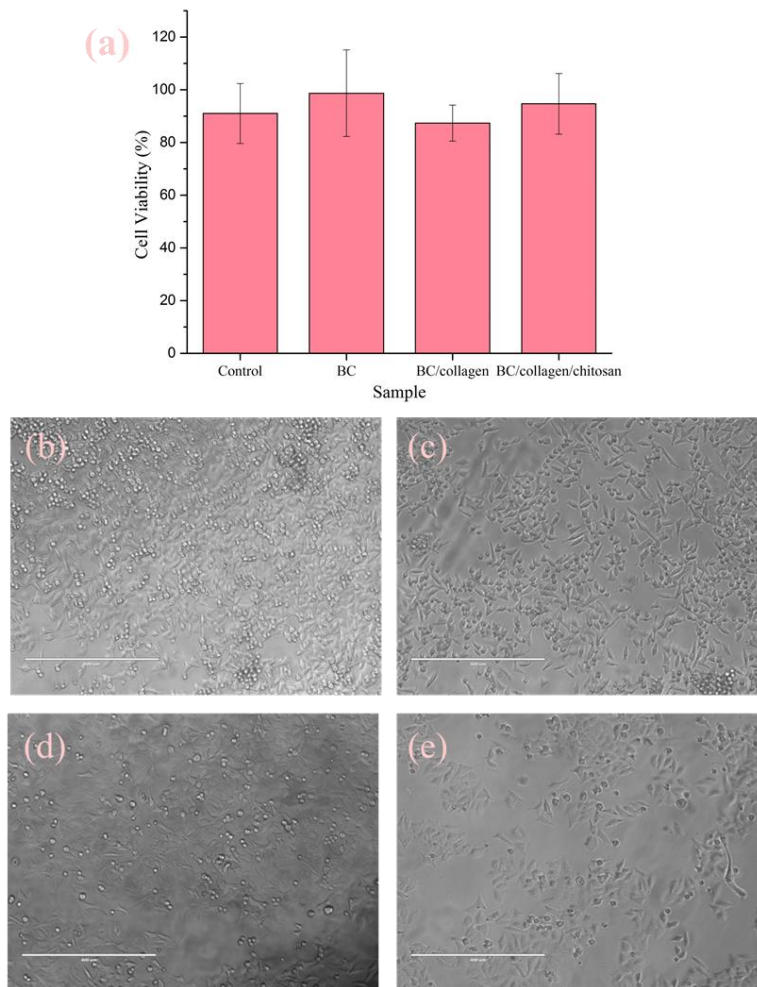


Figure 7. (a) Cytotoxicity test results and (b-e) optical photomicrograph of wound cells at 10x magnification: (b) Control, (c) BC wound dressing, (d) BC/collagen wound dressing, and (e) BC/collagen/chitosan wound dressing.

### Histological Analysis

Histological analysis shown in Figure 8 staining was performed with H&E to evaluate burn healing progress with various treatment. The area of the skin that was intentionally injured appeared to be regenerated after 3 weeks of treatment. The wound area of all subjects in all groups was seen to have formed epidermal and dermal layers, although there were differences in the thickness and structure of the tissue formed. H&E staining showed that the regeneration of the epidermal layer in the untreated group (control (-)) was very slow and only observed to occur at the wound margin area. This may be the result of the migration of keratinocytes to the wound edges. More blood vessels were found in the control (-) group, which implied that the initial stage of wound healing, such as proliferative stage, occurred differently from the other two groups (BC/collagen and BC/collagen/chitosan) that were already in the remodeling stage, where the blood vessels had subsided. The epidermis layer of the wound treated with BC was seen to be thicker than that treated with BC/collagen/chitosan. This showed that the stage of wound healing wrapped with chitosan BC/collagen was faster, where epithelial formation increased but the epidermis remained in its original thickness.

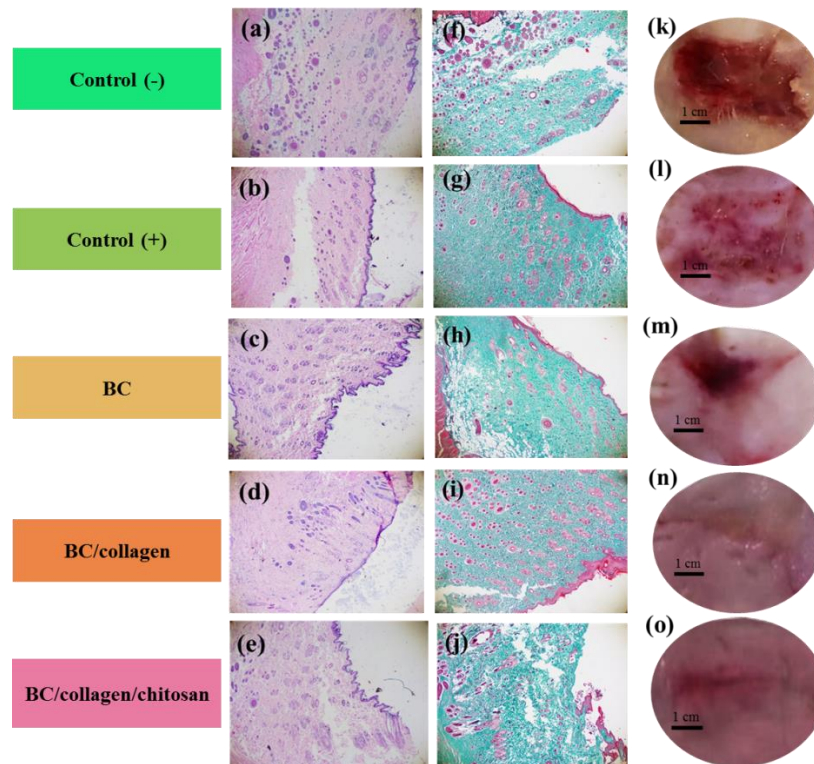


Figure 8. Histopathological observations of skin tissue on day 21 with various treatments (a) control (-); (b) control (+); (c) BC; (d) BC/collagen and (e) BC/collagen/chitosan, Masson trichrome staining of skin tissue on day 21 with various treatments (f) control

(-); (g) control (+); (h) BC; (i) BC/collagen and (j) BC/collagen/chitosan, photo of granulation of burned skin tissue on day 21 (k) control (-); (l) control (+); (m) BC; (n) BC/collagen and (o) BC/collagen/chitosan.

### **Analysis of Masson's trichrome stain and wound granulation**

Masson's trichrome stain is a stain that specifically identifies collagen. In wound tissue that has healed, the formation of collagen fibers needs to be assessed to observe the formation of new epithelial tissue in the epidermis. Collagen is the main component of the skin matrix. The process of analyzing collagen fibers based on histopathological images is very common [38], [39], [41]. The higher number of collagen fibers and matured fibers in the sample group treated with BC/collagen/chitosan showed a better wound healing process.

The granulation tissue shown in Figure 8 is very important to evaluate the wound healing process. It provides information deep into the damaged tissue, prevents bacterial invasion, and prepares the matrix for cell proliferation and migration. Re-epithelialization is also an important factor for wound closure [39], [41]. Therefore, granulation tissue thickness and epithelial formation were observed in this study. Thicker granulation tissue and better epithelial formation were observed in rats which burns were treated with BC/collagen/chitosan wound dressing compared to rats receiving other wound dressing treatment. This finding indicated that BC/collagen/chitosan wound dressing supported the wound healing process. Figure 8 (k) showed that the wounds were not closed in the rats in control (-) group. This happened because the newly formed epidermis on the injured skin is very vulnerable as the dermal matrix is still immature. Newly formed epidermis is important to protect the wound area and support the healing process. The presence of wound dressings acted as the replacement of the epidermal tissue and protected the immature wound skin tissue.

### **Conclusions**

BC/collagen/chitosan wound dressing was successfully made by collagen *in-situ* followed by chitosan *ex-situ* impregnation into BC fibers. FTIR analysis showed that the functional groups of BC, chitosan, and collagen biopolymer were observed in BC/collagen/chitosan wound dressing bands. *In-vitro* test on a living cells showed that BC/collagen/chitosan hydrogel was acceptable to be used as a burn wound dressing since it is confirmed as non-toxic materials. *In-vivo* tests revealed that the addition of chitosan and collagen into the BC fiber effectively plays an active role in inhibit bacterial growth and support the wound healing process on second degree burn, respectively. The histological observation of a burnt tissue treated with BC/collagen/chitosan wound dressing also showed a fully formatted epithelial layer and finer scars on granulation tissue.

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supervision: S.G., S.I., T.T., I.R., A.G., and A.G.S; validation: S.G., S.I., T.T., I.R., A.G., and A.G.S.; visualization: K.M.P.; writing—original draft: K.M.P.; writing—review and editing: K.M.P., S.G., S.I., T.T., I.R., S.S., A.G., and A.G.S. All authors have read and agreed to the published version of the manuscript.

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## Declaration of interests

- The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
- The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.