Macromolecular Research Article

DOI 10.1007/s13233-022-0005-0 www.springer.com/13233 pISSN 1598-5032 eISSN 2092-7673

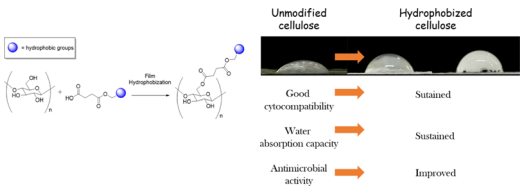
Performance Improvement of Hydrophobized Bacterial Cellulose Films as Wound Dressing

Katlyn Bazoli dos Santos[1]
Gustavo Eiji Higawa[2]
Karen Stefany Conceição[3]
Denise Coutinho Endringer[4]
Elisangela Flavia Pimentel Schmitt[4]
Lorena Martins Xavier[4]
Marcio Fronza[4]
Alessandra Stevanato[2]
Cesar Augusto Tischer[3]
Renato Márcio Ribeiro-Viana*[,1,2]

[1] Programa de Pós-graduação em Ciência e Engenharia de Materiais (PPGCEM), Universidade Tecnológica Federal do Paraná, UTFPR-Ld, CEP 86036-370, Londrina, PR, Brazil
[2] Departamento Acadêmico de Química, Universidade Tecnológica Federal do Paraná, UTFPR-Ld, CEP 86036-370, Londrina, PR, Brazil
[3] Departamento de Bioquímica e Biotecnologia, Universidade Estadual de Londrina, UEL, CEP 86051-980, Londrina, PR, Brazil
[4] Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Vila Velha - UVV, CEP 29102-920, Vila Velha, ES, Brazil

Received August 17, 2021 / Revised October 24, 2021 / Accepted October 29, 2021

Abstract: In the wound dressing research field, there is a continuous search for high-quality materials which present properties superior to those already used. Bacterial cellulose films are recognized as being effective, but their performance can still be further enhanced. On the other hand, wound dressings which present surfaces modified with hydrophobic molecules, such as dialkyl carbamoyl chloride, appear to be an alternative material, acting as antimicrobial dressings. Based on that, this paper describes the synthesis of small hydrophobic molecules based on inexpensive alcohols, such as octyl and benzyl alcohol, conjugated to the hydroxyl groups of bacterial cellulose. The films were prepared using ultrasound irradiation and characterized *via* infrared, as well as for their wettability and water absorption capacity, which showed greater contact angles and similar moisture retention when compared to unmodified films. Morphological aspects of modified films were analyzed by scanning electron microscope (SEM) and minimal modification in the structure was found. The hydrophobized cellulose films showed cytocompatibility with fibroblasts, and antimicrobial activity when compared to native bacterial cellulose films, by reducing the bacterial load up to 75%. This type of modification on these films is of interest in order to prepare films with better properties as dressings based on bacterial cellulose.



Keywords: wound dressing, chemical modification, bacterial cellulose, hydrophobic, biomaterial.

1. Introduction

The global market currently has various types of wound dressings, including natural or synthetic polymers and combinations thereof, with many types implemented, such as films, foams, hydrocolloids and hydrogels. In some of these, adjuvants such as drugs, growing factors, peptides and other bioactive substances may be added to speed up the healing process.[1]

The presence of bacteria in the wounds is a factor which delays the healing process, in addition to presenting the risk of starting septicemia.[2] Thus, the use of antimicrobial agents such as antibiotics[3] and silver[4] have been described, and also commercialized in dressings for use on infected wounds.

Another method that has been studied in the literature is the passive removal of bacterial content from the wound to the dressing.[5-7] This approach is based on the enhancement of the surface hydrophobic balance of the dressing, which interacts better with most bacteria cells; thus, causing the removal of these microorganisms. In addition, it is hypothesized that by using this mechanism the natural selection pressure is also low, as there is minimal bacterial death.

Dressings that use this mechanism are described as dialkyl carbamoyl chloride (DACC)-covered systems, which have been shown to be an interesting approach for reducing bacteria, although there are no studies showing whether the same result can be achieved *via* the use of other substances.[7] It is interesting to note that other studies describe the role of hydrophobic molecules in the antimicrobial activity as a direct action of killing the bacteria by disrupting its membrane and not by a passive removal of them.[8,9]

Cellulose is the most abundant, renewable and biodegradable natural polymers found on Earth. One special type, bacterial cellulose (BC), has drawn attention in the medical field due to its biomedical applications, and has been used to cover the surface of medical devices, such as stents, and in wound care. BC is chemically identical to cellulose produced by plants, but is

Acknowledgment: The authors would like to thank the Spectroscopy Laboratory at the State University of Londrina (SPEC-UEL-CT INFRA 2009-01.10.0534.01), the Multiuser Laboratory at the Federal University of Technology - Paraná - Londrina campus - for the analyses performed and Scanning Electron Microscopy Laboratory at State University of Maringa (Dr Eduardo Radovanovic). The authors would also like to acknowledge the financial support received from CNPq - Brazilian National Counsel of Technological and Scientific Development (423643/2018-5), CAPES and Araucária Foundation for Scientific and Technological Development of Paraná State.

***Corresponding Author:** Renato Márcio Ribeiro-Viana (renatoviana@utfpr.edu.br)

free from lignin and hemicellulose.[10-12] Furthermore, it presents different morphological aspects, with a higher degree of crystallinity and nanometric fibers, and is, thus, considered a nanomaterial. All these characteristics contribute to its use in the medical field, in addition to its presenting one of the lowest cytotoxicity levels among biopolymers.

In the wound dressing field, bacterial cellulose films present exceptional performance in the healing process. In addition, they do not adhere to wound beds; thus, facilitating dressing changes. However, bacterial cellulose is far from being perfect when considering all the desired aspects of an ideal dressing.[1]

Considering this, many research groups have implemented modifications to bacterial cellulose, particularly at the surface,[13-15] seeking to contribute additional features to this polymer. An antimicrobial action is one such desirable characteristic. Cellulose has no antibacterial activity besides physical protection; thus, it does not work properly on infected wounds. Some attempts to give cellulose antimicrobial characteristics have been described, such as delivery of antimicrobial molecules,[16-20] but this kind of approach usually leads to an increase in bacterial resistance, albeit at a low level due to topical delivery.

An interesting alternative to produce cellulose films with antimicrobial activity, but without the release of antibiotics, was described by Orlando *et al*.[21] In this study, bacterial cellulose film was modified with small organic compounds that contained positive charges and hydrophobic chains. This modified film showed antimicrobial activity and maintained its biocompatibility, therefore indicating that this strategy is valid in the search for antimicrobial films that do not release antibiotics.

The purpose of this study was to examine the enhancement of biocompatibility of cellulose dressings due to a decrease in hydrophilic/hydrophobic balance, and to test the antimicrobial activity due the presence of hydrophobic molecules on the surface of the films. In order to achieve this, films with reduced hydrophilic/hydrophobic balances were prepared. Cellulose surfaces were modified with small hydrophobic molecules and tested for achievement of this property, and resulting antimicrobial activity and cytocompatibility.

2. Experimental

2.1. Materials

Reagents were purchased from Sigma-Aldrich and Fluka and used without purification. Dichloromethane was dried under 3 Å molecular sieves for 48 h prior to use. The reactions under ultrasound irradiation were performed in an ultrasound cleaner - model *Ultra Sônico* - USC - 3300 (220 W, 40 kHz).

2.2. Methods

2.2.1. Production of bacterial cellulose

The Acetobacter xylinum ATCC 23769 (Gluconacetobacter xylinus ATCC 23769) bacterial strain was cultivated in a glucose medium based on the Hestrin–Schramm culture medium. Cellulose was produced as described previously.[22] The bacterial cellulose film was purified by immersion in an aqueous solution of 0.1 mol L^{-1} sodium hydroxide for 1 d. The films were repeatedly rinsed with deionized water until reaching pH 7.

2.2.2. Analytical methods

2.2.2.1. Liquid-state NMR analysis

^{1}H and ^{13}C spectra were recorded on a Bruker Avance III 400 MHz spectrometer, operating at a frequency of 400.6 MHz for ^{1}H and 100 MHz for ^{13}C, using a 5 mm inverse probe, BBI, at 25 ℃. Chemical shifts (δ) for ^{1}H spectra are expressed in ppm and calibrated according to the residual solvent signal ($CHCl_3$; δ = 7.26 ppm; HOD: δ = 4.76 ppm). Chemical shifts (δ) for the ^{13}C spectrum are expressed in ppm and calibrated according to the solvent signal ($CDCl_3$; δ = 77.00 ppm).

2.2.2.2. Fourier transform infrared (FT-IR) spectroscopy analysis

Infrared spectra were recorded on a Perkin Elmer Spectrum Two FT-IR spectrometer, coupled to the attenuated total reflectance accessory (ATR/FTIR), with measurements performed within the range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1}. Sixteen scans were performed for each sample, with a resolution of 1 cm^{-1}.

2.2.2.3. Contact angle measurements

Contact measurements were made by depositing 1 μL of deionized water on the surface of previously-dried films, which were glued onto double-sided tape, and their behavior was continuously captured using a digital optical microscope (UC500X). The contact angle was estimated by taking a static image after 10 s of contact between the water drop and the film surface. The image was digitally treated for grayscale mode using the GIMP 2.0 software). Finally, the estimates were manually performed using contour lines in each captured image, with angles determined using ImageJ software. All measurements were performed at room temperature (25 ℃) and in triplicate.

2.2.2.4. Water absorbance capacity

For water absorption tests, the membranes were dried in the desiccator prior to testing. Then, the dry membranes were submerged in 5 mL of distilled water for 24 h. After this period, the excess surface water was removed with the aid of a sieve, and their respective masses were noted. Subsequently, the membranes were kept in the oven at 70 ℃ for 1 h and the masses (dried) were determined. This test was carried out in triplicate for three different films for each sample. The result was expressed as mean and standard deviation. One-way ANOVA was applied, and differences were considered in cases where $p < 0.005$. The water absorbance capacity was determined by the equation:

$$\% \text{ moisture} = [(W_{wet} - W_{dry})/W_{dry}] \times 100 \quad (1)$$

Where W_{wet} is the mass of the wet film and W_{dry} is the mass of the dried film.

2.2.2.5. SEM analysis

The morphology of the cellulose films was evaluated using Electron Microscopy of Scanning (SEM) using a Shimadzu equipment, model SS-550. Before 35 analyses, a thin layer of gold was depos-

ited on the surface of the sample for make it electrically conductive. The images were obtained by applying a voltage of electron acceleration between 10 to 12 kV.

2.2.3. Synthesis

2.2.3.1. Synthesis of monooctylsuccinate

A total of 0.45 g of succinic anhydride (4.5 mmol), 0.391 g of n-octanol (3 mmol), 0.168 g of *N*,*N*-dimethylaminopyridine (DMAP) (3 mmol) and 6 mL of anhydrous dichloromethane were added to a 25 mL round-bottom flask. The reaction was kept for 2 h under constant agitation at room temperature. After the consumption of the alcohol, as observed by thin layer chromatography (TLC) (8:2 Hex:EtOAc, $KMnO_4$ solution used as stain), the mixture was evaporated and rinsed with dichloromethane, and then separated into two phases *via* liquid-liquid extraction, and extracted three times with dichloromethane. The organic phase was rinsed with 0.1 mol L^{-1} HCl, then with distilled water until its pH reached close to neutral. The organic phase was dried over Na_2SO_4 and concentrated to produce an oil (0.56 g) with 82% yield.

^{1}H NMR (400 MHz, $CDCl_3$, δ) - 4.15-3.09 (m, 2H), 2.70-2.55 (m, 4H), 1.70-1.50 (m, 1H), 1.40-1.20 (m, 8H), 0.90-0.85 (m, 6H).

^{13}C NMR (100 MHz, $CDCl_3$, δ) - 177.8, 172.31 (C=O), 67.2 (CH_2O), 38,7, 30.31, 28.9 (CH_{2succ}), 23.7, 22.9, 14.0, 10.9.

IR spectrum: 3400-2400 (O-H stretching, carboxylic acid), 2950, 2924, 2833 (C_{sp3}-H stretching), 1748 (C=O ester), 1709 (C=O carboxylic acid), 1157 (C-O stretching).

2.2.3.2. Coupling of monooctylsuccinate to bacterial cellulose film (BC-L1)

First, the films (2 cm × 2 cm) were rinsed with ethanol and dichloromethane. 0.030 g of bacterial cellulose film (0.185 mmol - AGU), 0.085 g of monooctylsuccinate (0.37 mmol), 0.0467 g of diisopropylcarbodiimide (DIC) (0.37 mmol), 0.011 g of DMAP (0.095 mmol) and 2 mL dichloromethane were added to a falcon tube. The vessel was submitted to ultrasound irradiation for 1.5 h at 40 ℃. After this period, the films were rinsed three times with ethanol and vortexed for 3 min, followed by three rinses with distilled water for the same period of time for each rinse. The films were left to dry at room temperature.

2.2.3.3. Synthesis of monobenzylsuccinate

0.5 g of succinic anhydride (5 mmol), 0.541 g of n-octanol (5 mmol), 0.61 g of DMAP (5 mmol) and 6 mL of anhydrous dichloromethane were added to a 25 mL round-bottom flask. The reaction was kept under constant agitation at room temperature for 2 h. After the consumption of the alcohol, as observed by TLC (8:2 Hex:EtOAc, $KMnO_4$ solution used as stain), the mixture was evaporated and rinsed with dichloromethane, then separated into two phases in a liquid-liquid extraction and extracted three times with dichloromethane. The organic phase was rinsed with 0.1 mol L^{-1} HCl, and then with distilled water until its pH was close to neutral. The organic phase was dried over Na_2SO_4 and concentrated to produce a solid material (0.87 g) in 83% yield.

^{1}H NMR (400 MHz, $CDCl_3$, δ) - 7.35 (s, 5H), 5.14 (s, 2H), 2.75-2.64 (m, 4H).

^{13}C NMR (100 MHz, $CDCl_3$, δ) - 178.1(C=O), 171.9 (C=O), 135.6 (C_{ar}), 128.5 (C_{ar}), 128.1 (C_{ar}), 66.6 (CH_2O), 28.86 (CH_{2succ}).

IR spectrum: 3380-2180 (O-H stretching, carboxylic acid), 3028, 3067 (C_{sp2}-H stretching), 2937, 2885 (C_{sp3}-H stretching), 1735 (C=O ester), 1701 (C=O carboxylic acid), 1644 (C=C stretching), 1170 (C-O stretching).

2.2.3.4. Coupling of monobenzylsuccinate to bacterial cellulose film (BC-L2)

First, the membranes (2 cm × 2 cm) were rinsed with ethanol and dichloromethane. 0.030 g of bacterial cellulose film (0.185 mmol - AGU), 0.085 g of monobenzylsuccinate (0.37 mmol), 0.0467 g of DIC (0.37 mmol), 0.011 g of DMAP (0.095 mmol) and 2 mL of dichloromethane were added to a falcon tube. The vessel was submitted to ultrasound irradiation for 1.5 h at 40 ℃. After this period, the films were rinsed three times with ethanol and vortexed for 3 min, followed by three rinses with distilled water for the same period of time for each rinse. The films were dried at room temperature.

2.2.4. *In vitro* cell assays

2.2.4.1. Fibroblast growth test:

2.2.4.1.1. Cell culture

Fibroblast cells (L929) (ATCC® CCL1TM) were cultured with Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 IU mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin (Sigma-USA), at 37 ℃ in a humidified atmosphere containing 5% CO_2. Fibroblasts were disaggregated by trypsinization with 0.05% Trypsin/0.02% EDTA (Gibco, Grand Island, NY, USA).

2.2.4.2. Assessment of cell viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method was used for the assessment of cell viability.[23] After 24 h of incubation of the L929 fibroblast cells, at a density of 5 × 10^5 cells mL^{-1} on the films, 100 µL of MTT (5 mg mL^{-1}) was added and allowed to incubate for a further 2 h. Then, the MTT was aspirated and 100 µL of dimethyl sulfoxide (DMSO) was added. The absorbance was measured at a wavelength of 595 nm using a microplate reader (Molecular Devices, Spectra Max 190, USA). Cell viability was calculated as a percentage of surviving cells in relation to the negative DMSO control. The result was expressed as mean and standard deviation. One-way ANOVA was applied followed by Tukey's post-hoc test, and differences were considered in cases where $p < 0.005$.

2.2.4.3. Antibacterial activity

The antibacterial activity of the bacterial cellulose film samples was assessed using the agar disc diffusion method with determination of zones of inhibition,[24] and estimated by counting the number of cells per mL in a Neubauer chamber. The *Pseudomonas aeruginosa* PAO1 gram-negative pathogenic bacterium, containing 4 colonies, was incubated in Luria-Bertani (LB) medium at 200 rpm, at 37 ℃ for 24 h. The films were prepared in the form of a 10 mm diameter round disc (BC-L1, BC-L2, BC and Cutimed® Siltec Sorbact commercial dressing - hydrophobic area only), sterilized under ultraviolet light for 10 min and then carefully placed on the surface of Mueller-Hinton agar (MH) containing the bacteria. They were subsequently incubated at 37 ℃ for 24 h.

After that time, the films were removed, washed with distilled water (1 mL) and the number of bacteria was counted in a Neubauer chamber using an optical microscope. The bacterial load reduction (R%) was expressed by the equation:

$$R\% = [|(N_{BC} - N_{sample})|/N_{BC}] \times 100 \quad (2)$$

Where N_{bc} is number of colonies for the unmodified bacterial cellulose and N_{sample} is number of colonies for any sample.

3. Results and discussion

The strategy developed in this study takes into consideration the chemical functionality of the bacterial cellulose surface, in order to produce a less hydrophilic contact environment of the film. To achieve this, small hydrophobic organic molecules were synthesized and then coupled to cellulose films in the heterogeneous phase, according to Figure 1.

Two succinyl monoesters were prepared by coupling low-cost, hydrophobic alcohols (octyl and benzyl) to the succinic group, and then chemically attaching them to the films *via* ester bonds. Initially, the selected alcohol was reacted with a small excess of succinic anhydride in the presence of DMAP. After observing that the alcohol was consumed, the reaction was interrupted by addition of water and the product was collected in the organic phase as oils, in high yields (80%). The compounds were identified *via* ^{1}H (Figure 2) and ^{13}C NMR (Figures S1 and S2 - Supplementary material) and IR spectroscopy (Figures S3 and S4 - Supplementary material). In the ^{1}H NMR spectra of both compounds (Figure 2), the relationship of 2:4 of carbinolic hydrogens and the two methylenes of the succinic group could be identified, showing the correct coupling of succinic group to the respective alcohol. Besides these signals, in the spectrum A of Figure 2, there are three signals between 1.7 and 0.75 ppm with integral of 15 hydrogens corresponding to the other hydrogens of the aliphatic chain of octyl group. In the spectrum B of Figure 2, besides the signals of methylenes of succinic between 2.75 and 2.64 ppm and the benzylic methylenes at 5.14 ppm, there is a singlet at 7.35 ppm corresponding the aromatic hydrogens with integral of five.

The next step was to couple the hydrophobic succinyl monoesters to the cellulose surface. A methodology was developed to perform reactions mostly on the films, without changing the bulk polymer, by using ultrasound radiation. The reaction occurs in a heterogeneous medium using any ordinary solvent.[25] To promote the reactions, coupling reagents such as DIC and DMAP were dissolved in anhydrous dichloromethane and submitted to convectional ultrasound irradiation. A control reaction was performed by using the same reagents and conditions, without the use of the coupling agent DIC (Figure S5 - Supplementary material). After 1.5 h, the irradiation source was stopped, then the films were rinsed and analyzed *via* IR spectroscopy (Figure 3).

In the IR spectra for both reactions, a similar pattern of bands could be observed. In addition to the typical bands found for cellulose, for instance 3000 cm^{-1} related to O-H stretching, 2980 cm^{-1} corresponding to C-H stretching and 1200 cm^{-1} for C-O bonds, a new signal appeared in both spectra. A band at 1720 cm^{-1}, typical of ester carbonyls, could be observed in the spectra, demonstrating the presence of the carbonyl hydrophobic esters in the film. Considering that only the films reacting in the presence of the coupling agent DIC showed this new band after the rinsing step, the carbonyl band was considered as a sign of successful coupling of these molecules to the cellulose films.

In order to characterize the morphology of the surface of the films and to observe if the chemical reactions had an impact on its structure, analyzes were carried out by SEM. Figure 4 shows SEM images with lower and higher magnification of BC-L1(Figure 4(A) and (B)), unmodified films (Figure 4(C) and (D)) and BC-L2 (Figure 4(E) and (F)). All of them have a flat and fibrillar network randomly distributed on the surface, typical of bacterial cellulose nanofiber strands. It is also observed the well-ordered three-dimensional network and in several superimposed layers. In general, the fibril structure is very similar between BC and BC-L1 films, with a diameter in the range of approximately 44 to 53 nm for both. On the other hand, the structure of BC-L2 film appears more cohesive, smooth and dense, with slightly larger diameters, in the range of 50 to 60 nm. Interestingly, the BC-L1 film has pores in its network, randomly distributed, similar to those found in the unmodified film. The BC-L2 structure has considerably fewer pores compared to other films. These data indicate that the performed chemical modifications smoothly modified

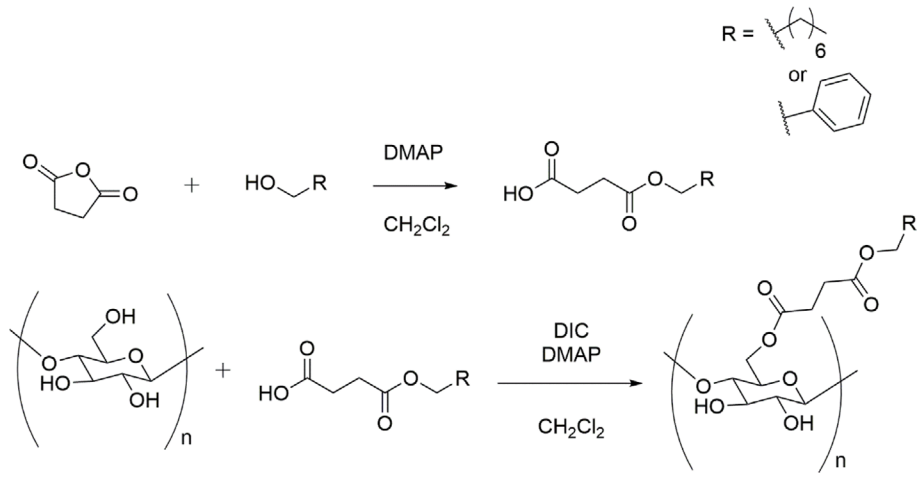


Figure 1. General strategy for the functionalization of bacterial cellulose (BC) films with hydrophobic groups.

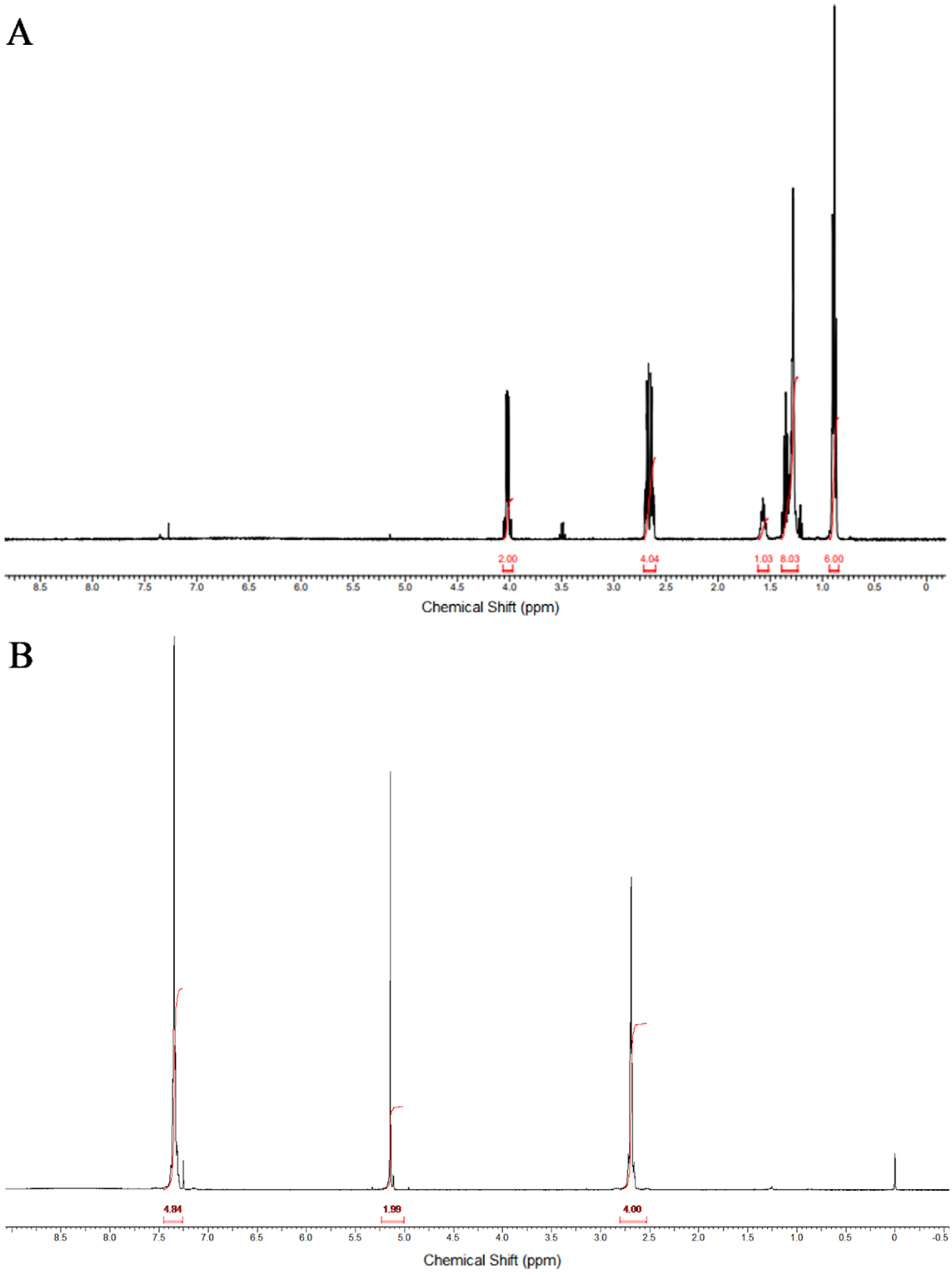


Figure 2. ^{1}H NMR spectra of (A) monooctylsuccinate and (B) monobenzylsuccinate.

the microstructure of BC-L2. The reaction carried out with the aromatic compound may have slightly altered the arrangement on its surface (BC-L2) due to the insertion of these hydrophobic groups, while the microstructure of BC-L1 is similar to the BC.

To measure the relevance of these chemical modifications in altering the hydrophilic/hydrophobic balance of the material, a wettability test (Figure 5) was carried out using water as a solvent, and the contact angle was measured. By obtaining a static image after 10 s of contact between the drop of water and the surface, the angles were determined, and measurements were averaged and compared to the unmodified film as shown on Table 1.

Formally, hydrophobic materials are considered those where water drops on their surfaces have contact angles greater than

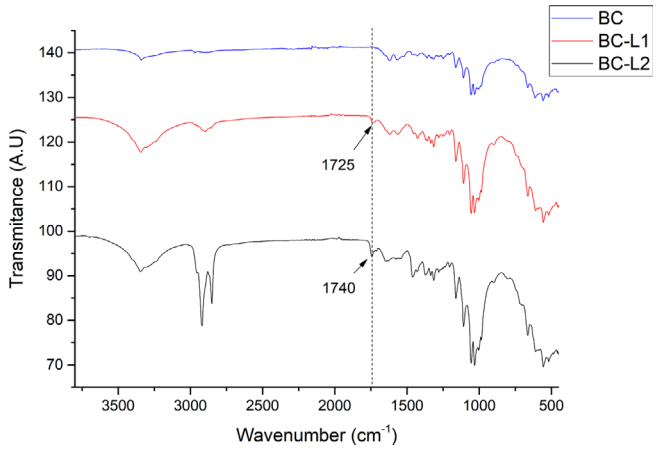


Figure 3. Infrared spectra of BC (blue line), BC-L1 (red line) and BC-L2 (black line).

90°. According to Table 1, it was found that the functional groups introduced increased the hydrophobicity of the medium when compared to the observed contact angle found for unmodified cellulose. Therefore, it is understood that these changes have increased the surfaces' hydrophobic character, although they are still considered hydrophilic. It is important to note that hydrophobic structures, especially those containing long carbon chains, are reported to have antimicrobial activity by interacting with the bacterial cell wall and driving these cells to death.[8,9]

Another important characteristic of an ideal wound dressing is the water absorbance capacity, due to the importance of retaining the excess exudate that occurs in some wounds. Bacterial cellulose films are recognized for absorbing many times their own weight in water, and although this feature can still be improved, it is considered adequate in these films. For this reason, the water absorbance capacity of these modified films was evaluated to check whether or not the chemical modification performed interfered with this parameter (Table 1). The values observed for the three films showed no statistical difference, indicating that the modified films can still absorb liquid from the medium. It is interesting to note that the insertion of hydro-

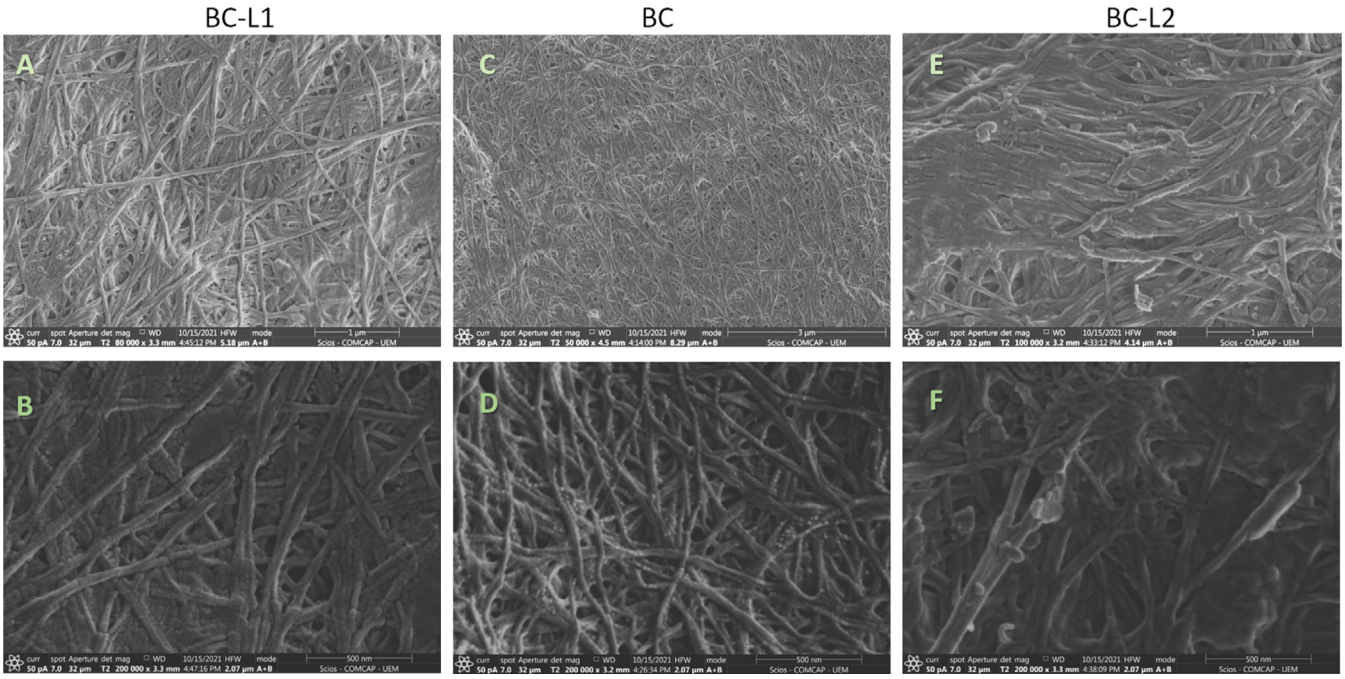


Figure 4. The SEM micrographs of (A,B) BC-L1, (C, D) BC and (E,F) BC-L2.

Table 1. Data from contact angles, water absorbance capacities and fibroblast growth assays of BC, BC-L1 and BC-L2

Membrane	Contact Angle (° ± MD)	Water absorbance capacity (% ± MD)	Fibroblast growth assay
BC	52.7 ± 2.1^{a}	551.19 ± 0.13^{a}	Confluence ~60-80% Viability 83.07% ± 3.24^{a}
BC-L1	66.5 ± 4.1^{b}	563.17 ± 1.32^{a}	Confluence ~60-80% Viability 97.51 ± 5.73^{a}
BC-L2	81.2 ± 3.2^{c}	659.71 ± 1.66^{a}	Confluence ~60-80% Viability 90.58 ± 4.61^{a}

For each columm, means followed by the same letter are not significantly different (P>0.05 ANOVA followed by Tukey's post-hoc test).

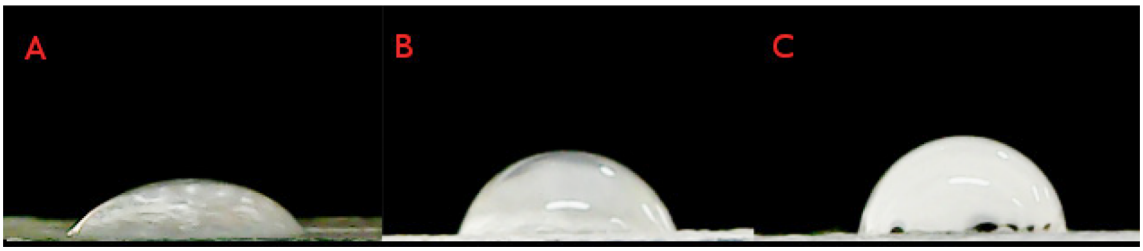


Figure 5. Picture of 1 μL of water on the surface of (A) BC, (B) BC-L1, and (C) BC-L2.

phobic chemical groups by the technique described in this paper is able to change the hydrophilic/hydrophobic balance of the surface; however, the surface still retains the ability to retain water over time. It is thought that this is due to the functionalization occurring more superficially, which allows the water, after first contact on the surface, to enter the more hydrophilic layers of the film. This feature, water absorption, is desirable in dressings, as it reduces the frequency of changes and keeps the wound bed moist. The ability to keep water in a dressing is directly linked to the speed of re-epithelialization and protection against contamination by microorganisms.[26] In the case of modified films, it is interesting to note that although they present a first hydrophobic layer in contact with water, they still maintain this characteristic in a statistically similar way to the unmodified film.

These films were analyzed for fibroblast cell growth in order to verify their growth and cytotoxicity (Table 1).

According to the data, it is observed that the confluence, that is, the growth of cells on the surface of the films, was similar among all films. It is understood that the modifications made did not change how the cells spread over the films. Although an increase in the viability of the cells could be observed, statistically all the films have the same performance considering this parameter. Currently, it is understood that cytocompatibility is governed by a series of factors and their combinations, such as morphology, functional groups present and hydrophilic/hydrophobic balance.[27] In several cases, surface modifications of materials with an ideal hydrophobic balance have already been reported, wherein cytocompatibility for tissue cells and antimicrobial activity were also reported.[28]

Considering that these films were as cytocompatible as the unmodified bacterial films, they were submitted to an antibacterial assay in order to observe their behavior towards bacteria. *Pseudomonas aeruginos*a was chosen as the bacterial model, due to its importance in skin infections.

In the antimicrobial activity inspection model used, two parameters can be observed: zone of inhibition and number of bacteria on the film surface. The samples used were modified bacterial cellulose (BC-L1 and BC-L2), unmodified bacterial cellulose film, and Cutimed®Siltec Sorbact wound dressing (hydrophobic area only) (Figure 6).

No sample presented an inhibition zone for bacterial cell growth. This result is in line with what was expected, as with the chemical modifications made to the films it was not expected that any type of substance would be released in the medium.

Regarding the count of bacteria present in the films, the following values were observed: 8×10^3 cells mL^{-1} for unmodified bacterial cellulose, 15×10^3 cells mL^{-1} for commercial dressing,

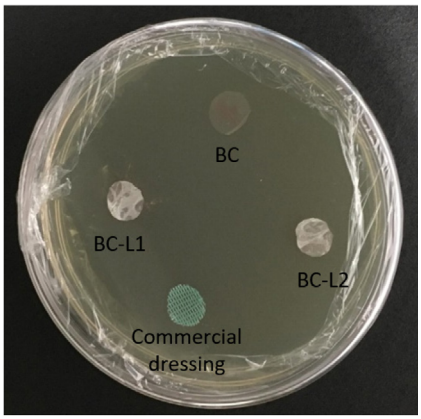


Figure 6. Antimicrobial assay of bacterial cellulose (BC), BC-L1, BC-L2, and commercial dressing films.

6×10^3 cells mL^{-1} for BC-L1, and 2×10^3 cells mL^{-1} for BC-L2 film.

Considering the unmodified bacterial cellulose as a reference in this test, the modified films showed a lower quantity of cells present on their surfaces, while the commercial dressing presented a higher quantity of cells. It represents a reduction of bacterial load (R%) in contact with the films by 25% and 75% for BC-L1 and BC-L2, respectively, in comparison with unmodified bacterial cellulose. Considering the hypothesis of passive removal of bacterial load as mentioned for the commercial dressing, the modified films did not show an increased capacity to retain bacteria on their surface; however, this result can be understood as an antimicrobial action, i.e., inhibition of bacterial cell growth on its surface, which is also desirable for the final application of these dressings. On the other hand, due to the experimental model, it was not possible to confirm the claim of the commercial dressing's ability to remove bacterial cells from the wound. The hydrophobic layer of the commercial dressing consists of a checkered weave containing empty spaces, which ends up being permeated by liquid from the medium where it is located. When the film was removed from the plate, it was possible to observe the accumulation of liquid in its weft. This may justify the increase in bacteria retention on its surface, indicating that this is a macromolecular effect in this experiment, specifically the retention of liquid medium containing bacteria, and not an effect of cell removal due to intermolecular interactions. In fact, according to a previous study,[7] the effect of the bacterial load reduction factor is not well observed in the data obtained for the Sorbact Cutmed dressing. In this study, the bacterial growth in this dressing is greater than in the control group. Thus, a comparison of these films in this model only allows us to observe

that fewer bacteria survived (antimicrobial effect) upon contact with the modified bacterial cellulose films.

4. Conclusions

Cellulose films were functionalized with small hydrophobic organic molecules and then characterized. Both films became more hydrophobic, although still remained a hydrophilic nature and retained their ability to absorb water, despite the chemical changes. These films decreased the population of *Pseudomonas aeruginosa* on contact, compared to unmodified bacterial cellulose films and a hydrophobic commercial wound dressing, thus showing an antimicrobial effect. The modified films showed cytocompatibility for fibroblasts indicating that the hydrophobic surface interacts well with those cells. This type of modification can improve the use of bacterial cellulose as a dressing by optimizing its characteristics for this purpose and being an alternative for further studier in infected wounds.

Disclosure information: The data of this work were deposited in the form of a patent by the authors and institutions under the number BR 10 2020 020709 1.

Supporting Information: Information is available on the ^{13}C and IR characterization of the synthesized molecules, in addition to the IR spectrum of the films that were reacted under conditions of absence of DIC coupling agent. The materials are available *via* the Internet at http://www.springer.com/13233.

References

(1) I. Sulaeva, U. Henniges, T. Rosenau, and A. Potthast, *Biotechnol. Adv.*, **33**, 1547 (2015).
(2) S. Homaeigohar and A. R. Boccaccini, *Acta Biomater.*, **107**, 25 (2020).
(3) F. Aavani, S. Khorshidi, and A. Karkhaneh, *J. Med. Eng. Technol.*, **43**, 38 (2019).
(4) K. Kalantari, E. Mostafavi, A. M. Afifi, Z. Izadiyan, H. Jahangirian, R. Rafiee-Moghaddam, and T. J. Webster, *Nanoscale*, **12**, 2268 (2020).
(5) P. Chadwick and K. Ousey, *J. Wound Care*, **28**, 370 (2019).
(6) T. Wadström, S. Björnberg, and S. Hjertén, *Acta Pathol. Microbiol. Scand. Ser. B Microbiol.*, **93B**, 359 (1985).
(7) H. Braunwarth and F. H. H. Brill, *Wound Med.*, **5**, 16 (2014).
(8) Z. Zheng, Q. Xu, J. Guo, J. Qin, H. Mao, B. Wang, and F. Yan, *ACS Appl. Mater. Interfaces*, **8**, 12684 (2016).
(9) M. C. Wiener, P. S. Horanyi, T. Om, and T. Om, *Proc. Natl. Acad. Sci.*, **108**, 10929 (2011).
(10) L. Fu, J. Zhang, and G. Yang, *Carbohydr. Polym.*, **92**, 1432 (2013).
(11) N. Shah, M. Ul-Islam, W. A. Khattak, and J. K. Park, *Carbohydr. Polym.*, **98**, 1585 (2013).
(12) M. Shoda, Y. Sugano, N. Petersen, and P. Gatenholm, *Appl. Microbiol. Biotechnol.*, **91**, 1277 (2011).
(13) H. Abushammala and J. Mao, *Molecules*, **24**, 2782 (2019).
(14) Y. Habibi, *Chem. Soc. Rev.*, **43**, 1519 (2014).
(15) F. G. Blanco Parte, S. P. Santoso, C.-C. Chou, V. Verma, H.-T. Wang, S. Ismadji, and K.-C. Cheng, *Crit. Rev. Biotechnol.*, **40**, 397 (2020).
(16) J. Wu, Y. Zheng, W. Song, J. Luan, X. Wen, Z. Wu, X. Chen, Q. Wang, and S. Guo, *Carbohydr. Polym.*, **102**, 762 (2014).
(17) I. Bernardelli de Mattos, S. P. Nischwitz, A.-C. Tuca, F. Groeber-Becker, M. Funk, T. Birngruber, S. I. Mautner, L.-P. Kamolz, and J. C. J. Holzer, *Burns*, **46**, 918 (2020).
(18) M. I. N. Ahamed, S. Sankar, P. M. Kashif, S. K. H. Basha, and T. P. Sastry, *Int. J. Biol. Macromol.*, **72**, 680 (2015).
(19) M. L. Cacicedo, G. Pacheco, G. A. Islan, V. A. Alvarez, H. S. Barud, G. R. Castro, *Int. J. Biol. Macromol.*, **147**, 1136 (2020).
(20) H. Namazi, R. Rakhshaei, H. Hamishehkar, and H. S. Kafil, *Int. J. Biol. Macromol.*, **85**, 327 (2016).
(21) I. Orlando, P. Basnett, R. Nigmatullin, W. Wang, J. C. Knowles, and I. Roy, *Front. Bioeng. Biotechnol.*, **8**, 1 (2020).
(22) F. D. E. Goelzer, P. C. S. Faria-Tischer, J. C. Vitorino, M. R. Sierakowski, C. A. Tischer, *Mater. Sci. Eng. C*, **29**, 546 (2009).
(23) T. Mosmann, *J. Immunol. Methods*, **65**, 55 (1983).
(24) CLSI, *Performance Standards for Antimicrobial Susceptibility Testing. Informational Supplement*, Clinical & Laboratory Standards Institute.
(25) S. Birkheur, E. Laureto, R. V. Fernandes, C. Tischer, A. P. Butera, and R. M. Ribeiro-Viana, *Mater. Res.*, **23**, e20200197 (2020).
(26) S. Kaewnopparat, K. Sansernluk, and D. Faroongsarng, *AAPS PharmSciTech*, **9**, 701 (2008).
(27) K. Khanmohammadi Chenab, B. Sohrabi, and A. Rahmanzadeh, *Biomater. Sci.*, **7**, 3110 (2019).
(28) R. Song, Y. Zhang, Q. Huang, Y. Yang, L. Lin, J. Liang, R. Hu, G. Rui, and C. Lin, *ACS Appl. Bio Mater.*, **1**, 1056 (2018).

