Crosslinked Bacterial Cellulose Hydrogels For Biomedical Applications

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Crosslinked bacterial cellulose hydrogels for biomedical applications

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Abstract

The skin, fundamental barrier that protects internal tissues, prevents pathogen invasion, and maintains the body fluid equilibrium, may be compromised upon traumas, such as incisions and burns. The healing process of such wounds is costly and usually hindered by the patient’s physiological conditions, associated diseases, inflammation and external factors, namely bacterial infections. Recently, increasing attention has been given to bacterial cellulose-based membranes to be applied as dressings for healing purposes. Bacterial cellulose is an attractive biomaterial due to its unique structural characteristics such as high porosity, high water retention capacity, high mechanical strength, low density, and biodegradability. One drawback of bacterial cellulose hydrogels is that, after the first dehydration, the water retention capacity is hindered. In this work we produced, modified, and characterized hydrated and dehydrated BC membranes. Two crosslinking methods were adopted (using citric acid and epichlorohydrin as crosslinking agents), and the results obtained from the characterizations such as water retention capacity, mechanical properties or contact angle were compared to those of unmodified bacterial cellulose. We demonstrate that the cross-linked bacterial cellulose membranes present physical properties suitable to be used as surgical and burn wound dressings when hydrated, or as exuding wound dressings, diapers dressing or sanitary pads when dehydrated.

Keywords
biomaterials, biomedical applications, bacterial cellulose, wound dressings, physical characterization.
Introduction

Cellulose is the most abundant natural polymer in the world and of great economic importance being a linear homopolysaccharide composed by D-glucopyranose units linked by glycosidic bonds (Fernandes et al. 2017). The largest source of this material is the cell wall of plants, but it is also produced by fungi, protozoa and prokaryotes (Morgan et al. 2016). Much of the cellulose by-products, such as paper, textiles, and construction materials, are removed from cotton and wood. But issues of sustainability and preservation of the environment have led to the search for alternative non-conventional materials, such as bacterial cellulose (BC). For many industrial applications, vegetable cellulose is inconvenient, due to its association with other biopolymers such as hemicellulose and lignin (Martinez-Sanz et al.). The separation of these materials requires the use of chemicals responsible for higher costs, wastes and more processing time. BC is also an attractive biomaterial due to its unique structural characteristics such as high porosity, high water retention capacity, high mechanical strength even in the hydrated state, low density, and biodegradability (Portela et al. 2019).

BC is free from other biopolymers and can be purified by simple processes. Another advantage of bacterial cellulose is the possibility of synthesis in a short time, independent of long and extensive agroforestry crops, by different genera of microorganisms. The genus *Komagataeibacter* is the most studied, from which the species *K. rhaeticus* stands out for its high production capacity from different carbon sources (glucose, fructose, molasses, glycerol, or other organic substrates) and nitrogen (Nguyen et al. 2022), which is an important advantage of this microorganism in industrial application (Czaja et al. 2006). Under static cultivation conditions, a BC-biofilm of tunable thickness is formed to maintain high oxygenation of the cells near the surface, and to serve as a protective barrier against drying and radiation. BC is formed by very small fibers, on a nanometric scale (Portela et al. 2019). Chemically the BC structure is identical to vegetal cellulose, being composed by β-1-4-linked glucosidic chains. These chains are organized into moderately crystalline BC fibers containing hydroxyl groups on their surfaces. These BC fibers are composed by microfibril units with diameters of around 3 nm, having themselves diameters in the range of 10-130 nm (Martinez-Sanz et al.). These randomly oriented fibers form a non-woven, highly hydrated gel during the aqueous fermentation conditions. This fact provides BC with superior properties compared to vegetable cellulose. Ribbon-shaped microfibers provide bacterial cellulose with peculiar mechanical properties, such as high tensile strength, elasticity, durability, and high-water retention capacity. This biopolymer can absorb over 200 times its own mass in water. BC is biocompatible, inert, non-toxic, and non-allergenic and thus its use as dressings results in better healing. It provides a moist environment for wounds, presents adequate structure and mechanical robustness, in addition to being selectively permeable. It is also promising for the incorporation of antimicrobial agents and can be used in several fields of health sciences, in applications such as structures for bone regeneration, drug delivery systems, new vascular grafts, or supports for tissue engineering (Mbituyimana et al. 2021). BC has already been used quite successfully in wound-healing applications, proving that it could become a high-value product in the field of biotechnology (Fursatz et al. 2018). Wound
dressing cellulose hydrogels should ideally display high porosity, with a nanopore structure and should keep the wound moist during the healing process (Capes, Ando, and Cameron 2005). Hydrated dressings of BC have proven healing potential, however, due to the water content, the accumulation of secretion in exudative injuries may occur. For these exuding wounds, as well as for diapers dressing and sanitary pads, dehydrated BC membranes might be used. Unmodified BC have a high-water retaining capacity (> 200x its own weight) and a low water release (Schrecker and Gostomski 2005). However, upon dehydration, the structure of BC collapses due to the evaporation of water, reducing its ability to re-swell. To revert this drawback, one possible solution is the crosslinking of the BC prior to the first dehydration of the membranes (Geravand et al.). Several other modifications have been proposed to counteract the disadvantageous consequences of the first BC dehydration, as for example, the production of BC-fibrin or BC-gelatin composites, resulting in improvement of their physical properties (Chang et al. 2012; Meftahi et al. 2018).

In this work, chemical modifications were performed on bacterial cellulose, by crosslinking the biopolymer with two different crosslinkers, namely Citric Acid (CA) and Epichlorohydrin (ECH), to increase its water retention capacity after the first dehydration. These modified membranes were characterized, and their properties were compared with unmodified BC.

Materials and Methods

Bacterial Cellulose production

The *Komagatetbacter rhaeticus* strain DSM-16663 was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and reconstituted accordingly to the DSMZ instructions. The strain was grown in DSMZ medium 1044 (glucose 50.0 g/L, yeast extract 5.0 g/L and agar 15.0 g/L, pH 6.5). The plates were incubated at 28 °C during 2-3 days.

To produce BC, single colonies were used to inoculate the growth media (yeast extract 4.0 g/L, anhydrous disodium phosphate 2.0 g/L, heptahydrated magnesium sulfate 0.8 g/L and ethanol 20 g/L) (De Salvi et al. 2014). The cultures were grown statically at 28 °C until the BC pellicles reached the desired thickness, for approximately 10-15 days.

After the 10-15 days of incubation, the BC discs were removed from the culture, washed twice with distilled autoclaved water to ensure the complete removal from the BC matrix of bacterial cells, cell debris, and remaining compounds of the culture. After this first step, the membranes were washed in absolute ethanol for two hours with gentle agitation and again twice in hot distilled autoclaved water. The final step consisted in immersing the BC disks statically in 0.1 M NaOH solution, for 48 h. After this alkaline treatment, the BC disks were again washed twice with autoclaved water until a neutral pH of 7 was achieved. After the washing procedure, the BC disks were stored in the hydrated form (in Milli-Q water) at 4 °C.

Bacterial Cellulose crosslinking
Considering that the water retention capacity of the bacterial cellulose is reduced after the first dehydration, meaning that after drying for the first time the BC will not be able to absorb the same amount of water with respect to its own weight, two methods of BC crosslinking were used to counteract this fact. By crosslinking the BC, a 3D structure is promoted that reduces the collapse of the pore structure, and thus sustains a higher value of water retention capacity of the BC membranes. BC crosslinking is typically obtained by linking at least two hydroxyl groups of single cellulose molecules or two or more hydroxyl groups of adjacent cellulose molecules, resulting in a stiffer polymer and preserving its 3D structure (Qi et al. 2016).

Two methods of crosslinking were applied, one using citric acid and the other epichlorohydrin as the crosslinking agents as can be seen in Fig. 1.

**Fig. 1** Crosslinking reaction between the hydroxyl groups of the bacterial cellulose (A) Citric acid and (B) Epichlorohydrin

**Citric acid crosslinking**

Citric acid (CA) is a tricarboxylic acid based on propane-1,2,3-tricarboxylic acid with a hydroxy substituent at position 2. Citric acid is a very suitable crosslinker for wound dressing, not only due to its non-toxicity, but also due to the stable crosslinking bonds that it forms with cellulose. Moreover, CA is widely accessible and very low cost, which makes it a very interesting reagent for industrial applications (Frone et al. 2020). To perform the BC crosslinking with CA, a mixture of sodium dihydrogen phosphate (H$_2$NaO$_4$P) and sodium hydrogen carbonate (NaHCO$_3$) (1:1 mass ratio) was used as catalyst. Aqueous solutions containing different amounts of CA (10%, 20%, 30% and 50% weight percentage with respect to the hydrated BC membrane), and catalyst (50% weight percentage with respect to CA) were prepared by magnetic stirring at room temperature. The
unmodified BC membranes were added to these aqueous solutions and incubated under magnetic stirring at 40 °C for 24 hours. The BC membranes were then crosslinked under reflux at 150 °C for 75 minutes. Subsequently, the membranes were washed at least 3 times, for 30 minutes each, with Milli-Q water to remove the residual H$_2$NaO$_4$P and NaHCO$_3$, and the unreacted CA. The membranes were then kept at 4 °C until further use. The crosslinking reaction between the hydroxyl groups of BC and citric acid is shown in Fig. 1A.

**Epichlorohydrin crosslinking**

Epichlorohydrin (ECH) is an epoxide compound, based on 1,2-epoxypropene in which one of the methyl hydrogens is substituted by chlorine. It has moderate toxicity and has been described that short-term inhalation of this compound might cause irritation to the eyes, respiratory tract and skin and might be a human carcinogen. However, if ECH fully reacts with BC, it becomes inoffensive (Schio et al. 2021). To perform the BC crosslinking with ECH, aqueous solutions containing different amounts of ECH (0.5%, 1%, 2%, 5% and 10% weight percentage with respect to the hydrated BC membrane) were prepared by magnetic stirring at room temperature. The unmodified BC membranes were added to the aqueous solutions and were crosslinked under reflux at 60 °C for 120 minutes. The pH value of the aqueous solutions was adjusted to 9 with a 0.1 M solution of NaOH. Subsequently, the membranes were washed at least 3 times, for 30 minutes each, with Milli-Q water to remove the residual NaOH and unreacted ECH. The membranes were then kept at 4 °C until further use. The crosslinking reaction between the hydroxyl groups of the bacterial cellulose and Epichlorohydrin is shown in Fig. 1B.

**Water loss rate**

The water loss assays were conducted by placing BC samples in a ventilated oven at 35 °C and by weighting them at periodic time intervals.

**Water uptake rate and water retention capacity**

The hydrophilic properties of BC membranes were studied through the water absorption capacity as a function of time. The water absorption capacity depends on the degree of interaction between water and the cellulose molecules. Furthermore, the water absorption capacity is related to the structure of the BC membrane, since the larger is the size of the pores in the membrane, the greater is the water absorption capacity and, consequently, the greater is the degree of water retention. To test the water retention capacity (maximum water absorption) of the different membranes, crosslinked with different amounts of each crosslinker, the membranes were initially weighted after dehydration and 48 hours after being placed in Milli-Q water, *i.e.*, after full hydration.

**FTIR-ATR characterization**

To characterize the chemical structure of the produced membranes, Fourier transform infrared spectroscopy – attenuated total reflectance (FTIR–ATR) analysis was performed. The identification of specific functional groups reveals chemical changes that occurred in the unmodified structure. The FTIR–ATR spectroscopy characterization of the BC membranes was conducted using an
attenuated total reflectance (ATR) sampling accessory (Smart iTR) equipped with a single bounce diamond crystal on a Thermo Nicolet 6700 Spectrometer. The spectra were acquired with a 45° incident angle in the range of 4500-540 cm\(^{-1}\) and with a 4 cm\(^{-1}\) resolution.

Mechanical properties of the dehydrated membranes

The mechanical tensile tests were carried out using a static-dynamic miniature testing machine inspect micro LC 100 N from Hegewald & Peschke Meß-und Prüftechnik GmbH with a 100 N load cell. Rectangular pieces of the BC membranes with dimensions 0.5×3.0 cm\(^2\) were cut radially from the membrane disks. At least 3 tests were conducted for each membrane. The rectangular samples were tested uniaxially in tension with a strain rate of 0.5 mm.min\(^{-1}\) along their widest dimension, at room temperature. The initial gauge length \(l_0\) for each sample was 10 mm, the width of the test samples was 5 mm and the thickness of the membranes used for the tensile tests were in the range of 50-100 µm when dehydrated. From these tensile tests, mechanical properties such as the ultimate tensile strength (UTS), maximum strain and the Young’s modulus (elastic modulus), were determined.

Mechanical properties of the hydrated membranes

The rheological characterization of BC membranes was performed with a Modular Compact Rheometer MCR 502 (Anton Paar, Madrid, Spain), using a parallel-plate geometry with a sandblasted surface, with a diameter of 25 mm. Oscillatory shear measurements were performed to evaluate the storage \(G'\) and the loss \(G''\) moduli within the linear viscoelastic regime (LVR) in which the moduli are strain independent. Measurements were carried out under a constant strain of 0.1% in the angular frequency range of 0.6 to 100 rad/s, at two different temperatures, 25 ºC and 37 ºC. The BC membranes were produced in containers with similar diameter (25 mm) as the one of the rheometer geometry to assure a correct fit and avoid sample cut. Sample thicknesses were obtained in the range of 1.5±0.7 mm. Before loading the BC samples in the rheometer plate, the membrane water excess was removed by a brief both side deposition on filter paper.

Contact angle measurements

To determine the water contact angles formed on the surface of BC membranes, measurements were performed using an OCA20 contact angle measuring instrument (DataPhysics Instruments GmbH, Filderstadt, Germany). Probe liquid micro drops (volume \(\approx 5 \mu\text{L}\) of Milli-Q water) were generated with an electronic micrometric syringe and deposited on the sample surface. A sequence of images was obtained The contact angle was determined at the moment of drop deposition and settlement (\(\tau = 0\) s) and its evolution was followed for 120 s with a system video camera and recorded at rate of 2 frames per second. The contact angle was determined by fitting the shape of the drop (in the captured video image) to the Young-Laplace equation, which relates the interfacial tension to the shape of the drop. A total of six drops of the probe liquid were dispensed and each drop was placed in a different region of the membrane. All measurements were performed at room temperature, using dry membranes of 1.0×2.0 cm. The results correspond to the average of six independent measurements. The probe liquid used was ultrapure water. Image acquisition, analysis and contact
angle determination were performed using the SCA20 v.4.3.12 and v.4.3.16 software (DataPhysics Instruments GmbH, Filderstadt, Germany).

SEM analysis

Scanning Electron Microscopy (SEM) images of the surface morphology of the membranes were obtained using a SEM DSM962 model from Zeiss. Gold was deposited on the samples by sputtering in an Ar atmosphere, using a 20 mA current, for 30 s at a deposition rate of 3 Å.s⁻¹. Images were obtained for an acceleration voltage of 5 kV.

Results and discussion

Bacterial cellulose membranes produced by the species K. rhaeticus were crosslinked with two different compounds (citric acid (CA) and epichlorohydrin (ECH)) using different percentages, to determine the crosslinking procedure that results in the best physical properties of the membranes for biomedical applications.

Water retention capacity

To estimate the water retention capacity the following expression was used, $H_{2}O\% = \frac{m_h - m_d}{m_h}$, in which $m_h$ is the hydrated mass of the membrane (BC+H₂O), and $m_d$ is the dehydrated mass of the membrane (only BC).

In Fig. 2 A and B, the maximum water retention capacity of the several studied samples, unmodified and crosslinked with different percentages of both crosslinkers ECH and CA, are presented. The error associated to the presented values was found to be smaller than 1%.
Fig. 2  Water retention capacity (in weight percentage) of samples crosslinked with ECH (A) and CA (B) as a function of the amount of crosslinking agent.

This work from hereon was focused on two type of bacterial cellulose samples: BC crosslinked with 2% ECH and BC crosslinked with 50% CA, which properties were interpreted, comparing them with the ones of unmodified BC. These two samples (BC crosslinked with 2% ECH and BC crosslinked with 50% CA) are the ones for each crosslinking method, that present the lowest reduction in the water retention capacity, when compared with the unmodified BC (prior to the first de-hydration). The sample crosslinked with 2% ECH underwent a water retention capacity reduction of 22.3% while the sample crosslinked with 50% CA underwent a water retention capacity reduction of 12.7%. Being so, the sample that showed the highest water retention capacity, proceeding from the dehydrated state, was the sample crosslinked with 50% CA.

Water uptake rate

To measure the water uptake rate, disks with a diameter of approximately 50 mm were used. After dehydration, they were initially weighted and placed in Milli-Q water and left at room temperature. At different pre-established times, the discs were removed using tweezers, carefully placed on absorbent paper, to remove the water excess at the surface and then weighted. The samples were weighted from $t = 0$ until $t = 1440$ min and the results obtained for three different samples were plotted in Fig. 3.
Fig. 3  Water uptake assays of BC membranes, normalized BC+H₂O mass (normalized by its final value).

The data presented in Fig. 3 was calculated by dividing the time dependent mass values of BC+H₂0 by their respective final mass value (fully hydration) for normalization. The water uptake rate was approximately linear during the first 120 minutes for all studied samples, approximately 0.002 g/min (per final weight of 1g of BC+H₂0) for the unmodified sample, approximately 0.003 g/min (per final weight of 1g of BC+H₂0) for the sample crosslinked with 2% ECH and approximately 0.006 g/min (per final weight of 1g of BC+H₂0) for the sample crosslinked with 50% CA.

Water loss rate

The data presented in Fig. 4 A was calculated by dividing the time dependent values of BC+H₂0 mass by its initial value for normalization. There was no measurable difference in the water loss rate between samples from unmodified BC and crosslinked BC (with AC and ECH). This is in accordance with the fact that the assay started with the BC membranes fully hydrated (Fig. 4 B), with the pores strained to their maximum dimensions, so that the access of the water molecules to the atmosphere was approximately the same for all samples. Close to the dried state (Fig. 4 C), the water loss rate was expected to be different from sample to sample and slightly higher in the crosslinked samples due to their structure, but the weight values obtained at this stage of the assays were so small (≈ 0.5 mg), that eventual differences were not observable.

Average values of the mass obtained for the samples tested at each time point were plotted in Fig. 4 A.
During the first 300 minutes, a water loss rate of approximately 0.003 g/min per initial weight of 1 g of BC+H₂O, was observed. After 300 minutes, the water loss rate was dramatically reduced, and a constant mass value (BC without water) was obtained only after 24 hours of the beginning of the assay. Similar results were obtained for unmodified BC (Kalyani and Khandelwal 2021).

FTIR-ATR characterization

FTIR spectroscopy was used to identify the functional groups of the modified and unmodified BC membranes produced. In the IR spectra obtained for the prepared membranes (Fig. 5) the characteristic peaks of cellulose were observed.

A broad absorption peak of –OH stretching vibration at 3332 cm⁻¹, due to the intermolecular hydrogen bonds (Kalyani and Khandelwal 2021), was observed for the three membranes. The peak around 2890 cm⁻¹, that corresponds to the aliphatic –CH stretching vibration characteristic of carbohydrates was also present in all the samples, being less intense in the ECH crosslinked sample. In the three spectra, the several peaks in the region between 1000-1200 cm⁻¹ are characteristic of C=O groups of the primary hydroxyl strong stretching vibration, that may be attributed to the
cellulose structure (Madivoli et al. 2016). The peak at 1641 cm\(^{-1}\) corresponds to the \(-\text{CH}_2\) bending vibration, being more intense in the CA crosslinked sample. The peaks associated to C–OH stretching and C–O–C bending vibrations that occur at 1031 cm\(^{-1}\) and 1107 cm\(^{-1}\) were also observed for all the membranes produced (Brandes et al. 2016). The spectral signatures of BC and of the modified BC with 2\% ECH form were highly overlapped (Udoetok, Wilson, and Headley 2018). The peak at 1735 cm\(^{-1}\), typical of ester carbonyl groups, was present in the BC modified by citric acid (Madivoli et al. 2016; Geravand et al. 2021a).

Mechanical properties of the dehydrated membranes

The mechanical properties of bacterial cellulose depend on the producing strain, as well as on the fermentation conditions. For instance, the degree of crystallinity of BC, strongly influences its mechanical properties (Chen et al. 2017). The BC mechanical properties have already been determined for single isolated fibers, evidencing the extraordinary specific tensile strength (normalized by specific density). The specific tensile strength of the BC fibers can achieve 598 MPa.g\(^{-1}.\)cm\(^3\), which is considerably higher than the novel lightweight steel (227 MPa.g\(^{-1}.\)cm\(^3\)) (Wang et al. 2017).

In this work, the mechanical properties were determined not for isolated fibers but for the dehydrated membranes, using uniaxial tensile testing. In Fig. 6, plots of the typical stress-strain behavior obtained for each membrane are shown. The differences in the mechanical behavior between the three studied membranes were obvious. The more pronounced differences were the higher tensile strength of the crosslinked samples and consequently their lower plastic deformation.

![Fig. 6](image)

**Fig. 6** Plot of the stress-strain curves obtained in the tensile tests for BC membranes: unmodified BC; crosslinked with 50\% CA and crosslinked with 2\% ECH.

In Table 1, the average values of the mechanical properties obtained for the different BC membranes are displayed and were extracted from the tensile tests.

| Table 1 | Mechanical properties of the bacterial cellulose membranes |
|-----------------|-----------------|-----------------|
| Young’s modulus (GPa) | Ultimate tensile strength (MPa) | Maximum strain (%) |
|-----------------|-----------------|-----------------|
|                 |                 |                 |

12
In Fig. 6, the crosslinking of the BC membranes induced a stiffer and more fragile behavior, when compared with the unmodified membrane. The unmodified BC samples attained a high value of maximum strain (39%), while for the crosslinked samples, this value decreased to 7.2% or 3.6% for the crosslinking with 2% ECH and 50% CA, respectively. This stiffer and more fragile behavior was not only evident in the strain but also in the ultimate tensile strength (UTS) and the Young’s modulus. The unmodified BC samples presented an UTS of 33.9 MPa and a Young’s modulus of 1.62 GPa, while the samples crosslinked with 2% ECH presented an UTS of 162.2 MPa and a Young’s modulus of 2.77 GPa and the sample crosslinked with 50% CA presented an UTS of 72.7 MPa and a Young’s modulus of 2.89 GPa. Similar mechanical properties have been described for the unmodified BC membranes (Chen et al. 2021; Menegasso et al. 2022; Nakagaito, Iwamoto, and Yano 2005). These obvious differences in behavior are expected in general, when comparing a non-crosslinked polymer with the same polymer after crosslinking. The crosslinking of the BC membranes hinders or prevents the movement of the BC molecules (chains’ mobility) that is necessary for the existence of plastic deformation. In fact, the unmodified BC membranes presented a high plastic deformation while the crosslinked samples displayed an almost exclusive elastic behavior, with a higher elastic modulus, almost twice as higher as the one obtained for the unmodified BC. Similar mechanical properties have been described for the unmodified BC (Huang et al. 2019).

In the dehydrated state, the unmodified BC sample was much more ductile than the crosslinked ones, which were more brittle, although for the proposed application (wound dressings) all samples presented suitable properties, being mechanically robust and resilient.

### Mechanical properties of the hydrated membranes

The viscoelastic properties of BC membranes can be observed in Fig. 7, where the storage $G’$ and loss $G’’$ moduli are represented in function of angular frequency for unmodified BC, with Citric acid crosslinking (50% CA) and with Epichlorohydrin crosslinking (2% ECH) membranes, at 25 °C and 37 °C. For all the BC membranes we found higher values for $G’$ than $G’’$. 

| Material       | UTS (MPa)  | Young’s Modulus (GPa) |
|----------------|------------|------------------------|
| Unmodified BC  | 33.9 ± 0.2 | 1.62 ± 0.01            |
| 2% ECH         | 162.2 ± 0.6| 2.77 ± 0.02            |
| 50% CA         | 72.7 ± 0.3 | 2.89 ± 0.02            |
Fig. 7  Viscoelastic properties: storage $G'$ (full symbols) and loss $G''$ (empty symbols) moduli in function of angular frequency for BC membranes: unmodified BC; with Citric acid crosslinking (50% CA) and with Epichlorohydrin crosslinking (2% ECH); represented data are an average of three measurements performed over the same membrane and error bars are within each symbol; measurements performed at temperatures 25 ºC (A) and 37 ºC (B).

Knowing that the higher the $G'$ value, the more pronounced are the elastic properties, and the higher the $G''$, the more pronounced are the viscous properties. The storage modulus, $G'$, is a measure of the energy stored and reflects the solid-like component or elastic behavior of the material. The results obtained for all the BC samples, showed that $G' > G''$, which indicates a prevalence of the elastic component, revealing a pronounced solid-like behavior with a gel-like behavior, as expected. These results are comparable with the viscoelastic properties described in the literature for unmodified BC membranes (Chen et al. 2021). The BC membranes produced with 2% ECH crosslinking showed higher viscoelastic properties than the ones produced with 50% CA crosslinking. This behavior is consistent with the higher mechanical performance observed in the 2% ECH membranes during the tensile strength characterization.

Moreover, the crosslinked membranes, 50% CA and 2% ECH, presented higher sensitivity to temperature, in the probed range, showing lower viscoelastic properties at the highest temperature.
(see Fig. 7), while BC membranes showed almost unchanged viscoelastic behaviors for $G'$ and $G''$, for both temperatures tested.

Contact angle measurements
The effectiveness of the crosslinking on the wettability of BC membranes was investigated by measuring the static water contact angle ($\theta$) (Huhtamäki et al. 2018). The wettability of a solid surface is assessed by the contact angle determined according to Young’s equation:

$$\gamma_{LV} \cos(\theta) = \gamma_{SV} + \gamma_{SL}$$

Where $\gamma_{LV}$, $\gamma_{SV}$ and $\gamma_{SL}$ are the interfacial tension between the liquid and vapor, solid and vapor, and solid and liquid, respectively (Park and Seo 2011).

The incorporation of different surface chemical functionalities may affect surface wettability, charge, roughness, and porosity (Gonçalves et al. 2015; Leal et al. 2020). The static water contact angle values for BC at 0 s, 20 s and 120 s were 76.9° ± 4°, 75.7° ± 4° and 72.1° ± 4°, respectively. BC crosslinked with 2% ECH and BC crosslinked with 50% CA, presented a decrease in the static water contact angle when compared with the unmodified BC (Fig. 8 A). The static water contact angles of BC crosslinked with 2% ECH at 0 s, 20 s and 120 s, were 65.1° ± 3°, 56.3° ± 3° and 50.5° ± 3°, respectively and of BC crosslinked with 50% CA at 0 s, 20 s and 120 s, were 44.1° ± 2°, 40.5° ± 2° and 40.6° ± 2°. As observed in Fig. 8 A, all the contact angles obtained for the 3 samples were lower than 90°, which is characteristic of hydrophilic surfaces (Rbihi et al. 2020). It is evident that membranes produced using both methods of crosslinking described in this paper, when compared to the unmodified BC, present an improvement in the wettability capacity (Fig. 8 B). The differences observed after crosslinking can be explained by modifications in surface chemistry, such as intermolecular interactions, (hydrogen and van der Waals bonds), static electricity or hydrophobicity induced by the crosslinking processes (Boonchai et al. 2021).
Fig. 8  Wettability measurements: (A) Photos acquired immediately after droplet deposition (0 s) and after 120 s using the OCA20 software for the water contact angle of the unmodified BC, 2% ECH and 50% CA membranes. (B) Plot of water contact angle for unmodified BC, 2% ECH and 50% CA membranes versus time. The contact angle values presented are an average of six independent measurements with standard deviation below 4°.

SEM analysis

Observations were performed both for the membrane surfaces and their cross-sections. Since the membranes were dehydrated during the SEM observations, the pore structure that expands during the hydration and that is responsible for the large water uptake was not visible on any of the observed sample’s cross-section, so the images are not presented. The evaluation of the surface morphologies of the membranes shown in Fig. 9, did not show observable differences induced on these surfaces by the crosslinking processes, when compared with the unmodified BC sample. The images reveal a typical nonwoven nanofibrous morphology that is liquid and gas permeable and hydrophilic, all due to the fiber composed membrane morphology, presented in Fig. 9.
**Fig. 9** SEM pictures of the membranes surface. (A) unmodified BC; (B) BC crosslinked with 50% CA and (C) BC crosslinked with 2% ECH

**Conclusions**

The hydrophilic character and the BC membrane’s high swelling ratio allow, if used in a hydrated state, the maintenance of a moist environment at the wound site, sometimes necessary to enhance recovery and healing. The hydrophilic character of the BC membranes, in particular if crosslinked, enables the reversible swelling and de-swelling of the membrane, when used in the dehydrated state, which allows its application on heavily exudating wounds.

Chemically, BC is equivalent to vegetal cellulose, being free of undesirable by-products, such as hemicellulose, pectin, or lignin. BC is not only biodegradable and non-toxic, but also presents a notable advantage in regard to vegetal cellulose, which is its high grade of intrinsic purity, that enables its use with minimal post-processing.

By crosslinking the BC membranes, the decrease in the water retention capacity after the first dehydration was reduced. The efficiency of the crosslinking was evident for the maintenance of the water retention capacity as well as for the mechanical properties both in the hydrated and dehydrated states.

The two crosslinking methods, with CA and with ECH, resulted in differences in terms of mechanical properties and water uptake capacity, although these were not very significative. The CA crosslinking showed advantages in the water uptake capacity and in the fact that CA is a harmless compound, entitling it as better suited for biomedical applications.

This works demonstrates that these BC membranes present physical properties suitable to be used as surgical and burn wound dressings if in the hydrated state, or as exuding wound dressings, diapers dressing or sanitary pads, if in the dehydrated state.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Pedro Marques de Almeida and Rita Gonçalves Sobral contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ana Patrícia Almeida; João Saraiva; Gonçalo Cavaco; Raquel Portela and Catarina Leal. The first draft of the manuscript was written by Pedro Marques de Almeida and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.