Biomacromolecules

Subscriber access provided by UNIV OF BARCELONA

Article

Bacterial Cellulose-Chitosan paper with antimicrobial and antioxidant activity

L. Verónica Cabañas Romero, Cristina Valls, Susana Valeria Valenzuela, M. Blanca Roncero, Pilar Diaz, Francisco I. Javier Pastor, and Josefina Martinez

Biomacromolecules, **Just Accepted Manuscript** • DOI: 10.1021/acs.biomac.0c00127 • Publication Date (Web): 12 Mar 2020

Downloaded from pubs.acs.org on March 19, 2020

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Published by American Chemical Society. Copyright © American Chemical Society. However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

Bacterial Cellulose-Chitosan paper with antimicrobial and antioxidant activity

*L. Verónica Cabañas-Romero[1], Cristina Valls[2], Susana V. Valenzuela[1,3], M. Blanca Roncero[2], F.I. Javier Pastor[1,3], Pilar Diaz[1,3] and Josefina Martínez[1,3]**

[1] Department of Genetics, Microbiology and Statistics, Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028, Barcelona, Spain

[2] CELBIOTECH_Paper Engineering Research Group, EGE Department, Universitat Politècnica de Catalunya, Barcelona Tech, 08222, Terrassa, Spain

[3] Institute of Nanoscience and Nanotechnology (IN2UB), Diagonal 645, 08028, Barcelona, Spain

*Corresponding author: Josefina Martínez. Department of Genetics, Microbiology and Statistics, Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain. e-mail: jmartinez@ub.edu. Phone: +34 934034625. FAX: +34 934034629

Keywords: Bacterial cellulose paper; chitosan; nanocomposites; antimicrobial; antibiofilm

ABSTRACT

The production of paper based bacterial cellulose-chitosan (BC-Ch) nanocomposites was accomplished following two different approaches. In the first, BC paper sheets were produced and then immersed in an aqueous solution of chitosan (BC-ChI); in the second, BC pulp was impregnated with chitosan prior to the production of the paper sheets (BC-ChM). BC-Ch nanocomposites were investigated in terms of physical characteristics, antimicrobial and antioxidant properties, and ability to inhibit the formation of biofilms on their surface. The two types of BC-Ch nanocomposites maintained the hydrophobic character, the air barrier properties, and the high crystallinity of the BC paper. However, BC-ChI showed a surface with a denser fiber network and with smaller pore than BC-ChM. Only 5% of the chitosan leached from the BC-Ch nanocomposites after 96 h of incubation in an aqueous medium, indicating that it was well retained by the BC paper matrix. BC-Ch nanocomposites displayed antimicrobial activity, inhibiting growth and having killing effect against the bacteria *S.aureus* and *P.aeruginosa,* and the yeast *C.albicans*. Moreover, BC-Ch papers showed activity against the formation of biofilm on

their surface. The incorporation of chitosan increased the antioxidant activity of the BC paper. Paper based BC-Ch nanocomposites combined the physical properties of BC paper and the antimicrobial, antibiofilm and antioxidant activity of chitosan.

INTRODUCTION

Bacterial cellulose (BC) is a polysaccharide, synthesized and extruded outside the cell by some microorganisms, especially from the genera *Komagataeibacter*. The biopolymer is composed of units of glucose lineally linked by β(1→4)–glycosidic bonds. Although identical to cellulose of plant origin in terms of molecular formula, BC presents unique properties that make it superior for many applications. Unlike vegetable cellulose, which is always bound to hemicellulose and lignin requiring subsequent refining treatments, BC is synthesized chemically pure. BC displays a high degree of polymerization and crystallinity, great mechanical strength, and a high water-holding capacity.[1] BC is also biodegradable and biocompatible.[2] Microorganisms produce cellulose as a three-dimensional open porous network of nanofibers, providing a large surface area. Moreover, cellulose contains available hydroxyl groups in its surface that facilitate the possibility of molecular adsorption by the formation of hydrogen bonds and electrostatic interactions. These structural and mechanical features are important for the practical application of BC as the supporting matrix for the preparation of new composite materials.[3,4] Because of these properties, in recent years there has been an increased

interest in commercial applications of bacterial cellulose. Important examples include supports for proteins, cell cultures and microorganisms; products for temporary skin and tissue replacement; material for headphone and loudspeaker membranes, food packing, and edible films. [1,5,6]

Chitosan is an amino-polysaccharide obtained by alkaline deacetylation of chitin, which is extracted from marine natural sources such as crustacean shells. Chemically, chitosan is a copolymer composed of β (1,4)-glucosamine and N-acetyl-D-glucosamine units. It is biodegradable, nontoxic and possesses reactive amino groups. Moreover, chitosan displays intrinsic antimicrobial activity, which depends on the molecular weight and the degree of deacetylation of the polymer as well as on the type of microorganism.[7] Also, chitosan is considered a secondary antioxidant because it can chelate the metal ions involved in the catalysis of an oxidant reaction due to the presence of active hydroxyl and amino groups in the polymer chains.[8] Owning to its properties, chitosan is considered a versatile material that participates in multiple applications, which include the formation of biodegradable films, blends, coatings, composites and nanocomposites; as a flocculating agent in wastewater treatment; in the generation of chitosan-based membranes for water

purification; as an additive for food packages or food preservation; and in wound dressing.[4,9–14]

The blending of polymers to produce composites materials with new properties has received extensive attention as a strategy for supporting new applications.[4,15–17] The combination of chitosan with BC has been successfully described for biomedical and packaging applications. [4,9,10] In those works, the matrix of BC was in form of native never-dried membrane or in form of film. Recently, the production of paper from BC pulp has been reported.[18] BC paper sheets combine the attributes of BC nanofibers with the stiffness and physical properties of paper, showing remarkable mechanical characteristics and barrier properties to water and oil. [18] Some studies have shown how chitosan-coated vegetable paper has impaired paper properties, such as its resistance to water or steam transfer. [11,19] However, to our knowledge, no work has yet been reported regarding the combination of BC paper and chitosan.

The aim of this work was to compare two different procedures to blend BC with chitosan in order to obtain a novel nanocomposite based on BC paper and investigate its physical characteristics and its antimicrobial, antioxidant, and antibiofilm properties. The study is

framed in the research area of bioactive papers with potential applicability in the design of biomedical devices and in the field of packaging of food and high value goods.

EXPERIMENTAL

Microbial strains

The cellulose producing strain was *Komagataeibacter xylinus* CECT 7351. Antimicrobial activity was tested against *Staphylococcus aureus* CECT 234, *Pseudomonas aeruginosa* PAO1 CR32 and *Candida albicans* CECT 1001. Strains were obtained from the Spanish Type Culture Collection (CECT).

Production of Bacterial Cellulose

To produce bacterial cellulose, *Komagataeibacter xylinus* was grown on the Hestrin and Schramm (HS) medium, containing 20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 1.15 g/L citric acid, 6.8 g/L Na_2HPO_4, pH 6. [20] Suspensions of *K. xylinus* were used to inoculate 10 cm–Petri dishes containing 40 mL of HS medium that were statically incubated at 25–28 °C for 7 days. After incubation, bacterial cellulose pellicles generated in the air/liquid interface of the culture media were harvested, rinsed with water, and

incubated in NaOH (1%) at 70 ºC overnight to remove the bacteria. Finally, the BC pellicles were thoroughly washed in deionized water until the pH reached neutrality. To obtain the BC pulp, pellicles were mechanically cut into small pieces (1 cm^2 approximately) and disrupted with a homogenizer (CAT Unidrive X1000 Homogenizer, Germany) at 20000 rpm for 10 m.

Preparation of Bacterial Cellulose/Chitosan nanocomposites

To obtain the composites of BC paper containing chitosan, two approaches were followed: the formation of BC paper followed by the impregnation by immersion of the paper sheets with chitosan; and the impregnation in mass of the BC pulp with chitosan followed by the formation of paper sheets.

Impregnations were carried out with the water soluble derivative Methyl Glycol Chitosan (FUJIFILM Wako Pure Chemical Corporation, MW: $(375,2)_n$) with a ratio of 0.3 mg of chitosan / mg of dry bacteria cellulose.

The impregnation of the previously produced BC paper was done by immersing pieces of 1 cm^2 of paper in a 3 mg/mL of an aqueous solution at pH 6 and incubating overnight

at room temperature. After incubation, the paper sheets were washed with deionized water to remove poorly attached chitosan. To perform the impregnation in mass, BC pulp and chitosan at 3mg/mL (final concentration) were mechanically mixed in a blender (proBlend6 3D, Philips-Spain) until homogenization (500 rpm, 5 m). The mixture was incubated overnight at room temperature and, after incubation, washed with deionized water. The pulp of BC impregnated with chitosan was then used to produce paper sheets.

Paper sheets were produced following the ISO–5269:2004 standard method using a Rapid–Köthen laboratory former (Frank–PTI, Germany). The sheets were sterilized by autoclave at 121 ºC for 20 minutes, dried at 45 ºC and storage at room temperature until further use. Three types of papers were obtained: Bacterial Cellulose (BC), Bacterial Cellulose Chitosan nanocomposite by immersion (BC-ChI) and Bacterial Cellulose Chitosan nanocomposite by impregnation in mass (BC-ChM).

Physical and barrier properties

Paper sheets were conditioned at 23 ºC and 50% of relative humidity for at least 24 h before physical testing, as indicated in ISO 187 (1990). Basic physical and barrier

properties were measured according with standards indicated in parenthesis as follow: grammage (ISO 536, 2012), thickness (ISO 534:2005), density (ISO 534:2005), Bendtsen roughness (ISO 8791-2:2013), Bendtsen air permeance (ISO 5636-5:2003), and water drop test (WDT) (Tappi T835 om-08). At least five measurements per sample were made and averaged. Statistical analysis of the results was performed. To determinate the Water Absorption Capacity (WAC), BC and BC-Chitosan nanocomposites were immersed in deionized water for 24 hours at room temperature. Then, the weight of the swollen pieces was measured.

Water absorption capacity was calculated as follows:

$$Water\ Absorption\ Capacity\ = \frac{\mathrm{Wf} - \mathrm{Wi}}{\mathrm{Wi}}$$

where Wi is the initial weight of dried sample and Wf is the weight of sample in swollen state.

Scanning Electron Microscopy (SEM)

Surface morphology of BC-Chitosan nanocomposites was analyzed by SEM (JEOL JSM 7100 F, Tokyo, Japan) using a LED filter. Samples were graphite coated using a Vacuum Evaporator EMITECH K950X, France.

X-Ray diffraction (XRD)

X-ray diffraction patters of the samples were obtained with a PANalytical X'Pert PRO MPD Alpha1 powder diffractometer (Malvern Panalytical B.V., Nederlands) in Bragg-Brentano $\theta/2\theta$ geometry of 240 millimeters of radius. The radiation was Cu $K\alpha_1$ (λ = 1.5406 Å) at 45 kV – 40 mA, focalizing Ge (111) primary monochromator, with sample spinning at 2 revolutions per second, fixed divergence slit of 0.25° was used. The measurement range (2θ) was from 2° to 50° with step size of 0.033° and measuring time of 100 s per step. The samples were placed, over zero background Silicon single crystal sample holders, to stay flat in the measure of the possible, using when necessary Silicon paste and/or small scotch pieces. The crystallinity index (CrI) of the samples was calculated from the XRD spectra using the following equation, based in the Peak Height method. [21]

$$CrI(\%) = \frac{\mathrm{I}_{002} - \mathrm{I}_{\mathrm{AM}}}{\mathrm{I}_{002}} x100$$

where I_{002} is the maximum intensity of the lattice diffraction and I_{AM} is the minimum in intensity at 2θ between 18º and 19º, which corresponds to the amorphous part of cellulose.

Fourier transform infrared spectroscopy (FTIR)

The infrared spectra of samples were obtained using FTIR spectroscopy (Fourier Transform IR spectrometer Perkin Elmer Frontier, Waltham, Massachusetts, U.S.). Spectra were obtained at wave numbers ranging 4000 to 400 cm^{-1} recorded at 4 cm^{-1} resolution.

Antimicrobial activity

The antimicrobial activity of BC-Ch composites was tested against the Gram-positive bacteria *Staphylococcus aureus*, the Gram-negative bacteria *Pseudomonas aeruginosa* and the yeast *Candida albicans*. To obtain the inoculum for the antimicrobial tests, the

strains were grown overnight in Luria Bertani (LB) broth at 37 ºC in shaking conditions. Then, these cultures were centrifuged for 5 minutes at 18407 RCF and the pellet resuspended in 0,3 mM KH_2PO_4 to remove the culture medium. BC-Chitosan nanocomposites were cut in squares of 1 cm^2 and sterilized prior to the assay. Two antimicrobial tests were performed, the *Drop over paper test* and the *Dynamic contact condition test*.

Drop over paper test

Three μl of the corresponding microbial suspension (about 10^5 microorganisms per mL) were inoculated over the 1 cm^2 BC-Chitosan composite placed on the surface of Tryptic Soy Agar (TSA) medium plates. The growth over a sample of BC paper was used as positive control. After overnight incubation at 37 ºC, the samples of composites and BC-paper were submerged in 1mL of 0.3 mM KH_2PO_4 and the microorganisms were detached by intense shaking. The metabolic activity of the resuspension was measured by the resazurin assay. In a medium with viable cells resazurin is reduced to resorufin and this reduction can be quantified by a fluorometer. [22]

For the assay, 30 µL of resazurin (7–Hydroxy–3H–phenoxazin–3–one–10–oxide) were added to 100 µL of each microbial resuspension in a 96–well plate. The plate was incubated at 37°C in dark conditions until the solution turned pink (approximately 10 minutes). Fluorescence was measured (λex 570nm, λem 600nm) with a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian Iberica, Spain). The difference between the metabolic activity of the microorganisms grown on BC-Chitosan composites and on BC paper was used to calculate the percentage of growth inhibition.

Dynamic contact condition test

This procedure was adapted from ASTM E2149–01:2001 (*Standard test Method for determining the antimicrobial activity agents under dynamic contact conditions*). Nine 1 cm^2 pieces of the BC-Chitosan composites were immersed in 5 mL of a suspension of a known concentration of microorganisms (approximately 10^7 UFC/ml) and incubated at room temperature while stirred. In each case, a control was run with the BC paper under the same conditions. The viable cells on the suspension were determined at different times (0, 1, 4 and 24 h). The percentage of reduction was calculated by the following equation:

$$Reduction(\%) = \frac{\text{viable CFU at t0} - \text{viable CFU at tx}}{\text{viable CFU at t0}} x\ 100$$

where t_0 is the time 0 h and tx is the time at which the percentage of reduction is calculated.

Antibiofilm activity

Antibiofilm properties of BC-Ch nanocomposites were assayed with *Pseudomonas aeruginosa*, a well-known biofilm producer. 1mL aliquots of a 1:100 dilution of an overnight culture of *P. aeruginosa* (about 10^6 bacteria) were pipetted into a 24-well plate where samples of BC-Ch and BC paper were previously placed. After overnight incubation at 37°C, the medium was removed, and the samples were rinsed three times with Phospate-buffered saline (PBS). Resazurin assay and SEM analysis were carried out on BC-Ch nanocomposites and BC paper.

Antioxidant activity

The antioxidant activity was assessed by a procedure that allows to determine the antioxidant capacity of insoluble components by quantification of the inhibition of 2,20-Azino-bis(3-ethylbenzothiazoline-6sulphonic acid radical ($ABTS^{\cdot+}$). [23–25] Firstly, 7 mM ABTS was oxidized by 2.45 mM of Potassium persulfate obtaining the $ABTS^{\cdot+}$ radical. Then, 1 cm^2 samples of BC-Chitosan nanocomposites and BC paper were placed in eppendorf tubes and 1mL of $ABTS^{\cdot+}$ was added. A tube without sample was used as the blank. The next step was vortex the tubes, centrifuged at 3381 x*g* and incubated them in dark conditions for 30 minutes. Finally, 900 µL of the liquid was pipetted in a cuvette and the final absorbance at 730 nm was measured in a T92+ UV Spectrophotometer (PG Instruments, UK). The antioxidant activity of the samples was expressed by the following equation

$$ABTS^{\cdot+}\ inhib(\%) = \frac{\mathrm{Ai} - \mathrm{Af}}{\mathrm{Ai}}\, x\, 100$$

Where Ai is the absorbance value of the blank and Af the absorbance value of the sample.

Determination of the concentration of chitosan

The method to measure chitosan was adapted from Badawy. [26] Briefly, samples were incubated with 0.5M $NaNO_2$ at 80 ºC for 30 m to complete the depolymerization-deamination reaction. Then, after raising the pH to 8, a 0.04 M thiobarbituric acid solution was added and the mix was incubated a second time at 80 ºC for 10 m. Finally, the absorbance was measured at 555 nm and the concentration of chitosan calculated based on a calibration curve.

RESULTS AND DISCUSSION

Production of Bacterial Cellulose-Chitosan nanocomposites

Figure 1 schematized the two approaches followed to produce BC-Chitosan nanocomposites: impregnation by immersion (BC-ChI) and impregnation in mass (BC-ChM). The produced paper sheets were dried and storage at room temperature, maintaining their properties for, at least, 12 months (results not shown). The amount of chitosan in the composites was estimated by subtracting the concentration of chitosan in the solution before and after the impregnation procedure. The amount of chitosan in the

wash liquids was considered in the calculations. BC-ChI and BC-ChM contained 625 ± 1.3 μg and 668 ±1.2 μg of chitosan per cm^2 of paper, respectively (A and B, Figure 1). These values correspond to 104 μg and 106 μg of chitosan absorbed per g of cellulosic matrix (dry weight), for BC-ChI and BC-ChM respectively, indicating that the ratio BC:Ch on the composites was about 10:1, and suggesting that chitosan was well incorporated into the nanofibers of cellulose network structure. In the mass impregnation process, the BC fibers were suspended in water, which would facilitate the access of the chitosan and favor its interaction with the cellulose molecules. In contrast, in the impregnation by immersion procedure, the molecules of cellulose could be less accessible to chitosan since BC fibers were compacted and dried during the process of papermaking. However, not substantial differences were found in the amount of chitosan loaded throughout the two approaches, suggesting that chitosan in BC-ChI composites not only coated the paper surface but penetrated the porous matrix of fibers of BC.

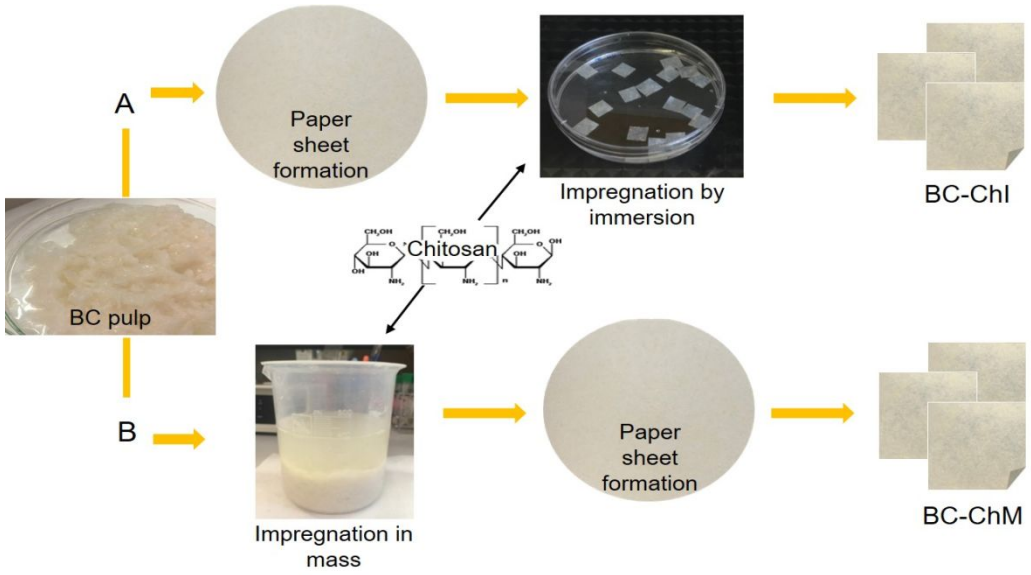

Figure 1. Scheme of the production of BC-Ch nanocomposites with the methods: A) impregnation by immersion and, B) impregnation in mass. Black arrows indicate when chitosan was added.

To assess whether the two types of composites had different retention capacity, the migration of chitosan from the cellulosic matrices was evaluated. BC-ChI and BC-ChM nanocomposites were immersed in distilled water and, at different times up to 96 h, aliquots samples were withdrawn and assayed for the presence of chitosan. No disintegration or erosion of the BC-Ch composites was observed during the migration experiments. Results indicated that after 96 h in water both types of nanocomposites retained more than 90 % of chitosan (Figure 2). Most of the release of chitosan took place during the first 2 h on contact with the aqueous solution and could be attributed to the

molecules that were loosely attached to the BC nanofibers. Less chitosan migrated from BC-ChM (3 %) than from BC-ChI (7 %), suggesting that in the course of the impregnation in mass, chitosan is trapped more effectively in the dense network of nanofibrils of BC than during the impregnation by immersion of the pre-formed paper sheet. Regardless, the results indicated that both BC matrices strongly retained the molecules of chitosan. Under these experimental conditions, the interactions between chitosan and BC can be explained by the overall opposite charge between cellulose (negative) and chitosan (positive) [27] as well as by the three-dimensional network of BC nanofibers that would entrap the molecules of chitosan. It is worth noting that the stabilization of chitosan into the BC matrices was achieved without the addition of chemical cross-linkers which could compromise the applicability of the composites by increasing their brittleness[28,29] or by weakening their antimicrobial ability[28]

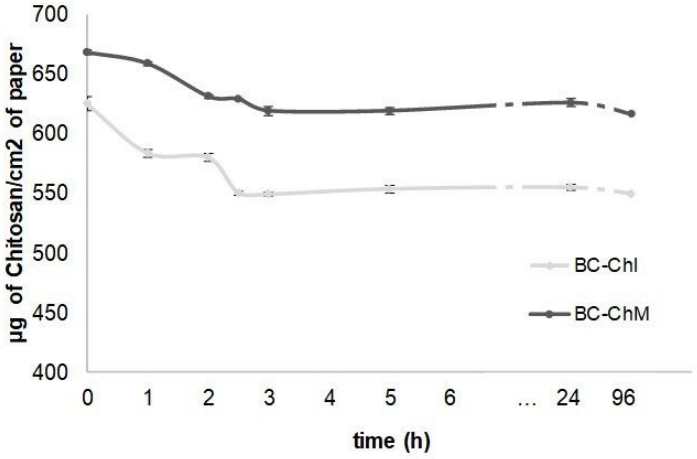

Figure 2. Retention of chitosan on the BC-Ch nanocomposites immersed in water. Results are expressed in µg of chitosan that remained per cm^2 of paper. The dark gray line corresponds to the BC-Ch nanocomposite by impregnation in mass and the light gray line to the BC-Ch nanocomposite by immersion. Values were expressed as means ± standard deviations and were analysed statistically by analysis of variance (ANOVA), $p \leq 0.05$ was considered statistically significant.

Bacterial cellulose membranes embedded with antimicrobial agents, such as chitosan, have been proposed for wound healing applications on the base of their progressive migration from the BC matrix towards the skin. [30,31] BC paper is a different kind of matrix that allowed the retention of chitosan and could expand the applicability of the BC-Ch composites. For example, chitosan has been proposed as a carrier for protein and enzyme immobilization owning to the availability of numerous amino and hydroxyl

groups.[32] The strong interaction between chitosan and the BC matrix would ensure permanent immobilization of the carrier and consequently would prevent the leaking of the bioactive molecules. In the packaging industry, one of the most appreciated properties is the retention of additives, preventing the transfer of active compounds from packaging materials to packaged goods. Furthermore, since chitosan is biocompatible, nontoxic and biodegradable, BC-Ch nanocomposites could be interesting for applications having to be in contact with food or pharmaceutical products. [33–35]

Characterization of the BC-Chitosan nanocomposites

FTIR analysis

FTIR spectra obtained from BC and BC-Ch nanocomposites are shown in Figure 3. The molecular structures of bacterial cellulose and chitosan are very similar, therefore, for chitosan, BC paper and BC-Ch nanocomposites characteristics peaks at 2893 cm^{-1} were attributed to aliphatic C-H stretching vibration.[10] The absorption band of chitosan at 1554 cm^{-1} was assigned to N-H bending of amide II. The strong band between 3500 and 3200

cm-1 corresponds to N-H and O-H stretching.[28,36] The band at 1645 cm^{-1} is due to amide I. The spectra of the BC-Ch nanocomposites are dominated by the cellulose component.

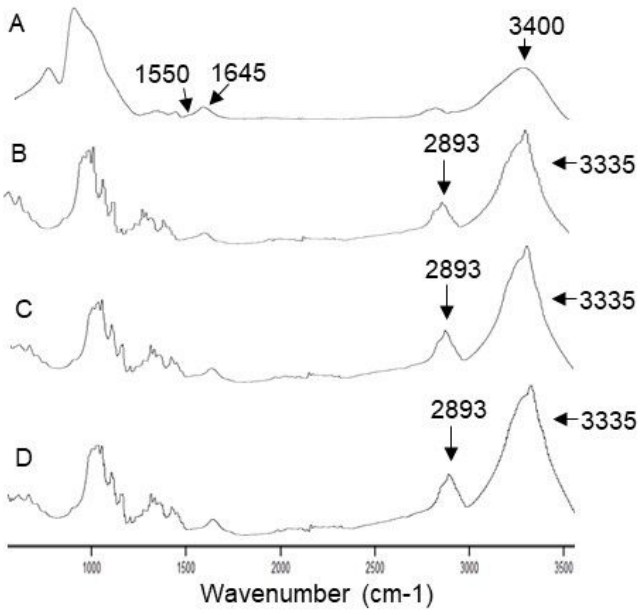

Figure 3. FTIR spectra of (A) chitosan, (B) BC, (C) BC-ChI and (D) BC-ChM

Structural analysis by SEM

SEM images of BC paper and BC nanocomposites are shown in Figure 4. Figure 4A shows the typical fibril network of BC with spaces randomly distributed through the matrix. The highly interweaved nanofiber network provides a large surface area and the porous

structure of the BC facilitates the bounding and entrapment of molecules. The images of BC-Ch nanocomposites (Figure 4B and 4C) reveled a homogeneous surface without aggregations, suggesting a correct dispersion of chitosan. Images 4B and 4C exhibit a layer coating the nanofibers of BC that could be attributed to the presence of chitosan molecules interacting with the BC fibers. Moreover, the addition of chitosan by the immersion method (BC-ChI; Figure 4B) rendered matrices with smaller pore, in agreement to what has been previously described for bacterial cellulose membranes.[10,36] The effect of chitosan on the surface of the BC matrix was more evident in the case of the nanocomposites obtained by the immersion method, which suggests that when impregnation is performed after paper formation chitosan molecules bind to a greater extent to the fibers of the surface of the paper.

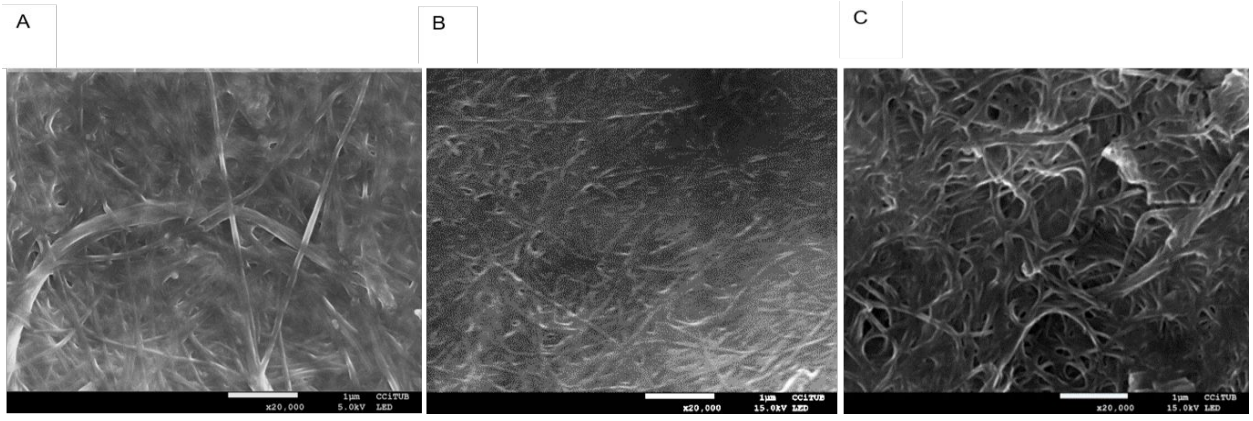

Figure 4. SEM images of A) BC, B) BC-Ch nanocomposite by immersion, C) BC-Ch nanocomposite by impregnation in mass. Magnification: 20000x

Physical and barrier properties

Table 1 summarizes the effect of the incorporation of chitosan into the network structure of the BC on certain physical and barrier properties of the resulting nanocomposite. No significant differences were observed between the results obtained for the BC-ChI and the BC-ChM composites (Table 1), suggesting that the two approaches to obtain the BC-Ch nanocomposites rendered similar matrices in terms of the characteristics assayed. However, some changes were observed on the BC paper after the binding of chitosan. As expected, the grammage, defined as the weight in grams of one square meter of paper, and the density of the BC-Ch composites were higher than those of BC paper, indicating a more closed structure. Chitosan also provided a slight increase in the smoothness of the paper surface, more obvious in the case of BC-ChI probably due to the differences on the methodology to produce the nanocomposites. In the case of BC-ChI, the chitosan was coated on the paper surface, being placed in the surface pores of the composite. Paper-like supports made of bacterial cellulose are characterized by excellent barrier properties to air, water and oil [18,37] which is important for applications that need impermeability, as for packaging material.[11] Results showed that the blend of chitosan and BC increased the impermeability to the air, indicated by the lower value in air permeance of the BC-Ch nanocomposites.

Table 1. Physical and barrier properties of BC paper and BC-Chitosan nanocomposites

Property	BC	BC-ChI	BC-ChM
Grammage (g/m^2)	50.67 (±0.6)	60.10 (±1.5)	63.08 (±3.9)
Thickness (μm)	171 (±16)	172 (±14)	171 (±10)
Density (g/cm^3)	0.295 (±0.004)	0.359(±0.031)	0.369(±0.042)
Bendtsen Roughness (mL/min)	4.3 (±0.6)	3.1 (±0.8)	3.6 (±0.8)
Bendtsen Air Permeance ($\mu m/Pa \cdot s^{-1}$)	1.24 (±0.19)	0.92 (±0.14)	1.19 (±0.13)
Water dropt test (WDT) (min)	34 (±3)	34 (±4)	32 (±4)

Hydrophylicity is a characteristic inherent to most matrices of polysaccharides. Bordenave et al., [11] reported that the association of paper produced from vegetal cellulose with chitosan was water sensitive and they improved the hydrophobic character of chitosan-coated vegetal papers after chemical modification of chitosan and bounding with fatty acids. However, BC paper features water and vapor barriers properties without the need of the addition of hydrophobic components or the chemical modification of the molecule.[18,37] The results obtained in this work indicated that the presence of chitosan

did not increase the wettability of the resulting BC-Ch nanocomposites estimated by WDT (Table 1), suggesting that the BC paper maintained its hydrophobic character.

Water absorption capacity (WAC) of BC-Ch nanocomposites was evaluated and compared with that of BC paper. The WAC of BC paper was about 6.5 times of its dry weight, while the WAC of BC-ChI and BC-ChM nanocomposites was 38 % and 25 % less, respectively, indicating that the addition of chitosan decreased the capacity of water absorption (Figure 5). The variation in the WAC between BC and BC-Ch papers could be explained by the electrostatic interaction between the amino groups of chitosan and hydroxyl groups of cellulose. Consequently, there are less hydroxyl groups available to interact with water molecules affecting the absorption behavior of the nanocomposites.[38]

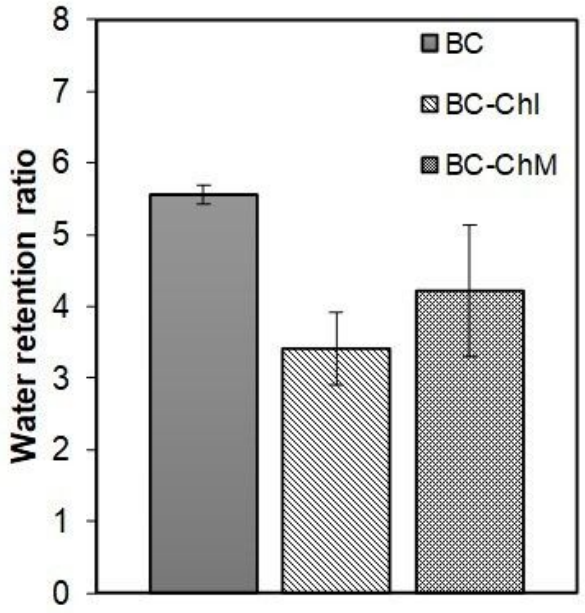

Figure 5 Water absorption ratio of BC paper and BC-Ch nanocomposites. Values were expressed as means ± standard deviations and were analysed statistically by analysis of variance (ANOVA), $p \leq 0.05$ was considered statistically significant.

X-Ray diffraction (XRD)

In order to compare the microstructural changes in the BC produced after the impregnation with chitosan, X-ray diffraction was used. The XRD diffraction patterns of the papers like supports are shown in Figure 6. The determination of crystallinity by the XRD using the peak height method described by Segal et al. [21] was chosen because it is one of the most widely used method to obtain semiquantitative amounts of amorphous and crystalline cellulosic components crystallinity index reference. The value of crystallinity obtained were: 94,5% for BC; 94,1% for BC-ChI; 93,9% for BC-ChM. When

chitosan is added, the crystallinity of the bacterial cellulose does not significantly change. Results on crystallinity suggested that the blending of BC and chitosan rendered composites of homogeneous structure, probably due to the high compatibility of both materials.[39]

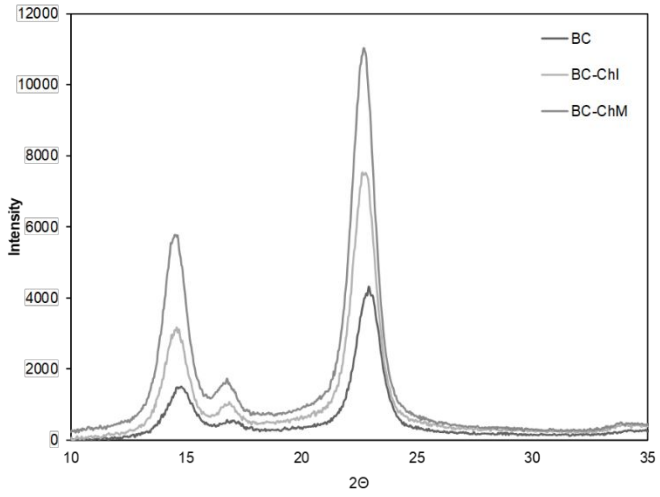

Figure 6 X-ray diffraction (XRD) patterns of BC, BC-ChI, BC-ChM nanocomposites.

Antimicrobial activity of the BC-Chitosan nanocomposites

The capability of the BC-Chitosan composites to inhibit the microbial growth by direct contact of the microorganism with their surface was assayed by the *drop over paper test*. Results showed that the three strains assayed were able to grow over BC paper, however,

when an equivalent inoculum was incubated over the BC–Ch nanocomposites, the activity declined (Table 2). The results indicated that the chitosan bound to the cellulose fibers forming the paper had a negative effect on the growth of the three strains assayed.

Table 2. Inhibition of microbial growth over BC-Ch nanocomposites surfaces

Strain	Inhibitory rate (%)*	
	BC-ChI	**BC-ChM**
Staphylococcus aureus	63	83
Pseudomonas aeruginosa	55	75
Candida albicans	18	38

* Results expressed as the percentage of reduction of the microbial activity with respect the activity over BC paper

The yeast *C. albicans* showed less sensitivity to chitosan than the bacteria strains. Moreover, chitosan was more active against the Gram-positive (*S. aureus*) that against the Gram-negative (*P. aeruginosa*) bacteria. The differences in the effectiveness of chitosan can be explained by its varied mechanisms of action as well as by the differences in the structure of the cell envelopes of the three microorganisms.[7] However, the exact mechanism of chitosan antimicrobial action it is not totally understood. Factors as MW and degree of acetylation of chitosan, and pH of the medium may influence its

antimicrobial action. Results suggested that the external lipidic membrane in Gram-negative could confer some protection hindering the access of chitosan to the cell. Nevertheless, in the literature there is not a general agreement regarding the degree of susceptibility of Gram-positive, Gram-negative and fungi to the chitosan.[40] While we could expect more chitosan to accumulate on the surface of the compounds obtained by the immersion procedure than in those obtained by mass impregnation, the results indicated that BC-ChM were more effective in preventing microbial growth on their surface. The SEM images of the BC-Ch nanocomposites (Figure 4) revealed that BC-ChM presented a less compact surface that would allow better contact of the bacteria with the nanofibers facilitating the action of chitosan.

One aspect to consider was that results stated above indicated that BC-Ch nanocomposites had less water absorption capacity than BC paper (Figure 5). Moreover, SEM analysis showed that chitosan covered the nanofibers and could be filling the matrix pores. These circumstances could be limiting the diffusion of water and nutrients dissolved in water during the *drop over paper test* analysis and artificially increase results of antimicrobial activity. To preclude this possibility, the biocidal ability of the BC-Chitosan

composites was assayed under dynamic liquid condition. Suspensions of the microorganisms on 0,3 mM KH_2PO_4 solution were incubated in contact with the BC-Ch composites and BC paper, at room temperature and lightly agitation. Viable cell counts were determined at different times, and the percentage of cell viability reduction was calculated (Table 3). Suspensions of the microorganisms in contact with samples of BC paper did not experiment a decrease of viability over 24 h incubation time (results not shown). However, the microorganisms in contact with BC-Ch nanocomposites showed a remarkable diminution of viability after one hour of incubation and, after 24 h hours, the reduction of viability was 100% (Table 3). These results indicated that BC-Ch composites, not only inhibited the microbial growth, but exhibited, also, strong biocidal activity against the tested strains. Moreover, the antimicrobial effectiveness of the two types of nanocomposites was similar, suggesting that this property did not depend on the procedure followed for the production of the nanocomposites.

Table 3. Viable cell counts (CFU/mL) and cell viability reduction (%) of microorganisms in dynamic contact with BC-ChI and BC-ChM composites.

Type of nanocomposite	Time (h)	Strains					
		S. aureus		*P.aeruginosa*		*C.albicans*	
		CFU/mL	% of reduction	CFU/mL	% of reduction	CFU/mL	% of reduction
BC-ChI	t_0	$9.70 \cdot 10^7$	0	$9.25 \cdot 10^7$	0	$4.05 \cdot 10^7$	0
	t_1	$4.20 \cdot 10^7$	57	$5.90 \cdot 10^7$	36	$2.06 \cdot 10^7$	49
	t_4	$8.35 \cdot 10^6$	91	$3.15 \cdot 10^6$	97	$9.10 \cdot 10^6$	78
	t_{24}	0	100	0	100	0	100
BC-ChM	t_0	$7.95 \cdot 10^7$	0	$5.00 \cdot 10^7$	0	$2.75 \cdot 10^6$	0
	t_1	$2.27 \cdot 10^7$	71	$2.95 \cdot 10^7$	41	$9.75 \cdot 10^5$	65
	t_4	$4.40 \cdot 10^6$	94	$2.00 \cdot 10^6$	96	$4.30 \cdot 10^5$	84
	t_{24}	0	100	0	100	0	100

Paper of BC is a matrix with great mechanical resistance and does not disintegrate in water, which allows its reusability. An interesting characteristic of the composites produced was to know if after being in an aqueous environment for a period and then dried retained their antimicrobial activity for further applications. To do this, samples of

BC-Ch and BC paper were incubated in water at room temperature for 24 hours. Then, the papers were dried and the *drop over paper test* was performed with a suspension of *S.aureus*. Both nanocomposites still showed antimicrobial activity after being in contact with water for 24 h and then dried (Table 4). BC-ChM and BC-ChI nanocomposites maintained 63% and 51% of its antimicrobial capacity compared to its initial antimicrobial activity (t_0, Table 4), respectively. BC paper did not show reduction of activity (results not shown). Differences in the results of the two types of composites were consistent with the fact that BC-ChM showed less migration of chitosan from the BC matrix than BC-ChI.

Table 4. Reduction of activity (%) of *S.aureus* in contact with both types of BC-Ch nanocomposites before (t_0) and after being in immersed in water for 24 h (t_{24})

Time (h)	BC-ChI	BC-ChM
t_0	63	83
t_{24}	32	52

Antibiofilm activity of the BC-Ch nanocomposites

Biofilms are microbial communities embedded in a self-produced matrix of extracellular polymers strongly attached to the surface of organic and inorganic materials. Biofilms increase the resistance of microorganisms to antimicrobial drugs and the immune system activity, and are difficult to eradicate with cleaning agents.[41,42] The prevention of biofilm formation is an important issue in

the development of new materials for biomedical, pharmaceutical and packaging application. Hence, the activity of the BC-Ch nanocomposites against the generation of biofilms on their surface was evaluated. To do that, samples of the BC-Ch nanocomposites and BC paper were immersed in a suspension of *P.aeruginosa* and incubated at 37 ºC, optimal growth temperature for the strain. After 24 h, samples were rinsed, dried, and analyzed by SEM. SEM images of the surface of the BC-Ch composites and BC paper are shown in Figure 7. It can be observed that BC paper, which did not contained chitosan, is covered by a material compatible with the existence of biofilm (Figure 7 A). However, in the images of BC-Ch nanocomposites, the typical network of BC nanofibers can be distinguished, suggesting that chitosan inhibited the generation of biofilm on the surface of the nanocomposites. Interestingly, this effect was more evident for BC-ChI than for BC-ChM, probably related with the higher smoothness of the surface of BC-ChI, which could hinder the adhesion of the bacteria.

The microbial activity of the samples was measure by the resazurin assay as an indication of the growth of *Pseudomonas* on their surfaces, and to confirm that material observed in SEM images could have a biological origin.

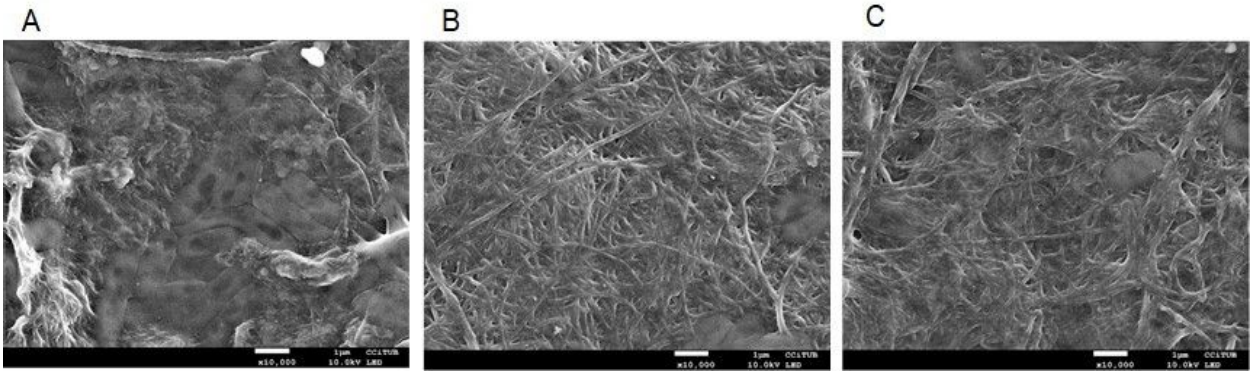

Figure 7. SEM images of biofilm on the surface of: A) BC paper, B) BC-ChI, C) BC-ChM. Magnification: 10000x

Results indicated the presence of microbial activity on the surface of the three types of analyzed samples: BC-ChI and BC-ChM composites and BC (Figure 8). However, BC-Ch nanocomposites displayed less activity than BC paper, in concordance with the presence of biofilm observed by SEM images. The reduction of activity was of 68% for the BC-ChI nanocomposite and 81% for the BC-ChM nanocomposite. These results were in agreement with the inhibition of *S.aureus* obtained by the *drop over paper test* and suggested that BC-ChM has enhance antimicrobial activity, but it is less efficient controlling biofilm formation than BC-ChI. The effectiveness of chitosan against biofilms has been documented. [43,44] The results of SEM analysis and microbial activity obtained in this work indicated that chitosan incorporated into the BC-Ch nanocomposites had a strong negative effect on biofilm formation on their surface.

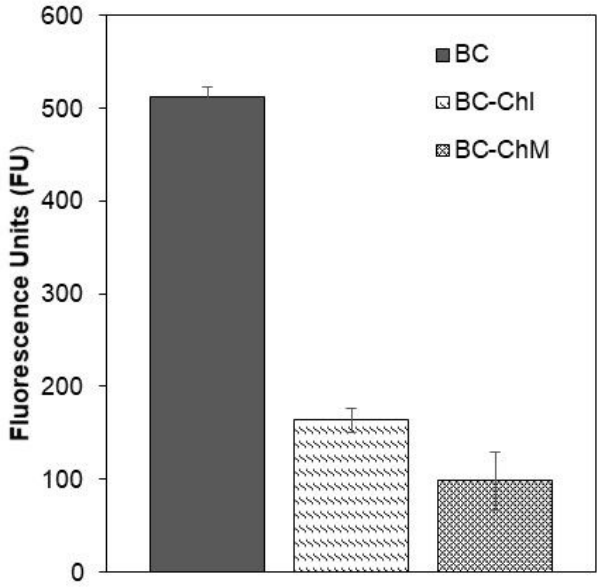

Figure 8. Microbial activity (FU) of the biofilm formed on the surface of BC-Ch and BC paper. Values were expressed as means ± standard deviations and were analysed statistically by analysis of variance (ANOVA), $p \leq 0.05$ was considered statistically significant.

Antioxidant activity of the BC-Chitosan nanocomposites

Antioxidant property of BC paper and BC composites containing chitosan was tested. As shown in Figure 9, BC paper showed some antioxidant activity. Previous studies have reported the presence of aldehyde groups in BC [18] and the antioxidant capability of those.[45] Moreover, when chitosan is impregnated to BC, the antioxidant activity in both types of BC-Ch nanocomposites increased. BC-ChI showed more antioxidant activity than BC-ChM, probably because the immersion method causes a greater chitosan load on the surface of the composite, favoring its exposure to the surrounding environment. Chitosan antioxidant activity is mainly attributed to NH_2 residues and secondarily to OH groups of chitosan which have the capacity to scavenge radicals.[8] One of the characteristics that make chitosan interesting for applications in medicine or in the food packaging industry is its

antioxidant activity [46], therefore, BC-Ch nanocomposites can be a suitable material for those kind of applications.

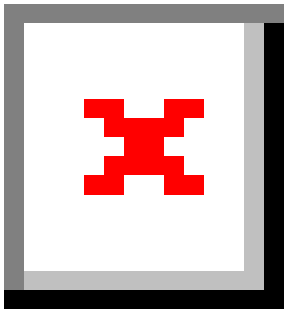

Figure 9. Antioxidant activity of the BC-Chitosan nanocomposites. Values were expressed as means ± standard deviations and were analysed statistically by analysis of variance (ANOVA), $p \leq 0.05$ was considered statistically significant.

CONCLUSIONS

In this work, the combination of BC and chitosan rendered composites of paper with improved physical, chemical and biological characteristics. The two impregnation methods tested allowed a stable binding of chitosan to the BC matrix without the requirement of crosslinking molecules, and produced BC-Ch nanocomposites with similar

characteristics. BC-Ch nanocomposites had the consistency and stiffness of paper and showed great durability, retaining their properties for a long time without the need of special storage in terms of temperature or humidity. They had good resistance to the passage of air and water. They exhibited antimicrobial activity against bacteria and yeasts, and prevented the formation of biofilm in their surfaces. Moreover, BC-Ch paper showed scavenging capacity of oxidizing radicals. The BC-Ch composites developed in this work generated paper-like supports, which can expand the previously described biomedical applications for chitosan embedded in never-dried BC membrane and BC film. Their physical and biological properties, and the organic nature of its components, make them suitable to be part of the design of environmentally friendly materials in the area of bioactive-paper.

ACKNOWLEDMENTS

This work was financed by the Spanish Ministry of Economy, Industry and Competitiveness, grant CTQ2017-84966-C2-2-R, FILMBIOCEL CTQ2016-77936-R

(funding also from the ‘‘Fondo Europeo de Desarrollo Regional FEDER”) and MICROBIOCEL (CTQ2017-84966-C2-1-R), and by the Pla de Recerca de Catalunya, grant 2017SGR-30, and by the Generalitat de Catalunya, “Xarxa de Referència en Biotecnologia” (XRB). L.V. Cabañas acknowledges a doctoral fellowship from Programa Nacional de Becas de Posgrado en el Exterior ¨Don Carlos Antonio López¨ del Ministerio de Hacienda del Paraguay. Special thanks are due to the Serra Húnter Fellow to Cristina Valls.

REFERENCES

(1) Klemm, D.; Heublein, B.; Fink, H.-P.; Bohn, A. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. *Angew. Chem., Int. Ed.* **2005**, *44* (22), 3358–3393. https://doi.org/10.1002/anie.200460587.

(2) Klemm, D.; Kramer, F.; Moritz, S.; Lindström, T.; Ankerfors, M.; Gray, D.; Dorris, A. Nanocelluloses: A New Family of Nature-Based Materials. *Angew. Chem., Int. Ed.* **2011**, *50* (24), 5438–5466. https://doi.org/10.1002/anie.201001273.

(3) Lee, K.-Y.; Buldum, G.; Mantalaris, A.; Bismarck, A. More Than Meets the Eye in Bacterial Cellulose: Biosynthesis, Bioprocessing, and Applications in Advanced Fiber Composites. *Macromol. Biosci.* **2014**, *14* (1), 10–32. https://doi.org/10.1002/mabi.201300298.

(4) Torres, F. G.; Arroyo, J. J.; Troncoso, O. P. Bacterial Cellulose Nanocomposites: An All-Nano Type of Material. *Mater. Sci. Eng. C* **2019**, *98*, 1277–1293.

https://doi.org/10.1016/j.msec.2019.01.064.

(5) Viana, R. M.; Sá, N. M. S. M.; Barros, M. O.; Borges, M. de F.; Azeredo, H. M. C. Nanofibrillated Bacterial Cellulose and Pectin Edible Films Added with Fruit Purees. *Carbohydr. Polym.* **2018**, *196*, 27–32. https://doi.org/10.1016/j.carbpol.2018.05.017.

(6) Lin, S.-P.; Loira Calvar, I.; Catchmark, J. M.; Liu, J.-R.; Demirci, A.; Cheng, K.-C. Biosynthesis, Production and Applications of Bacterial Cellulose. *Cellulose* **2013**, *20* (5), 2191–2219. https://doi.org/10.1007/s10570-013-9994-3.

(7) Rabea, E. I.; Badawy, M. E. T.; Stevens, C. V.; Smagghe, G.; Steurbaut, W. Chitosan as Antimicrobial Agent: Applications and Mode of Action. *Biomacromolecules* **2003**, *4* (6), 1457–1465. https://doi.org/10.1021/bm034130m.

(8) Crouvisier-Urion, K.; Bodart, P. R.; Winckler, P.; Raya, J.; Gougeon, R. D.; Cayot, P.; Domenek, S.; Debeaufort, F.; Karbowiak, T. Biobased Composite Films from Chitosan and Lignin: Antioxidant Activity Related to Structure and Moisture. *ACS Sustainable Chem. Eng.* **2016**, *4* (12), 6371–6381. https://doi.org/10.1021/acssuschemeng.6b00956.

(9) Wahid, F.; Hu, X.; Chu, L.; Jia, S.; Xie, Y.; Zhong, C. Development of Bacterial Cellulose/Chitosan Based Semi-Interpenetrating Hydrogels with Improved Mechanical and Antibacterial Properties. *Int. J. Biol. Macromol.* **2019**, *122*, 380–387. https://doi.org/10.1016/j.ijbiomac.2018.10.105.

(10) Lin, W.-C.; Lien, C.-C.; Yeh, H.-J.; Yu, C.-M.; Hsu, S. Bacterial Cellulose and Bacterial Cellulose–Chitosan Membranes for Wound Dressing Applications. *Carbohydr. Polym.* **2013**, *94* (1), 603–611. https://doi.org/10.1016/j.carbpol.2013.01.076.

(11) Bordenave, N.; Grelier, S.; Coma, V. Hydrophobization and Antimicrobial Activity of Chitosan and Paper-Based Packaging Material. *Biomacromolecules* **2010**, *11* (1), 88–96. https://doi.org/10.1021/bm9009528.

(12) Carvalho, T.; Guedes, G.; Sousa, F. L.; Freire, C. S. R.; Santos, H. A. Latest Advances on Bacterial Cellulose-Based Materials for Wound Healing, Delivery Systems, and Tissue Engineering. *Biotechnol. J.* **2019**, *14*, (12), 1900059. https://doi.org/10.1002/biot.201900059.

(13) Fortunati, E.; Mazzaglia, A.; Balestra, G. M. Sustainable Control Strategies for Plant Protection and Food Packaging Sectors by Natural Substances and Novel Nanotechnological Approaches. *J. Sci. Food Agric.* **2019**, *99* (3), 986–1000.

https://doi.org/10.1002/jsfa.9341.

(14) Fortunati, E.; Giovanale, G.; Luzi, F.; Mazzaglia, A.; Kenny, J.; Torre, L.; Balestra, G. Effective Postharvest Preservation of Kiwifruit and Romaine Lettuce with a Chitosan Hydrochloride Coating. *Coatings* **2017**, *7* (11), 196. https://doi.org/10.3390/coatings7110196.

(15) Chen, C. H.; Wang, F. Y.; Mao, C. F.; Liao, W. T.; Hsieh, C. D. Studies of Chitosan: II. Preparation and Characterization of Chitosan/Poly(Vinyl Alcohol)/Gelatin Ternary Blend Films. *Int. J. Biol. Macromol.* **2008**, *43* (1), 37–42. https://doi.org/10.1016/j.ijbiomac.2007.09.005.

(16) Grande, C. J.; Torres, F. G.; Gomez, C. M.; Troncoso, O. P.; Canet-Ferrer, J.; Martínez-Pastor, J. Development of Self-Assembled Bacterial Cellulose–Starch Nanocomposites. *Mater. Sci. Eng. C* **2009**, *29* (4), 1098–1104. https://doi.org/10.1016/j.msec.2008.09.024.

(17) Bonilla, J.; Fortunati, E.; Atarés, L.; Chiralt, A.; Kenny, J. M. Physical, Structural and Antimicrobial Properties of Poly Vinyl Alcohol–Chitosan Biodegradable Films. *Food Hydrocolloids* **2014**, *35*, 463–470. https://doi.org/10.1016/j.foodhyd.2013.07.002.

(18) Morena, A. G.; Roncero, M. B.; Valenzuela, S. V.; Valls, C.; Vidal, T.; Pastor, F. I. J.; Diaz, P.; Martínez, J. Laccase/TEMPO-Mediated Bacterial Cellulose Functionalization: Production of Paper-Silver Nanoparticles Composite with Antimicrobial Activity. *Cellulose* **2019**, *26* (16), 8655–8668. https://doi.org/10.1007/s10570-019-02678-5.

(19) Kjellgren, H.; Gällstedt, M.; Engström, G.; Järnström, L. Barrier and Surface Properties of Chitosan-Coated Greaseproof Paper. *Carbohydr. Polym.* **2006**, *65* (4), 453–460. https://doi.org/10.1016/j.carbpol.2006.02.005.

(20) Hestrin, S.; Schramm, M. Synthesis of Cellulose by *Acetobacter Xylinum* . 2. Preparation of Freeze-Dried Cells Capable of Polymerizing Glucose to Cellulose. *Biochem. J.* **1954**, *58* (2), 345–352. https://doi.org/10.1042/bj0580345.

(21) Segal, L.; Creely, J. J.; Martin, A. E.; Conrad, C. M. An Empirical Method for Estimating the Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. *Tex. Res. J.* **1959**, *29* (10), 786–794. https://doi.org/10.1177/004051755902901003.

(22) Mariscal, A.; Lopez-Gigosos, R.; Carnero-Varo, M.; Fernandez-Crehuet, J. Fluorescent Assay Based on Resazurin for Detection of Activity of Disinfectants against Bacterial Biofilm. *Appl. Microbiol. Biotechnol*. **2009**, *82* (4) 773–783.

https://doi.org/10.1007/s00253-009-1879-x.

(23) Serpen, A.; Capuano, E.; Fogliano, V.; Gökmen, V. A New Procedure To Measure the Antioxidant Activity of Insoluble Food Components. *J. Agric. Food Chem.* **2007**, *55* (19), 7676–7681. https://doi.org/10.1021/jf071291z.

(24) Valls, C.; Roncero, M. B. Antioxidant Property of TCF Pulp with a High Hexenuronic Acid (HexA) Content. *Holzforschung* **2013**, *67* (3), 257–263. https://doi.org/10.1515/hf-2012-0114.

(25) Cusola, O.; Valls, C.; Vidal, T.; Roncero, M. B. Conferring Antioxidant Capacity to Cellulose Based Materials by Using Enzymatically-Modified Products. *Cellulose* **2015**, *22* (4), 2375–2390. https://doi.org/10.1007/s10570-015-0668-1.

(26) Badawy, M. E. I. A New Rapid and Sensitive Spectrophotometric Method for Determination of a Biopolymer Chitosan. *Int. J. Carbohydr. Chem.* **2012**, *ID139328*, 1–7. https://doi.org/10.1155/2012/139328.

(27) Fernandes, S. C. M.; Oliveira, L.; Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P.; Gandini, A.; Desbriéres, J. Novel Transparent Nanocomposite Films Based on Chitosan and Bacterial Cellulose. *Green Chem.* **2009**, *11* (12), 2023–2029. https://doi.org/10.1039/b919112g.

(28) Liang, J.; Wang, R.; Chen, R. The Impact of Cross-Linking Mode on the Physical and Antimicrobial Properties of a Chitosan/Bacterial Cellulose Composite. *Polymers* **2019**, *11* (3), 491. https://doi.org/10.3390/polym11030491.

(29) Aryaei, A.; Jayatissa, A. H.; Jayasuriya, A. C. Nano and Micro Mechanical Properties of Uncross-Linked and Cross-Linked Chitosan Films. *J. Mech. Behav. Biom. Mater.* **2012**, *5* (1), 82–89. https://doi.org/10.1016/j.jmbbm.2011.08.006.

(30) Wei, B.; Yang, G.; Hong, F. Preparation and Evaluation of a Kind of Bacterial Cellulose Dry Films with Antibacterial Properties. *Carbohydr. Polym.* **2011**, *84* (1), 533–538. https://doi.org/10.1016/j.carbpol.2010.12.017.

(31) Kingkaew, J.; Kirdponpattara, S.; Sanchavanakit, N.; Pavasant, P.; Phisalaphong, M. Effect of Molecular Weight of Chitosan on Antimicrobial Properties and Tissue Compatibility of Chitosan-Impregnated Bacterial Cellulose Films. *Biotechnol. Bioprocess Eng.* **2014**, *19* (3), 534–544. https://doi.org/10.1007/s12257-014-0081-x.

(32) Orelma, H.; Filpponen, I.; Johansson, L. S.; Laine, J.; Rojas, O. J. Modification of Cellulose Films by Adsorption of Cmc and Chitosan for Controlled Attachment of Biomolecules.

Biomacromolecules **2011**, *12* (12), 4311–4318. https://doi.org/10.1021/bm201236a.

(33) VandeVord, P. J.; Matthew, H. W. T.; DeSilva, S. P.; Mayton, L.; Wu, B.; Wooley, P. H. Evaluation of the Biocompatibility of a Chitosan Scaffold in Mice. *J. Biomed. Mater. Res.* **2002**, *59* (3), 585–590. https://doi.org/10.1002/jbm.1270.

(34) Baldrick, P. The Safety of Chitosan as a Pharmaceutical Excipient. *Regul. Toxicol. Pharmacol.* **2010**, *56* (3), 290–299. https://doi.org/10.1016/j.yrtph.2009.09.015.

(35) Fortunati, E. *Multifunctional Films, Blends, and Nanocomposites Based on Chitosan: Use in Antimicrobial Packaging*. In: *Antimicrobial Food Packaging* **2016**, 467-477 Elsevier Inc https://doi.org/10.1016/B978-0-12-800723-5.00038-3.

(36) Phisalaphong, M.; Jatupaiboon, N. Biosynthesis and Characterization of Bacteria Cellulose-Chitosan Film. *Carbohydr. Polym.* **2008**, *74* (3), 482–488. https://doi.org/10.1016/j.carbpol.2008.04.004.

(37) Fillat, A.; Martinez, J.; Valls, C.; Cusola, O.; Valenzuela, S. V. Bacterial Cellulose for Increasing Barrier Properties of Paper Products. *Cellulose* **2018**, *25*, 6093–6105. https://doi.org/10.1007/s10570-018-1967-0.

(38) Requies, J.; Gabilondo, N.; Urbina, L.; Corcuera, M. A.; Retegi, A.; Guaresti, O.; Eceiza, A. Design of Reusable Novel Membranes Based on Bacterial Cellulose and Chitosan for the Filtration of Copper in Wastewaters. *Carbohydr. Polym.* **2018**, *193*, 362–372. https://doi.org/10.1016/j.carbpol.2018.04.007.

(39) Mishima, T.; Hisamatsu, M.; York, W. S.; Teranishi, K.; Yamada, T. Adhesion of β-D-Glucans to Cellulose. *Carbohydr. Res.* **1998**, *308* (3–4), 389–395. https://doi.org/10.1016/S0008-6215(98)00099-8.

(40) Goy, R. C.; Britto, D. De; Assis, O. B. G. A Review of the Antimicrobial Activity of Chitosan. *Polim.: Cienc. Tecnol.* **2009**, *19* (3), 241–247. https://doi.org/10.1093/jac/dkg286.

(41) Flemming, H.-C.; Wingender, J. The Biofilm Matrix. *Nat. Rev. Microbiol.* **2010**, *8* (9), 623–633. https://doi.org/10.1038/nrmicro2415.

(42) Costerton, J. W. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science* **1999**, *284* (5418), 1318–1322. https://doi.org/10.1126/science.284.5418.1318.

(43) Martinez, L. R.; Mihu, M. R.; Han, G.; Frases, S.; Cordero, R. J. B.; Casadevall, A.; Friedman, A. J.; Friedman, J. M.; Nosanchuk, J. D. The Use of Chitosan to Damage

Cryptococcus Neoformans Biofilms. *Biomaterials* **2010**, *31* (4), 669–679. https://doi.org/10.1016/j.biomaterials.2009.09.087.

(44) Campana, R.; Biondo, F.; Mastrotto, F.; Baffone, W.; Casettari, L. Chitosans as New Tools against Biofilms Formation on the Surface of Silicone Urinary Catheters. *Int. J. Biol. Macromol.* **2018**, *118*, 2193–2200. https://doi.org/10.1016/j.ijbiomac.2018.07.088.

(45) Zhang, L.; Ge, H.; Xu, M.; Cao, J.; Dai, Y. Physicochemical Properties, Antioxidant and Antibacterial Activities of Dialdehyde Microcrystalline Cellulose. *Cellulose* **2017**, *24* (5), 2287–2298. https://doi.org/10.1007/s10570-017-1255-4.

(46) Coma, V. Polysaccharide-Based Biomaterials with Antimicrobial and Antioxidant Properties. *Polim.: Cienc. Tecnol.* **2013**, *20* (2), 287–297. https://doi.org/10.4322/polimeros020ov002.

For Table of Contents Only