Cellulose

Laccase/TEMPO-mediated bacterial cellulose functionalization: production of paper-silver nanoparticles composite with antimicrobial activity

--Manuscript Draft--

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Corresponding Author:	Josefina Martinez, PhD University of Barcelona Barcelona, SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Barcelona
Corresponding Author's Secondary Institution:	
First Author:	A. Gala Morena
First Author Secondary Information:	
Order of Authors:	A. Gala Morena
	M.Blanca Roncero
	Susana V. Valenzuela
	Cristina Valls
	Teresa Vidal
	F.I. Javier Pastor
	Pilar Diaz
	Josefina Martinez, PhD
Order of Authors Secondary Information:	
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Abstract:	Bacterial cellulose (BC) was functionalized applying the Laccase/TEMPO oxidative treatment, leading to a five-fold increase of the concentration of carboxyl groups. Paper produced with this cellulose showed improved mechanical properties while maintaining barrier function against water and greases as compared to paper produced with non-oxidized BC. Also, the negative charge provided by the carboxyl groups on functionalized BC was used to generate silver nanoparticles (AgNPs), obtaining a BC paper and Ag composite. The presence of AgNPs in the composites was validated by SEM, EDS and ICP analysis, showing spherical, uniformly sized particles stabilized in the BC nanofibers matrix. Additionally, antimicrobial property of composites containing AgNPs was tested. The results showed the strong antimicrobial activity of the

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Response to Reviewers:	Author's response to the issues raised by reviewers Manuscript number: CELS-D-19-00522 Dear Editor, Please find below the answers (in red) to the issues raised by the reviewers to the manuscript entitled Laccase/TEMPO-mediated bacterial cellulose functionalization: production of paper-silver nanoparticles composite with antimicrobial activity. The questions raised by the reviewers have been carefully addressed and the corresponding corrections have been included in the revised manuscript. We thank the reviewers for helping to improve the manuscript. Reviewer #1: The manuscript "Laccase/TEMPO-mediated bacterial cellulose functionalization: production of paper-silver nanoparticles composite with antimicrobial activity" described the oxidation of bacterial cellulose (BC) by applying laccase and TEMPO, the paper produced from oxidized BC showed capacity for stabilizing silver nanoparticles (AgNPs) and thus showed antimicrobial activity. The manuscript was organized well and fit the scope of the journal of Cellulose, it is recommended that the manuscript can be considered for publication after minor revisions according to the following suggestions. 1. "laccase" should be included in the keywords The suggestion has been accepted and incorporated 2. All of "via" in the manuscript should be in italic font. The suggestion has been accepted and incorporated 3. As described in the introduction part of manuscript: "The hydroxyl groups and ether oxygen of the cellulose molecule anchor the silver ions via ion-dipole interaction and, once reduced, form stabilized nanoparticles in the fine nanofiber network (Maneerung et al. 2008; Pinto et al. 2009; Barud et al. 2011; Yang et al. 2012)." The description means that besides carboxyl groups, the hydroxyl groups can also stabilize silver nanoparticles. If so, Fig.1 should be modified to be more accurate. We modified the Figure 1 to be more specific 4. The manuscript showed that by laccase/TEMPO oxidation, the carboxyl groups was increased to 139.49μmol/g, is this the highest content of carboxyl groups, or it can be improved by optimizing the oxidation condition? It is the highest content of carboxyl groups under the used conditions. The optimization of the laccase/TEMPO oxidation procedure was conducted previously by the research group for vegetal cellulose (Quintana E, Roncero MB, Vidal T, Valls C (2017) Cellulose oxidation by Laccase-TEMPO treatments. Carbohydrate Polymers, 157, 1488–1495. https://doi.org/10.1016/j.carbpol.2016.11.033). This study showed that the concentration of carboxyl groups reached in this work was enough to generated Ag nanoparticles. 5. Table 1, the density of BC-ox paper is lower than that of BC paper, why? The density difference of the two samples is derived from the variability of the paper making process. This does not affect the discussion of the results of the properties evaluated, considering above all that the paper with lower density is the one produced with the oxidized cellulose.

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This manuscript describes the preparation and characterization of homogenised bacterial cellulose membranes that are oxidized using the laccase/TEMPO oxidative treatment. This bacterial cellulose functionalized with carboxyl groups allowed the attraction of silver ions via electrostatic assembly that are posteriorly reduced by thermal treatment. The authors made the characterization of the paper materials by several techniques namely SEM, EDS, ICP, physical and mechanical properties. Furthermore, they studied the respective antimicrobial activity against distinct types of Gram-positive and Gram-negative bacteria and fungi.

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Author's response to the issues raised by reviewers
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A. Gala Morena[a,c] M. Blanca Roncero[b], Susana V. Valenzuela[a,d], Cristina Valls[b], Teresa Vidal[b], F.I. Javier Pastor[a,d], Pilar Diaz[a,d] and Josefina Martínez*[a,d]

[a] Department of Genetics, Microbiology and Statistics, Faculty of Biology, Universitat de Barcelona, Av. Diagonal 643, 08028, Barcelona, Spain
[b] CELBIOTECH_Paper Engineering Research Group, EGE Department, Universitat Politècnica de Catalunya, Barcelona Tech, 08222, Terrassa, Spain
[c] Molecular and Industrial Biotechnology Group, Department of Chemical Engineering, Universitat Politècnica de Catalunya, Rambla Sant Nebridi 22, 08222 Terrassa, Spain
[d] Institute of Nanoscience and Nanotechnology (IN2UB), Universitat de Barcelona, Spain

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

A. Gala Morena angela.gala.morena@upc.edu (0000-0003-4470-8249)
M. Blanca Roncero blanca.roncero@upc.edu (0000-0002-2694-2368)
Susana V. Valenzuela susanavalenzuela@ub.edu (0000-0002-1684-9514)
Cristina Valls cristina.valls@upc.edu (0000-0003-2307-1779)
Teresa Vidal teresa.vidal@upc.edu (0000-0001-6269-4114)
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Pilar Diaz pdiaz@ub.edu (0000-0003-4008-0669)
Josefina Martínez jmartinez@ub.edu (0000-0002-2411-8188)

Abstract

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Keywords: Bacterial cellulose oxidation, laccase, bacterial cellulose paper, nanocomposite, silver nanoparticle, antimicrobial activity

Introduction

Bacterial cellulose (BC) is a biopolymer produced by some microorganisms, especially from the genera *Komagataeibacter*. In terms of chemical structure, BC is identical to the cellulose produced by vascular plants, composed by units of glucose linked by β(1→4)–glycosidic bonds. However, unlike vegetable cellulose, which is always bound to hemicellulose and lignin, BC is chemically pure (Chawla et al. 2009). The mechanical properties and microstructure of BC differ from those of vegetable cellulose. BC displays a higher degree of crystallinity, a higher

tensile strength, a higher water-holding capacity, and a finer three-dimensional nanofiber network (Yano et al. 2005; Lee et al. 2014). The structural features and mechanical properties are significant for practical application of BC. It can be used as a biomaterial for cosmetics (Hasan et al. 2012; UllahSantos et al. 2016) and medical devices (Gao et al. 2011; Bielecki et al. 2012; Nimeskern et al. 2013; Ul-Islam et al. 2015; Stumpf et al. 2018;) as a reinforcement of polymeric materials or paper (Miao et al. 2013; Fillat et al. 2018;), and as a material for food packaging (Spence et al. 2010; Wu et al. 2018).

The chemical modification of the molecule is frequently a prerequisite to provide new functions and applicability to cellulose (Rol et al. 2019). The functionalization of plant cellulose by the oxidation of the C-6 carbon of glucose unites is known to improve some physical characteristics of the paper such as the wet strength development (Kitaoka et al. 1999; Saito et al. 2005, 2006). The most common procedure to selective oxidation of C-6 primary hydroxyl to carboxyl or aldehyde groups in cellulose is through the radical 2,2,6,6–tetramethylpiperidine–1–oxyl (TEMPO) combined with NaBr/NaOCl under alkaline conditions (Saito et al. 2004; Gert et al. 2005). This is a well-established treatment widely used in vegetal cellulose (Isogai et al. 2011) and also has been recently attempted in bacterial cellulose (Lai et al. 2013; Feng et al. 2014; Pahlevan et al. 2018). The rate of these reactions is remarkably high, but the treatment presents some disadvantages such as the undesirable de-polymerization of the cellulose, the harsh conditions of the reaction, and the generation of chemical residues (Isogai et al. 2011). The use of enzyme technology in industrial processes can reduce its negative environmental and economic impact. The Laccase/TEMPO mediated oxidation operates in milder conditions than the TEMPO/NaBr/NaClO treatment generating less environmental harmful residues, and it has been successfully performed on vegetal cellulose (Aracri et al. 2011; Aracri and Vidal 2012; Aracri et al. 2012; Jiang et al. 2017; Quintana et al. 2017).

79 Once the cellulose is oxidized and new functional groups are created, compounds can be
80 added in order to provide new functionalities or to generate new composites (Johnson et al.
81 2011). Carboxyl groups have been used as host groups to introduce metal ions by an ion-
82 exchange reaction (Saito et al. 2005; Matsumoto et al. 2006;). Metal nanoparticles have been
83 proposed in different catalytic, photoelectric, magnetic, sensor, and biomedical applications
84 due to their electronic, optical, and chemical properties (Zhou et al. 2003; Sondi et al. 2004;
85 Jun et al. 2007; Wu et al. 2008). An essential issue with the synthesis and stabilization of metal
86 nanoparticles is their strong tendency to aggregate, losing their nanoscale characteristics. One
87 effective approach to prevent aggregation is the immobilization of the nanoparticles in a
88 polymeric insoluble matrix. The BC membranes have been used as nanoreactors for the
89 generation of silver nanoparticles (AgNPs). The hydroxyl groups and ether oxygen of the
90 cellulose molecule anchor the silver ions *via* ion-dipole interaction and, once reduced, form
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93 membranes to generate carboxyl groups has been reported to increase the bounding strength
94 between the cellulose fibers and the silver ions, achieving a higher yield and a more uniform
95 distribution of the metal nanoparticles (Ifuku et al. 2009; Jin Feng et al. 2014). Recently, the
96 generation of aldehyde groups in BC by the hybrid system Laccase/ TEMPO oxidation of BC to
97 obtain aldehyde groups has been reported (Zhou et al. 2017) and its capability of further
98 oxidation to carboxyl groups would be expected. Moreover, in the AgNPs/BC composites
99 described so far, the metal nanoparticles are contained in membranes of BC. The implantation
100 of Ag nanoparticles in a matrix that combine the high surface-to-volume ratio of the BC
101 nanofibers with the stiffness and the mechanical properties of paper would generate a
102 composite with extended applicability.

103 The purpose of this study was to develop BC paper with good mechanical properties from
104 bacterial cellulose oxidized with the milder condition treatment Laccase/TEMPO. Furthermore,

we investigated the suitability of the bacterial cellulose functionalized with carboxyl groups to obtain silver nanoparticles on a solid stiff organic matrix and their antimicrobial activity.

Experimental

Materials

Microbial strains *Komagataeibacter xylinus* CECT 7351, *Staphylococcus aureus* CECT 234, *Pseudomonas aeruginosa* PAO1 CR321, *Klebsiella pneumoniae* CECT 143 and *Candida albicans* CECT 1001 were obtained from the Spanish Type Culture Collection (CECT). Peptone, Yeast extract, Luria Bertani broth (LB), Triptone Soy Agar (TSA) and Bacteriologic Agar were purchased from Laboratiorios Conda. Citric acid and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Emsure. Glucose was purchased from PanReac. Silver nitrate, sodium hydroxide anhydrate pellet, sodium chloride and 2,2,6,6–tetramethyl–1–piperidinyloxy (TEMPO) and resazurin were purchased from Sigma Aldrich. Laccase from *Trametes villosa* with an activity of 746 U/mL was supplied by Novozymes.

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. Inoculum for culture was prepared by transferring *K. xylinus* cells grown on HS–Agar to HS liquid medium. After shaking vigorously, the resulting cell suspension was used to inoculate (1:40) 10 cm–Petri dishes containing 40 mL of HS medium. The cultures 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 purified by incubating them in 1 % NaOH at 70°C overnight. Finally, the BC pellicles were thoroughly washed in deionized water until the pH reached neutrality. To

obtain the bacterial cellulose suspension, pellicles were mechanically cut into small pieces and disrupted with a homogenizer (Homogenizing System UNIDRIVE X1000).

Laccase/TEMPO oxidation

Laccase/TEMPO oxidation was adapted from earlier studies carried out in vegetable cellulosic fibers (Aracri et al. 2012; Aracri et al. 2011; Quintana et al. 2017). The treatment was performed at room temperature in a 50 mM acetate buffer at pH 5, in the dark. TEMPO (8% w/w) and Laccase (60 U/dry gram of BC) were added to the 5% consistency BC suspension. The blend was mechanically mixed until the components were totally homogenized and then kept at room temperature for 24 h. After the treatment, the functionalized BC suspension was filtered and washed with deionized water. These oxidized BC samples were named as BC–ox.

Quantification of carboxyl and aldehyde groups

Carboxyl and aldehyde groups were measured in the initial and oxidized BC samples. Quantification of carboxyl groups (COOH) was performed by the methylene blue dye test. Briefly, this method is based on the following ion exchange reaction (Equation 1):

$$\mathrm{R-COOH + Mb^{+} \leftrightarrow R-COOMb + H^{+}} \quad (1)$$

where Mb^{+} represents the methylene blue ions in dye solution (Davidson 1948).

For the analysis, 0.05 dry grams of sample were suspended in 50 mL of a 0.2 mM solution of methylene blue. After 24 h of stirring in the dark, the sample was passed through a glass filter. The filtrate was centrifuged at 3,000 rpm for 20 minutes. The supernatant was diluted 1:25 and analyzed using UV spectroscopy (Type Evolution 600 BB, Thermo Scientific) at 664 nm. The concentration of carboxyl groups (µmol per dry gram of BC) was estimated through the Equation 2 and using a calibration curve:

$$\text{Concentration of COOH groups } (\mu\text{mol/g}) = \frac{(c - c') \cdot 0.05 \cdot 1000}{m + p - m'} \quad (2)$$

where *c* is the initial concentration of methylene blue, *c'* is the concentration of methylene blue after the reaction, *p* is the dry weight of the sample, *m* is the weight of the glass filter, and *m'* is the weight of the glass filter after the filtration.

Quantification of aldehyde groups (CHO) was performed by the methylene blue dye test, using 0.25 dry grams of sample. Prior to the measurement, the samples were introduced into 25 mL of sodium chlorite. The mixture was incubated for 24 h, stirring in the dark. The concentration of aldehyde groups can be determined by Equation 3:

$$\text{Concentration of CHO groups } (\mu\text{mol/g}) = \text{COOH}_{\text{AO}} - \text{COOH}_{\text{BO}} \quad (3)$$

where $COOH_{AO}$ is the content of carboxyl groups(μmol/g) after the oxidation with sodium chlorite and $COOH_{BO}$ is the content of carboxyl groups (μmol/g) before the oxidation with sodium chlorite.

BC paper sheet formation and physical and mechanical properties characterization

Bacterial cellulose sheets were produced using a Rapid–Köthen laboratory former (Frank–PTI) following the ISO–5269:2004 standard method. Sheets were conditioned at 23°C and 50% of relative humidity for at least 24 h before physical and mechanical testing, as indicated in ISO 187. Physical and mechanical properties were measured according with standards indicated in parenthesis as follow: density (ISO 534), brightness (UNE 57060), opacity (UNE 57063), water drop test (tappi T835 om-08), grease resistance (UNE 57071), and wet tensile index (ISO 1924-2).

Formation of composites of paper containing silver nanoparticles

Oxidized BC suspension was soaked in a 10 mM or 0.1 mM $AgNO_3$ solution in a 1:1 ratio (BC wet weight : $AgNO_3$ solution volume). The mixture was mechanically homogenized and incubated in the dark, at room temperature for 24 hours. After the incubation, the treated BC was rinsed with water and filtered through a glass filter to remove the excess of $AgNO_3$. Following the formation of BC paper sheets, a thermal treatment at 121°C for 20 minutes was applied to induce the reduction of Ag ions and promote the formation of AgNPs (Fig. 1). For simplicity, the composites of paper generated with 10 mM and 0.1 mM $AgNO_3$ will be referred as BC-ox-10Ag and BC-ox-0.1Ag throughout respectively.

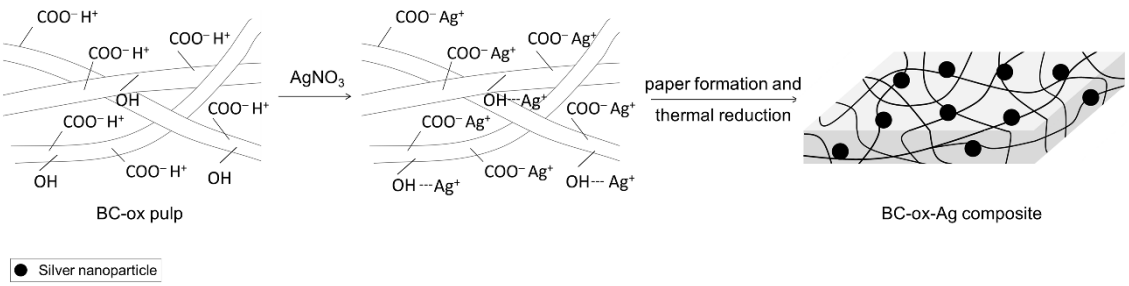

Fig. 1 Schematic model of silver nanoparticles generation in BC composites after oxidation treatment

Scanning Electron Microscope (SEM) and Energy Dispersive X–ray Spectroscopy (EDS) analysis

The presence of nanoparticles in BC-ox-Ag composites was verified by SEM (JSM 7100 F) using a LED filter and a backscattered electron detector (BED). EDS analysis was carried out to verify the chemical composition of the nanoparticles. The diameter of the nanoparticles was measured using the ImageJ software.

Ag migration from the composites

To measure the diffusion of silver from the BC matrix, the composites were cut into square pieces of 1 cm^2, immersed into 1 mL of deionized water, and incubated at room temperature while shaken at 1000 rpm during 24 h. Then, the composites were removed and the silver content in the water was analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The Ag content of the samples was analyzed both before and after the addition of HNO_3 at a final concentration of 1%. The acid dissolves the AgNPs to Ag ions prior to ICP-MS analysis.

Antimicrobial activity of the composites containing silver nanoparticles (BC-ox-Ag)

The antimicrobial properties of BC-ox-Ag composites were tested against the Gram-positive bacteria *Staphylococcus aureus*, the Gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumoniae,* and the yeast *Candida albicans*. To obtain the inoculum for the antimicrobial tests, the strains were grown overnight in LB broth at 37°C in shaking conditions. The overnight cultures were centrifuged for 4 minutes at 14000 *xg* and the pellet suspended in 0.3 mM KH_2PO_4 (hereinafter work solution) to remove the culture medium. Both, BC-ox-Ag composites and BC paper 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

3 µl of the corresponding microbial suspension (about 10^5 microorganisms per mL) were inoculated over the 1 cm^2 BC-ox-Ag composites placed on the surface of TSA medium plates. The growth over a sample of BC paper was used as positive control. After overnight incubation at 37°C, the microorganisms were detached from the composites and BC paper by intense shaking on the work solution, and the metabolic activity of the resuspension was measured by

the resazurin assay. For the assay, 50 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 with Varian Cary Eclipse Fluorescence Spectrophotometer. The difference between the metabolic activity of the microorganisms grown on BC-ox-Ag composites and on BC paper was used to calculate the percentage of growth inhibition.

Dynamic contact conditions test

This procedure was adapted from ASTM E2149–01 (*Standard test Method for determining the antimicrobial activity agents under dynamic contact conditions*). Nine 1 cm^2 pieces of the composites were immersed in 5 mL of a suspension of a known concentration of microorganisms 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 Equation 4:

$$\%\ \text{cell viability reducion} = \frac{\text{viable CFU at } t_0 - \text{viable CFU at } t_x}{\text{viable CFU at } t_0} \times 100 \qquad (4)$$

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

Results and discussion

Laccase/TEMPO oxidation

BC suspension was treated with Laccase/TEMPO to oxidize the hydroxyl groups of cellulose molecules and to introduce functional carboxyl groups. The enzyme Laccase catalyzes the oxidation of the TEMPO molecule. The oxidized TEMPO radical, in turn, oxidizes the primary alcohols in cellulose to carboxyl (COOH) and aldehyde (CHO) functional groups (Aracri et al.

2011). Fig. 2 shows the content of carboxyl and aldehyde groups of BC molecule before (BC) and after Laccase /TEMPO oxidation (BC-ox) with 8% TEMPO and 60 U/g Laccase.

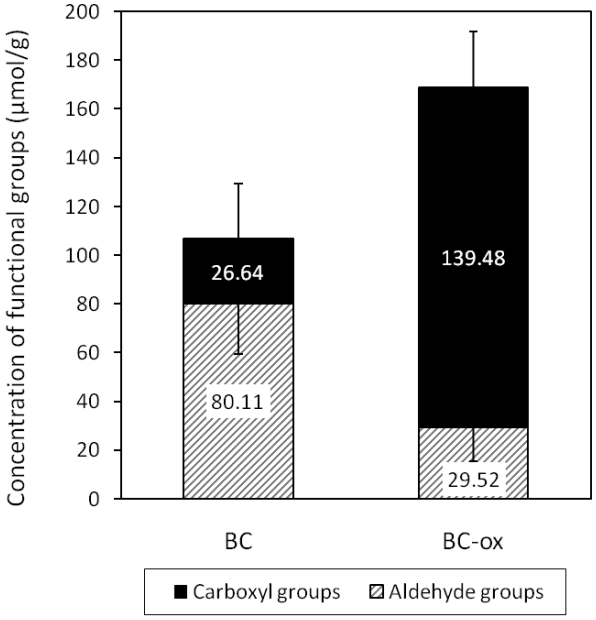

Fig. 2 Carboxyl and aldehyde groups (µmol/g cellulose) of bacterial cellulose (BC) and oxidized bacterial cellulose with the Laccase/TEMPO treatment (BC-ox)

Results showed that the amount of carboxyl groups increased from 26.6 µmol/g to 139.5 µmol/g after the oxidation, which is five times more than the initial value. The presence of carboxyl and aldehyde groups in the BC molecule before the Laccase/TEMPO treatment could be due to the oxidation of cellulose by unspecific physical factors, such as visible light (Tolvaj et al. 1995), or during the isolation and purification procedures (Jaušovec et al. 2015). After the treatment, the concentration of aldehyde groups decreased because part of these aldehyde groups was oxidized to carboxyl groups by action of Laccase/TEMPO. Thus, the results suggested that some of the carboxyl groups detected were induced from aldehyde groups initially present in BC, while other were generated *de novo* from new aldehyde groups which, in turn, were induced from primary alcohol groups present in BC.

Previous studies have shown that the TEMPO/NaBr/NaClO oxidation treatment of cellulose was efficient generating carboxyl groups. Milanovic et al. reported an eight-fold increase of the COOH amount in cotton fibers after TEMPO/NaBr/NaClO (Milanović et al. 2016), while Gehmayr et al. achieved an eleven-fold increase in ECF eucalyptus kraft pulp (Gehmayr et al. 2012). The TEMPO/NaBr/NaClO procedure has been also successfully attempted in nanofibrillated cellulose from different plant cellulosic fibers (Chen et al. 2017). The studies reporting bacterial cellulose oxidized with TEMPO/NaBr/NaClO treatment found an efficiency similar to that previously referred to cellulose from plant (Ifuku et al. 2009; Wu et al. 2018). The Laccase/TEMPO mediated oxidation operates in milder conditions than the TEMPO/NaBr/NaClO treatment generating less environmental harmful residues and it has been successfully applied in plant cellulose, although with less efficiency. Quintana et al. reported a 6-fold increase of COOH groups in a refined dissolving pulp from plant cellulose after Laccase/TEMPO oxidation (Quintana et al. 2017). Likewise, Patel et al. oxidized cotton linters by Laccase/TEMPO and the carboxyl group content was 9 times higher than in the control sample (Patel et al. 2011). However, other authors reported an increase of the COOH content of only up to 2 or 3 times (Aracri et al. 2012; Aracri and Vidal 2012; Jaušovec et al. 2015). While the Laccase/TEMPO procedure has been previously applied to generate aldehyde groups in BC membranes (Zhou et al. 2017), this work assessed the oxidation to carboxyl groups. The results were comparable to those obtained in plant cellulose after Laccase/TEMPO mediated oxidation.

Characterization of paper sheets produced with oxidized BC

After the oxidation of BC, paper sheets were produced and compared with paper made from non-oxidized BC in terms of physical and mechanical properties to verify if the Laccase/TEMPO oxidation treatment affected those properties. Results are shown in Table 1.

284 Table 1. Physical and mechanical properties of oxidized BC (BC–ox) paper sheets and BC papers
285 sheets

Property	BC paper	BC–ox paper
Density (g/cm^3)	0.78	0.51
Brightness (%)	52.0 ± 1.6	54.4 ± 1.9
Opacity (%)	66.3 ± 1.8	65.6 ± 1.4
Water dropt test (WDT) (s)	2355 ± 102.5	2397 ± 89.6
Grease resistance (s)	>1800	>1800
Wet tensile index (kN·m/kg)	11.1 ± 4.3	15.1 ± 0.3
Wet tensile strength development (W/D) (%)	13.8	22.3

286

287 The oxidative treatment of BC did not affect brightness and opacity of the paper. Moreover,
288 values of WDT and grease resistance were similar in both BC and BC-ox sheets, indicating that
289 water and grease barrier properties were not modified by the Laccase/TEMPO treatment.
290 However, the strength properties varied in the two types of paper. The wet tensile strength
291 development is the increase of tensile resistance in wet paper in relation to dry paper, and it is
292 also known as ratio of wet versus dry tensile index (W/D). The wet strength is one of the most
293 important properties of papers that must be in contact with liquids, such as tissue paper,
294 paper towels, filter paper, packaging papers, etc. Paper made from BC-ox showed a 22.3% wet-
295 to-dry (W/D) strength ratio, whereas for paper made from BC, this value was 13.8%. Thus, the
296 Laccase/TEMPO treatment allowed the improvement of the wet strength development by
297 62%. The reported W/D value of BC-ox paper is a significant improvement, paper with values
298 over 15% are considered to have excellent wet tensile strength properties (Scott 1996). The
299 increase of wet strength obtained in BC-ox paper could be attributed to the formation of
300 hemiacetal bonds in cellulose, as suggested by Aracri et al. (2011).

301

Production and characterization of BC-ox-Ag composites

The suitability of the functionalized BC on the generation of paper sheets containing silver nanoparticles (BC-ox-Ag composites) was tested. Suspensions of BC-ox were mixed with 10 mM or 0.1 mM $AgNO_3$ solutions as a source of Ag ions. In the proper conditions, it would be expected that the negatively-charged BC molecules functionalized with carboxyl groups attract the Ag^+ cations *via* electrostatic interactions. In addition, electron-rich oxygen atoms resulting from hydroxyl and ester of the BC molecule could also contribute to keep stable the Ag ions in the BC nanofibers matrix (i.e., by ion-dipole interaction) (Barud et al. 2011). Then, with the obtained BC-ox-Ag mix, paper sheets were produced, and heat (121°C, 20 min) was used to trigger the reduction of Ag ions and consequently the formation of nanoparticles (Maria et al. 2010). It has been described that the complex formed between Ag and carboxyl groups could promote particle nucleation, anchoring the growing nanoparticle (de Santa Maria et al. 2009). The tri-dimensional structure of BC nanofibrils with very high specific surface area would help to stabilize the particles preventing agglomeration.

BC-ox-Ag composites were analyzed by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS). Fig. 3 shows SEM images of the surface of BC paper and BC-ox-Ag composites produced with 10 mM $AgNO_3$ (BC-ox-10Ag) and 0.1 mM $AgNO_3$ (BC-ox-0.1Ag). Fig. 3a is an image of the surface of the paper produced with BC showing a typical network structure of ribbon-shaped cellulose fibrils of about 50-70 nm wide and several micrometers long. After treatment with 10 mM $AgNO_3$ and 0.1 mM $AgNO_3$, we observed the matrix of nanofibers with randomly distributed spherical nanoparticles attached to their surface (Fig. 3c and 3e, respectively). The same SEM fields observed with the BED–C filter showed the nanoparticles brightly highlighted (Fig. 3d and 3f), suggesting that they may be made of a high atomic weight element. As shown in Fig. 3c to 3f, both BC-ox-10Ag and BC-ox-

0.1Ag composites presented nanoparticles on the BC fiber surface, unlike on the BC paper (Fig. 3a and 3b).

The composition of the generated nanoparticles was further investigated by energy dispersive X–ray spectroscopy (EDS) analysis (Fig. 4). EDS of BC-ox-10Ag and BC-ox-0.1Ag composites indicated a strong signal corresponding to silver (white arrows in Fig. 4). Peaks of hydrogen, oxygen, and carbon in the spectrum correspond to components of the molecule of cellulose. The results confirmed that the spherical nanoparticles observed in SEM images of the BC composites were made of silver.

The average size of the nanoparticles was measured, resulting in diameters of 41.4 ± 2.4 nm and 51.4 ± 2.4 nm for BC-ox-10Ag and BC-ox-0.1Ag composites, respectively. The size of the nanoparticles was uniform in both cases. BC-ox-0.1Ag composites presented larger but less abundant nanoparticles than BC-ox-10Ag composites, as observed in microscope images (Fig. 3). These results could be explained by different dynamics in the silver nanoparticle formation depending on the ratio between silver ion concentration and the carboxyl groups available (Uddin et al. 2014). During the reduction process, the lowest concentration of Ag^+ (0.1 mM $AgNO_3$) could allow the nucleation of a limited number of stable clusters of metallic silver to form the nanoparticles. The remaining dissolved silver of the surroundings, that would not reach the nucleation threshold to cluster, would be absorbed into the growing nanoparticles (Perala et al. 2013) leading to larger sizes. In contrast, in 10 mM $AgNO_3$ solutions there would be enough concentration of silver ions to form of a larger number of stable clusters, but of smaller size.

The impregnation of BC membranes with Ag nanoparticles has been previously described to provide antimicrobial activity for wound healing applications (Inoue et al. 2010; UllahWahid et al. 2016; Chun-Nan Wu et al. 2018). In these studies, BC membranes were immersed in $AgNO_3$ solutions followed by the reduction of the Ag ion and the formation of the metal particles.

352 Often, additives such as protective colloids were used to control the formation and size
353 distribution of the particles (Maneerung et al. 2008; Jalili Tabaii et al. 2018). BC membrane
354 oxidized by the TEMPO chemical system has been used as a template to form AgNPs by
355 thermal (Ifuku et al. 2009) or chemical (Jin Feng et al. 2014) reduction.

356 In agreement with the results obtained, the procedure employed here was efficient in forming
357 spherical, uniformly sized AgNPs, without the inclusion of chemical reducing agents and
358 stabilizers. This improves both the environmental aspect of the process, avoiding the
359 secondary pollutants, and the reduction of the presence of residues in the nanocomposite that
360 could interfere in its applicability, especially related to fields such as biomedicine and catalysis.
361 Moreover, in this work AgNPs were generated in a dry, stiff, enduring paper with important
362 properties for applications such as biocatalysis, biosensors, or packaging.

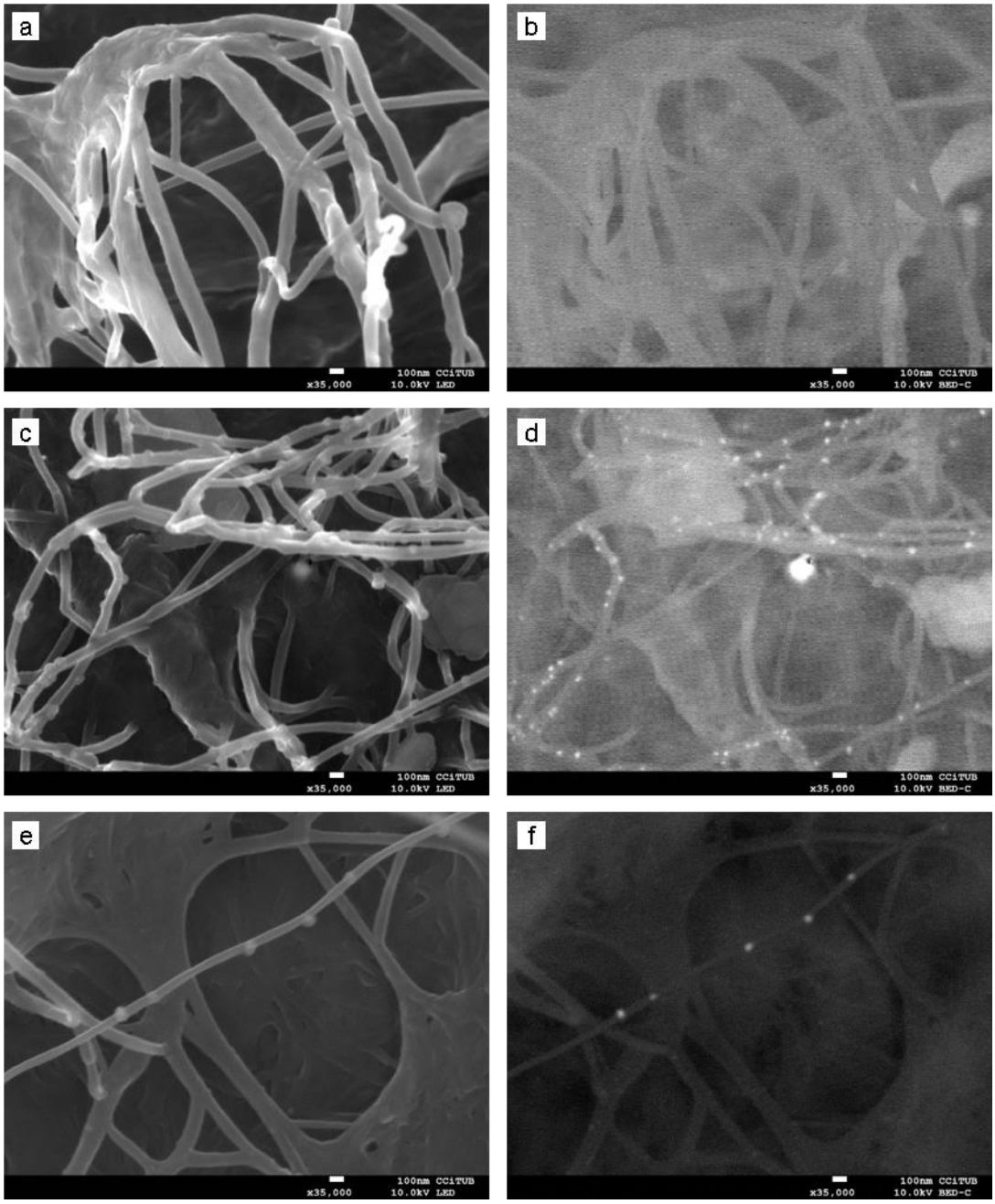

Fig. 3 SEM images of BC composites. (a) BC paper visualized with LED filter. (b) BC paper visualized with BED–C filter. (c) BC-ox-10Ag composites visualized with LED filter. (d) BC-ox-10Ag composites visualized with BED–C filter (e) BC-ox-0.1Ag composites visualized with LED filter. (f) BC-ox-0.1Ag composites visualized with BED–C filter

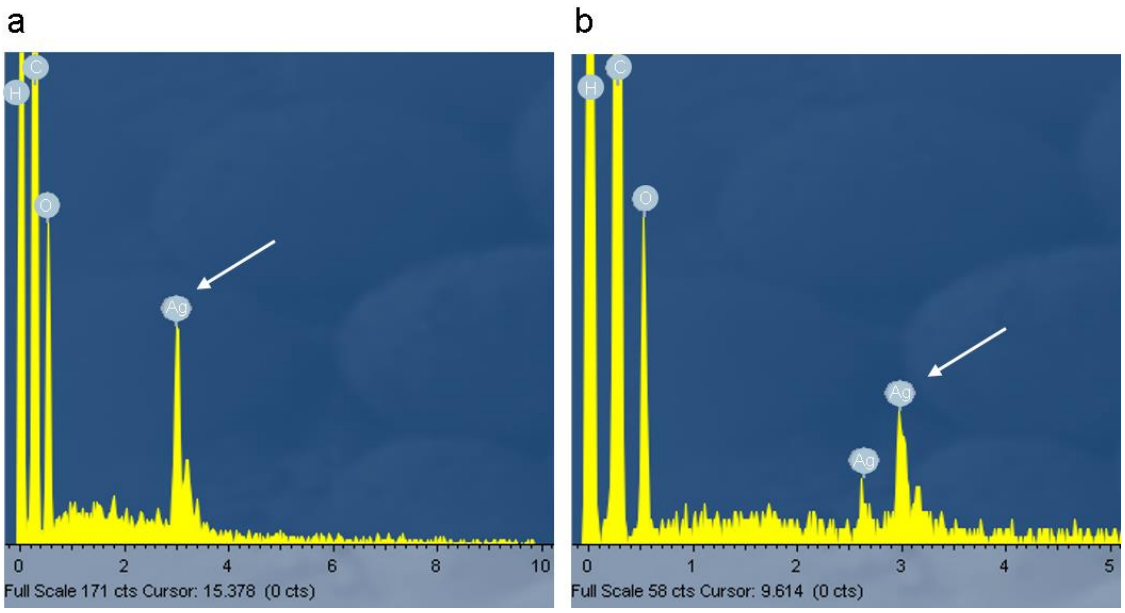

Fig. 4 Energy dispersive X–ray spectrometer (EDS) spectrum of silver nanoparticles in BC-ox-10Ag composite (a) and in BC-ox-0.1Ag composite (b). White arrows indicate the Ag peak in the spectrum

Silver release from BC-ox-Ag composites

The silver diffusion from the BC-matrix was analyzed to acquire further information regarding the properties of the composites. Composites which were not heat-treated to prevent the induction of AgNPs formation (BC-ox-10Ag-NR and BC-ox-0.1Ag-NR, in Table 2) were produced and compared with composites containing Ag nanoparticles (BC-ox-10Ag and BC-ox-0.1Ag, in Table 2). The composites were immersed and shaken in water for 24 h. Then, the silver content in the water was analyzed by inductively coupled plasma (ICP) (Table 2). Samples were analyzed both with and without the addition of HNO_3. Without the addition of HNO_3, the Ag released in form of ions was determined. The addition of HNO_3 allowed the digestion of the AgNPs to Ag ions prior to the ICP-MS analysis, thus retrieving the value of the total content of silver released by the matrix of the composites. The comparison of the values obtained for the same sample with and without the treatment with HNO_3 would allow the estimation of Ag released that was in form of nanoparticles.

386 Table 2. Silver migration from composites (ng Ag/mg composite)

Composite	Silver released in form of Ag ions	Total content of silver released
BC-ox-10Ag	4.65 ± 0.2	5.34 ± 0.3
BC-ox-10Ag-NR	16.42 ± 2.2	19.44 ± 3.7
BC-ox-0.1Ag	<0.1 (*)	0.23 ± 0.1
BC-ox-0.1Ag-NR	1.24 ± 0.2	1.92 ± 0.2

387 NR: not reduced. (*): Value below the detection limit of the method

388

389 Differences of the values comparing the silver released as ion with the total Ag release,
390 showing Table 2, were found not significant (t-Student, statistical confidence level 95 %). Thus,
391 for the composites containing AgNPs, the results indicated that most of the Ag present in the
392 analyzed samples was in the form of Ag ion, suggesting that silver was leached to the medium
393 in form of Ag ions diffused from the NPs inside the BC matrix, rather than in form of NPs.
394 Probably, the fine network of nanofibers of the BC matrix on the composite helps to stabilize
395 and to retain the NPs. Release of Ag from composites that were not submitted to heat
396 treatment, and therefore no NPs were generated (BC-ox-Ag NR), was greater than from its
397 counterpart composites (BC-ox-Ag) (Table 2), indicating that the chemical form in which the
398 silver was embedded in the BC matrix affected its diffusion into the surrounding aqueous
399 medium.

400 The fraction of silver released varied regarding the chemical form of Ag embedded in the BC
401 matrix (Fig. 5). For BC-ox-10Ag composites, 29 % of its silver content was diffused from the
402 matrix after 24 h immersed in the aqueous medium. However, the release of Ag increased to
403 94% for composites where the heat reduction treatment was not applied, indicating that the
404 reduction was necessary for the stabilization of Ag in the matrix, probably through the
405 generation of NPs.

406 Finally, we explored the importance of the BC oxidation on the silver release from the
407 composites prepared with non-oxidized BC pulp. As shown in Fig. 5, the diffusion of Ag from

non-oxidized composites (BC-10Ag) was significantly higher than that found in oxidized composites (BC-ox-10Ag). The difference could be attributed to the carboxyl groups induced by the laccase/TEMPO oxidation, which provided negative charges attracting Ag ions and promoting nucleation for NPs formation.

These results suggested that both steps, oxidation and heat treatment, were necessary to obtain BC composites containing silver nanoparticles stabilized in the matrix. The same conclusion was drawn from the results obtained using BC-ox-0.1Ag composite (data not shown). The stability of the nanoparticle inside the matrix as well as the diffusion of silver ions could have important implications for biomedical or food packaging applications (Marini et al. 2007; Kong et al. 2008; Maneerung et al. 2008).

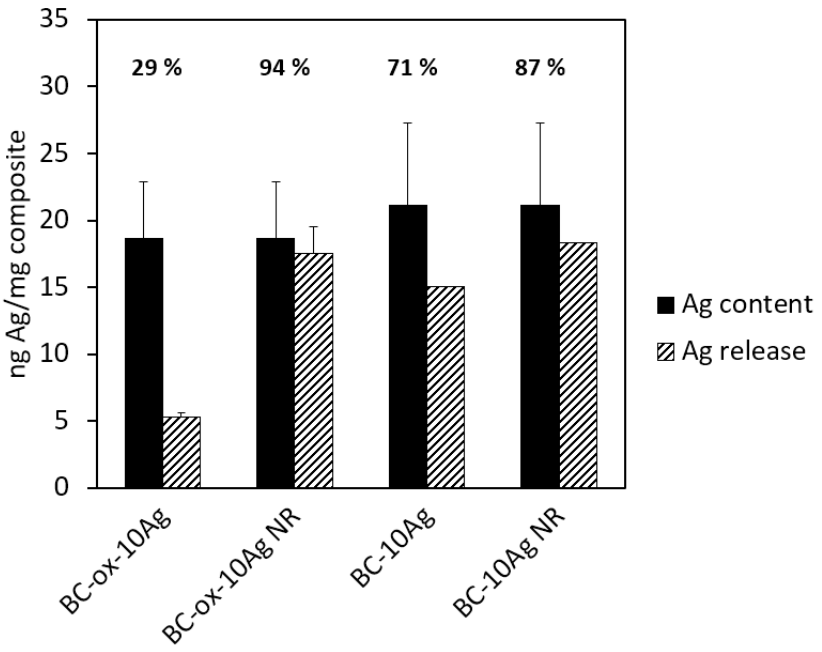

Fig. 5 Silver content and silver migration from the composites (ng Ag/mg composite). BC-ox-10Ag: composite produced with oxidized bacterial cellulose and 10 mM of silver nitrate; BC-10Ag: composite produced with non-oxidized bacterial cellulose and 10 mM of silver nitrate. NR: no thermal reduction applied. Percentages represent the fraction of silver released from each type of composite

425 **Antimicrobial properties of BC-ox-Ag nanocomposites**

426 The antimicrobial property of composites containing silver nanoparticles (BC-ox-Ag) was tested
427 against Gram-positive bacteria (*S. aureus*), Gram-negative bacteria (*P. aeruginosa*, *K.*
428 *pneumoniae*), and yeast (*C. albicans*).

429 The capability of the BC-ox-Ag composites to inhibit the microbial growth on their surface was
430 assayed by the *drop over paper test*. Microbial metabolic activity was not detected after
431 incubation in contact with BC-ox-10Ag and BC-ox-0.1Ag composites for any of the
432 microorganisms tested, while all four strains were able to grow in contact with the BC paper
433 sheet (results not shown).

434 To evaluate the bactericidal and fungicidal ability of the BC-ox-Ag composites under dynamic
435 liquid condition, suspensions of microorganisms were incubated in contact with the
436 composites. Viable cell counts were determined at different times, and the percentage of cell
437 viability reduction was calculated (Table 3). Suspensions of the microorganisms in contact with
438 samples of BC paper sheets did not experiment a decrease of viability over 24 h incubation
439 time (results not shown). The reduction of viability after one hour of incubation with BC-ox-
440 10Ag composites was over 90% for *S. aureus*, *P. aeruginosa* and *K. pneumoniae*, and complete
441 loss of bacterial viability was obtained after 4 h (Table 3). For *C. albicans*, total elimination of
442 10^6 CFU/mL was not achieved after 24 h in contact with BC-ox-10Ag composite. These results
443 demonstrated that BC-ox-10Ag composite presented strong biocidal activity against the tested
444 strains, being more effective for bacteria than for yeast.

445 BC-ox-0.1Ag composites presented antibacterial properties as well, although unevenly for
446 different types of microorganisms. Hence, contact with BC-ox-0.1Ag composites eliminated
447 Gram-negative bacteria *P. aeruginosa* and *K. pneumoniae,* and reduced the viability of the
448 Gram-positive *S. aureus* to 95 % after 24 h (Table 3). However, the viability of about 10^5
449 CFU/mL of the fungi *C. albicans* was not affected after 24 h. The differences observed between

the three types of microorganisms may be related to the structure of their cellular envelopes. Thus, to some extent, thicker cell walls, such as those of *S. aureus* and *C. albicans*, would protect the cell from the contact of silver ions with the cell membrane and their penetration into the cytoplasm (Feng et al. 2000). Evidently, BC-ox-10Ag composites presented a further pronounced antimicrobial property as they contain a larger amount of silver. BC membranes containing silver nanoparticles have previously been reported to present antimicrobial activity against *E. coli*, *S. aureus*, *K. pneumoniae* and *C. albicans* (Maneerung et al. 2008; Pinto et al. 2009; Shao et al. 2015; Jalili Tabaii et al. 2018). Silver has been used for centuries for the treatment of burns and wounds. It has been reported that silver ions bind to the thiol groups of proteins and the respiratory enzymes of the bacterial cell membrane (Liau et al. 1997; Feng et al. 2000). However, the mechanism for its antimicrobial action is not completely understood. Both silver ion and silver nanoparticles are toxic for microorganisms (Abdel-Mohsen et al. 2014), although some authors maintain that the antimicrobial effect of the nanoparticles derives from the release of silver ions (Lansdown 2006).

Table 3.Viable cell counts (CFU/mL) and cell viability reduction (%) of microorganisms in dynamic contact with BC-ox-10Ag and BC-ox-0.1Ag composites.

		BC-ox-10Ag		BC-ox-0.1Ag	
		CFU/mL	% reduction	CFU/mL	% reduction
S. aureus	t_0	$2.5 \cdot 10^7$	0	$1.3 \cdot 10^6$	0,0
	t_1	$1.2 \cdot 10^6$	95.2	$5.4 \cdot 10^5$	57.9
	t_4	0	100	$1.8 \cdot 10^5$	84.5
	t_{24}	0	100	$5.7 \cdot 10^4$	95.6
P. aeruginosa	t_0	$1.4 \cdot 10^6$	0	$3.5 \cdot 10^5$	0
	t_1	$1.3 \cdot 10^5$	90.7	$2.7 \cdot 10^5$	5.3
	t_4	0	100	$1.92 \cdot 10^5$	45.1
	t_{24}	0	100	0	100
K. pneumoniae	t_0	$6.6 \cdot 10^5$	0	$3.5 \cdot 10^6$	0
	t_1	$2 \cdot 10^3$	99.7	$2.7 \cdot 10^6$	22.8
	t_4	0	100	$2.2 \cdot 10^6$	34.9
	t_{24}	0	100	0	100
C. albicans	t_0	$1.4 \cdot 10^6$	0	$5.9 \cdot 10^4$	0
	t_1	$7.6 \cdot 10^5$	47.2	$8.4 \cdot 10^4$	0
	t_4	$8 \cdot 10^5$	44.4	$1 \cdot 10^5$	0
	t_{24}	$4.6 \cdot 10^5$	69.8	$1 \cdot 10^5$	0

BC-ox-Ag composites produced in this work presented strong antimicrobial activity due to their silver nanoparticle content. The toxic action of the nanoparticles can be exerted both by direct contact of the microorganisms with the surface of the composite, and by the release of Ag ions in aqueous conditions. The composites suitability to inhibit the microbial growth in their surfaces as well as to eliminate bacteria and fungi in aqueous surroundings is a fundamental aspect to consider for future applications.

Conclusions

In this work the production of BC and Ag nanoparticles composites with paper mechanical features and excellent barrier properties was achieved. Carboxyl groups induced by the Laccase/TEMPO oxidation of BC nanofibers enabled the interaction with Ag ions and the generation of silver nanoparticles after thermal induction. BC matrix allowed the stabilization of evenly sized and shaped nanoparticles. Composites had antimicrobial activity, showing great capability to both inhibit growth and kill Gram-positive bacteria, Gram-negative bacteria, and fungi. It is foreseeable that composites with nanoparticles of other metals can be obtained by following the same method described here. BC paper composites containing metal nanoparticles could be employed in catalytic, magnetic, conductive, and biomedical applications.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Fig1

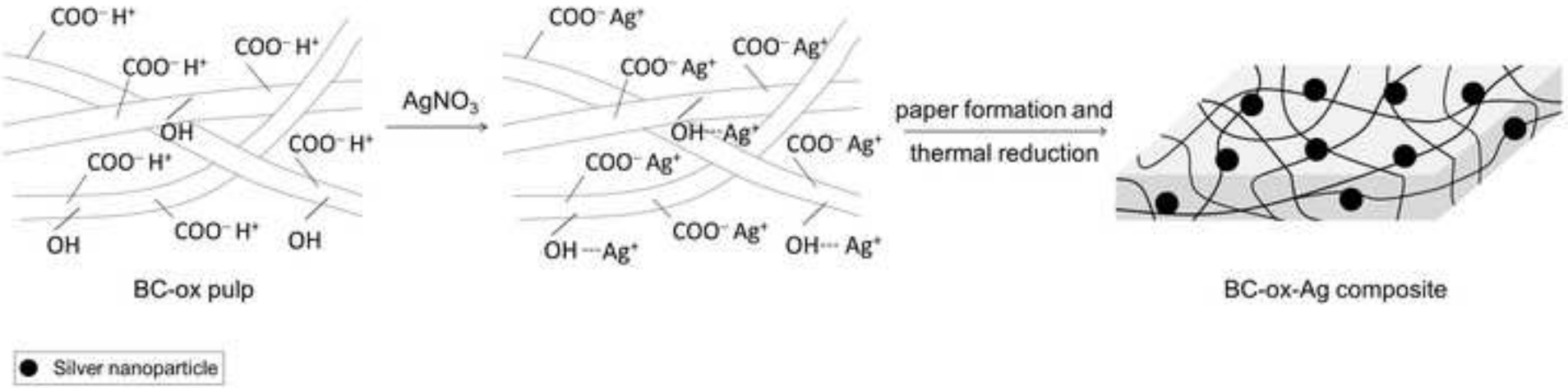

Fig 2

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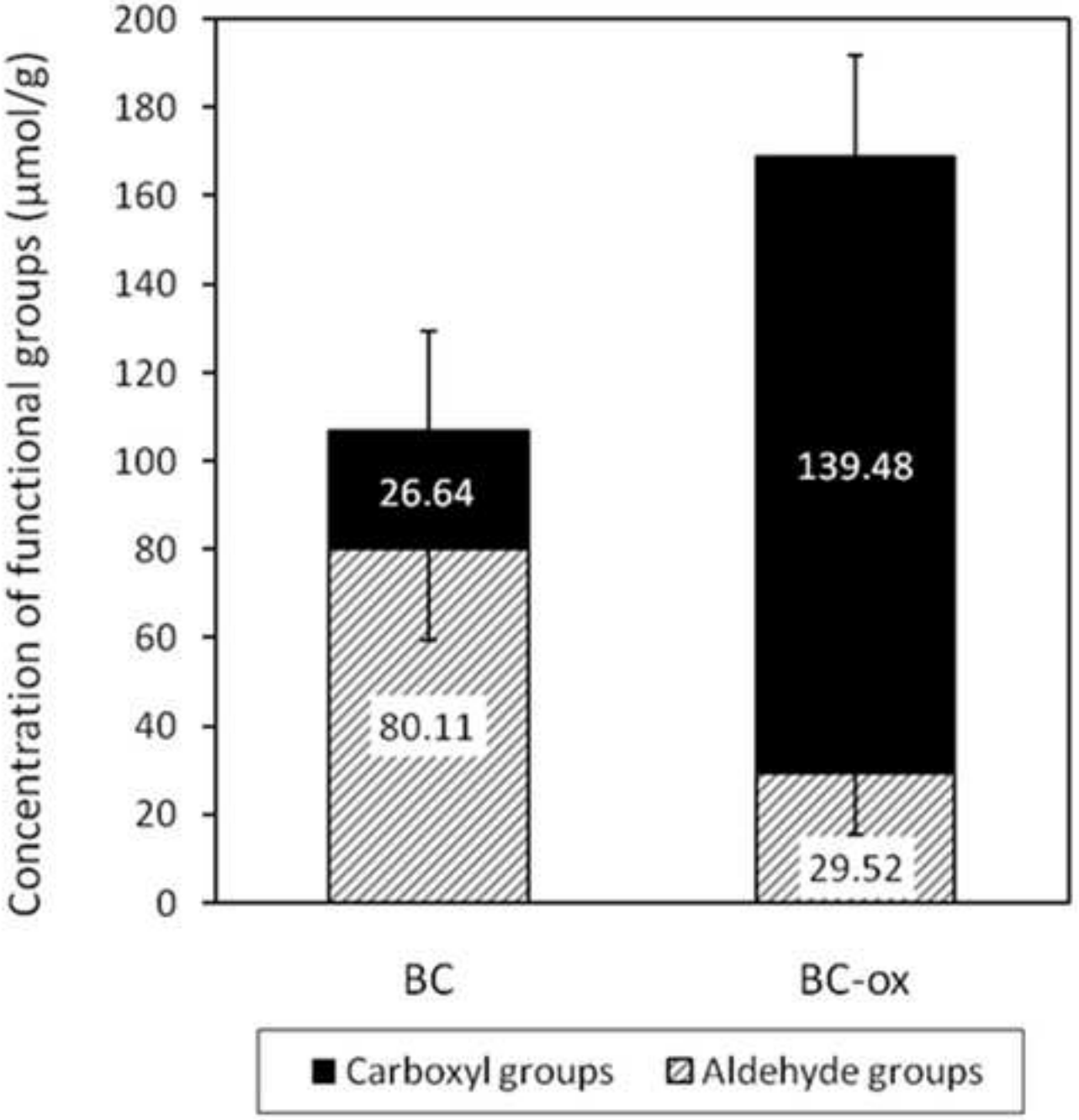

Fig 3

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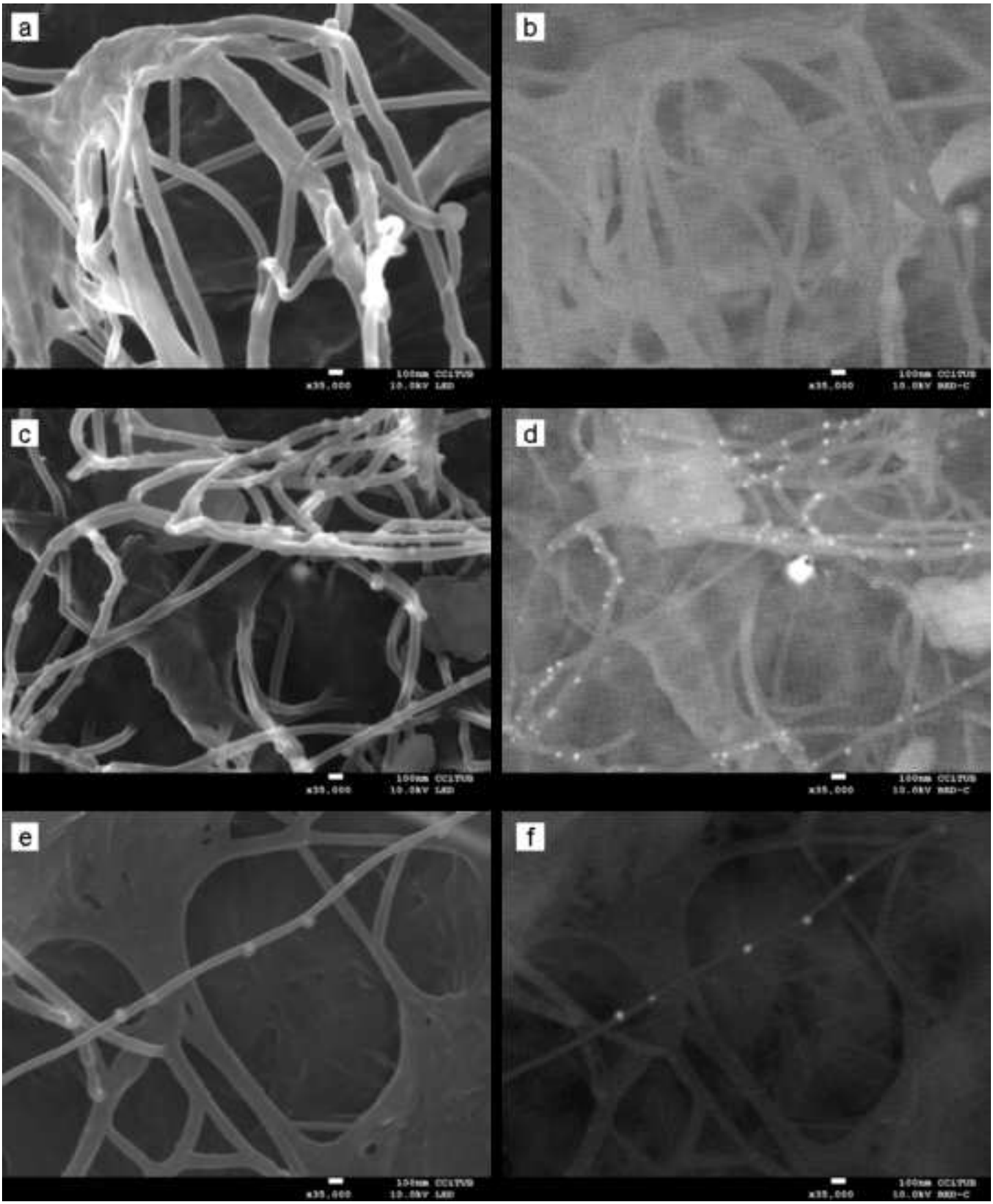


Fig 4

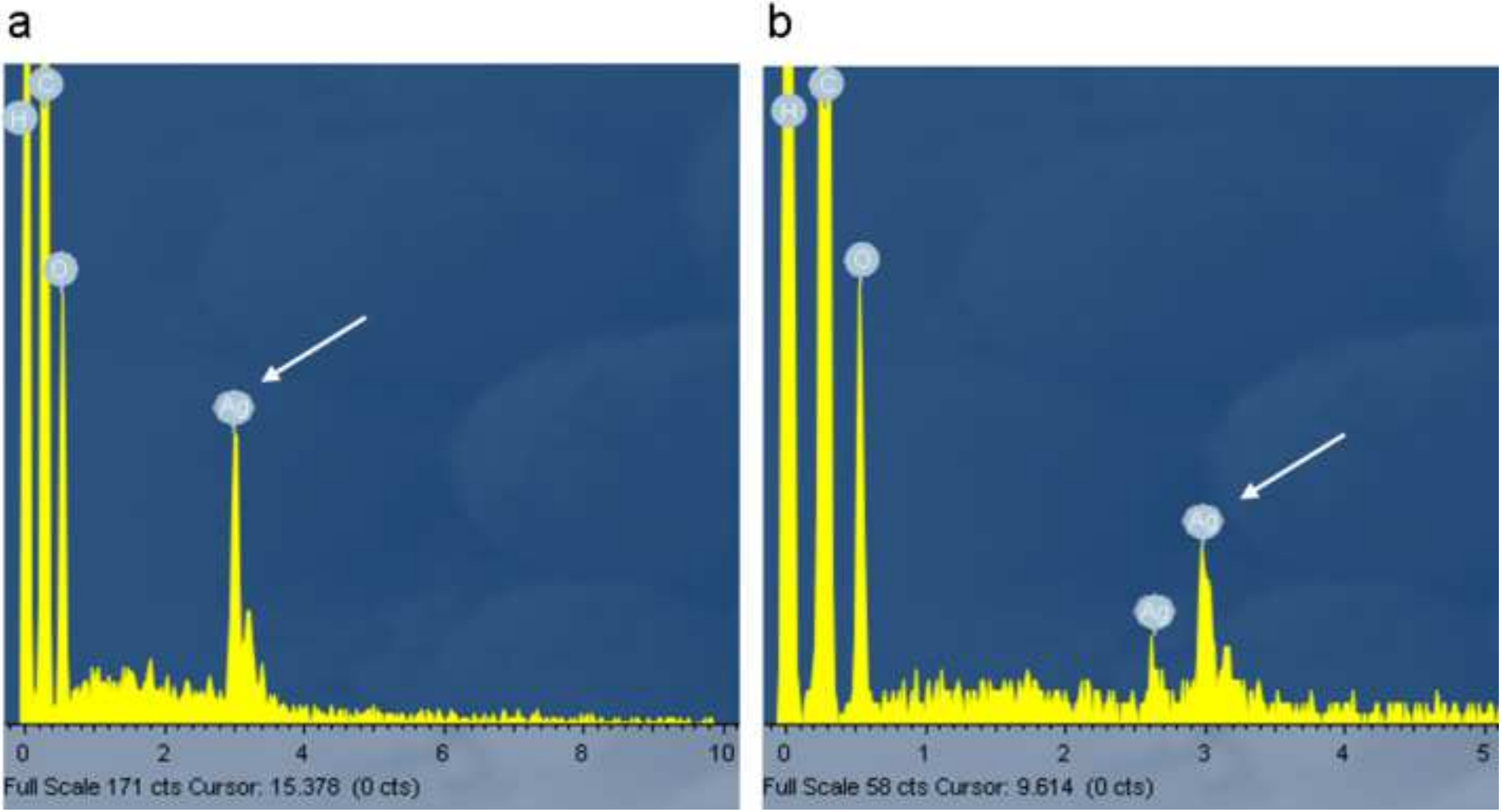
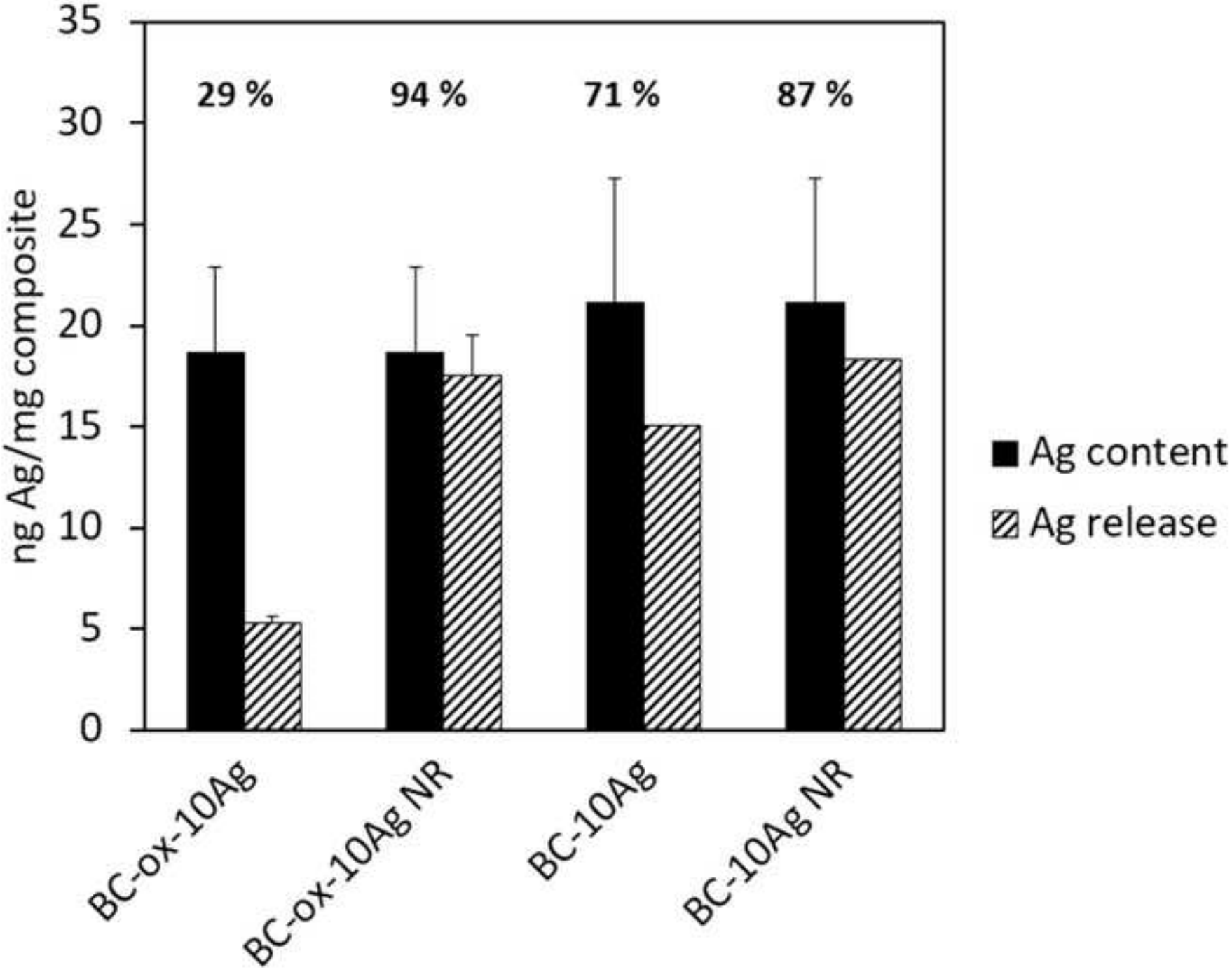


Fig 5



Graphical Abstract