Engineering in Life Sciences

Eng. Life Sci. **2017**, *17*, 1108–1117

www.els-journal.com

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Research Article

Ultrasound-assisted swelling of bacterial cellulose

Bacterial cellulose (BC) was obtained by static cultivation using commercial BC gel from scoby. BC membranes (oven dried and freeze-dried) were swelled with 8% NaOH, in the absence and in the presence of ultrasound (US), for 30, 60, and 90 min. The influence of swelling conditions on both physico-chemical properties and molecules entrapment was evaluated. Considering the highest levels of entrapment, an optimum swelling procedure was established: 8% NaOH for 30 min at room temperature in the presence of US. Native and PEGylated laccase from *Myceliophthora thermophila* was immobilized on BC membranes and a different catalytic behaviour was observed after immobilization. Native laccase presented activity values similar to published reports (5–7 U/gBC) after immobilization whereas PEGylated enzymes showed much lower activity (1–2 U/gBC). BC swelled membranes are presented herein as a potential support for the preparation of immobilized enzymes for industrial applications, like phenolics polymerization.

Keywords: Bacterial cellulose / PEG-FITC / Polymerization / Swelling / Ultrasound

Received: April 24, 2017; *revised:* June 19, 2017; *accepted:* July 13, 2017

DOI: 10.1002/elsc.201700085

1 Introduction

Bacterial cellulose (BC) is a promising natural polymer synthesized by acetic bacterium like *Aerobacter*, *Achromobacter*, *Gluconacetobacter xylinus* (*G. xylinum*) with unique structural, physical and chemical properties [1–3]. SCOBY, a symbiotic culture of bacteria and yeasts, has been receiving special attention as a by-product to produce BC. It is obtained from Kombucha fermentation and is comprised of acetic acid producing bacteria, mostly *Acetobacter xylinum,* ethanol fermenting yeasts, and a thick cellulose pellicle [2–6]. The chemical structure of BC is similar to the plant cellulose, with a significantly different fibrous nanostructure which confers it superior physical and chemical properties. It possesses excellent elasticity, biocompatibility, high purity, good mechanical properties, high hydrophilic nature, and liquid loading capacity [1]. These superior properties have made possible their use in a vast number of applications such as paper, textiles, pharmaceutical, and biomedical fields [3–6]. BC is an interesting material for use as a wound dressing as it provides a moist environment to a wound, resulting in better wound healing. Although BC itself has no antimicrobial activity that prevents wound infectivity, it can be attained by imparting silver nanoparticles into BC by immersing BC in silver nitrate solution [7]. Implantable biomaterials (*i.e.*, scaffolds) are also required; a novel approach has been undertaken to apply cellulose as a material entirely integrated into the body, as either bone or skin graft [8]. BC is one of the new biosorbents that is mostly abundant in nature, showing renewable, biodegradable, and biocompatible qualities. It has been used as bioadsorbent for dye decolorization as reported by Mohite and Patil, 2013 [4]. They used BC produced by *Gluconoacetobacter hansenii* to decolorize Aniline blue and obtained decolorization yields of up to 90% of 100 mg/L concentrations in 10 min at pH 7. Due to its high crystallinity, high water holding capacity, high porosity, and mechanical strength BC has also potential as bioadsorbent, offering application on heavy metal removal [9].

To date, BC has also received considerable attention as a novel cellulosic support material for enzyme immobilization. The nanostructured network of BC is expected to easily entrap enzyme molecules and, due to its large surface area, high mechanical strength, and porosity to promote high protein loading [3–6]. The three-dimensional porous structure of BC provides also high accessibility onto the active site through low diffusion resistance of the substrate [10–12]. BC structures are considered excellent supports for enzyme immobilization since they can enhance enzyme stability against pH and temperature changes [11].

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Abbreviation: BC, bacterial cellulose

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Different methodologies can be used for an effective enzyme immobilization on BC, namely physical adsorption [11, 12], entrapment, covalent binding or cross linking [13–17]. Wu et al. [15] investigated the conditions of glucoamylase immobilization on BC beads, using various immobilization techniques. The results showed that the epoxy method with glutaraldehyde coupling was the best method. To date only few works have reported the immobilization of laccases onto BC by physical entrapment techniques. Frazão et al. [18] used BC membranes produced by *Gluconacetobacter sacchari* to immobilize laccase from *Myceliophtora thermophila*. The immobilized laccase showed high operational stability, since it retained 86% of its initial activity after seven consecutive biocatalytic cycles of reaction with 2,2_-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid). Sampaio et al. studied the physical immobilization of a commercial laccase on bacterial nanocellulose (BNC) aiming to identify the laccase antibacterial properties suitable for wound dressings [11]. BC has been suggested to have a cage-like structure, which can efficiently entrap the enzymes with no need of covalent binding nor the use of chemical crosslinkers, like glutaraldehyde. Among the different enzymes that have been immobilized onto these supports, special attention has been devoted to laccase. It is a multicoper oxidase with low specificity that catalyzes the removal of a hydrogen atom from the hydroxyl group of methoxy-substituted monophenols, ortho- and para-diphenols, and also can oxidize other substrates such as aromatic amines, syringaldaine and non-phenolic compounds to form free radicals. They are also known catalyse reactions that lead to the generation of antimicrobial species [11].

The main aim of this study was to investigate the role of ultrasound on the swelling of BC, to improve molecules entrapment and laccase immobilization. The BC membranes were produced using commercial scoby (tea fungus) in Hestrin & Schramn (HS) medium. The medium cultivation conditions were optimized to obtain high production yields at the lowest processing costs. After production and bleaching, the BC samples were swelled under different conditions (time, ultrasound, temperature and state of samples). The influence of swelling was further studied during fluorescein-polyethylene glycol (PEG) entrapment and enzyme immobilization. Swelling process is known to play an important role on the enzymes entrapment and immobilization since it is mainly related to the network elasticity, to the extent porosity of the polymer and to the large water uptake capacity to absorb enzyme molecules [1–3]. An enhancement of the microfibrils and porous network of BC would increase its ability for enzyme molecules entrapment. Moreover, the increase of available hydroxyl groups on the surface of nanocellulose would promote an higher enzyme immobilization degree via chemical bonding and/or electrostatic adsorption. Swelled and non swelled BC samples were characterized by scanning electron microscopy (SEM), and Fourier transform infrared (FT-IR). The intrinsic properties of BC, namely mechanical strength, tridimensional nanostructure, high purity and increased water absorption make this material the ideal candidate for enzyme immobilization. Moreover, the hydroxyl groups available at the surface of BC will provide the enzyme immobilization via chemical bonds and electrostatic adsorption. Enzyme immobilization studies on BC samples were performed with native and chemically PEGylated laccase from *Myceliophthora thermophila*, being the enzyme activity evaluated after immobilization. This evaluation was undertaken by ABTS and catechol oxidation.

2 Materials and methods

2.1 Materials and equipment

Commercially available BC gel from scoby, tea fungus KOMBUCHA GET was obtained from Culver, CA, USA. Glucose and peptone were obtained from Merck Co. Ltd., Germany while yeast extract was obtained from Sigma Chemical Co (St. Louis, Mo, U.S.A.). Analytical grade sodium hydroxide hydrate pellet and sodium chloride was purchased from Sigma Chemical Co (St. Louis, Mo, U.S.A.). Analytical grade glacial acetic acid was purchased from Fisher chemical (Fair Lawn, N.J, U.S.A.). Methoxyl PEG fluorescein (5 kDa) was purchased from Nanocs Inc. (N.Y, USA). Phosphate buffer solution of pH 8.0 was prepared using potassium dihydrogen phosphate (KH_2PO_4) and sodium hydroxide (NaOH) from Sigma Chemical Co (St. Louis, Mo, U.S.A.). Commercial laccase (Novozym 51003) (EC 1.10.3.2) from *Myceliophthora thermophila* was obtained from Novozymes (Bagsveard, Denmark). 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), MES (2-[morpholino]ethanesulfonic acid), EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide), and Sulfo-NHS (N hydroxysulfosuccinimide) and bifunctional polymers such as poly(ethylene glycol) bis (amine) and α,ω-bis{2-[(3-carboxy-1-oxopropyl)amino]ethyl}polyethylene glycol were also purchased from Sigma Chemical Co (St. Louis, Mo, U.S.A.). Catechol was purchased from Sigma Chemical Co (St. Louis, Mo, U.S.A.).

Static cultivation of BC was conducted in an incubator (SI-600R, JEIO TECH Co., Daejeon, Korea). The swelling of BC was performed using an ultrasonic bath (VWR USC 200TH, VWR International, Malaysia) or an orbital agitator (AG-200-A, optic ivy men system, Comecta, S.A). The absorbance was monitored using UV-visible spectrophotometry (Multimode Microplate Reader Synergy™ Mx and Gen5™, BioTek, Instruments, Winooski, VT, U.S.A.). Fourier Transform Infrared Spectroscopy (FT-IR) analysis was carried out using a FT/IR-670 Plus spectrophotometer (Jasco International Co. Ltd., Tokyo, Japan). The internal morphology of BC samples was examined by scanning electron microscope (SEM, JSM-7600F, JEOL Korea Ltd., Japan).

2.2 Production of bacterial cellulose

The bacterial cellulose was produced using HS medium composed of: 20 g/L glucose, 5 g/L yeast extract, and 3 g/L peptone [10, 11]. Commercial BC gel from scoby was added to the culture medium in a liquor ratio of 1:5 (v/w). Static cultivation was conducted in the stationary phase at 26°C for eight days in an incubator (SI-600R, JEIO TECH Co., Daejeon, Korea). After cultivation, the BC was washed with distilled water at 100°C for 5 min. Afterwards the BC was bleached with 10% (v/v) of H_2O_2 solution at 90°C for 60 min in a water bath (110 rpm).

2.3 Swelling of bacterial cellulose

The BC was swelled with 8% of NaOH solution in a liquor ratio of 1:10 (v/w). The swelling was performed in samples at different conditions, wet and freeze-dried, in the absence and in presence of US. The ultrasonication was conducted in a sonicator bath at a fixed frequency 45 kHz and power intensity at 120 W. The samples swelled in absence of US were incubated in an orbital agitator of 50 rpm at room temperature. The effect of US and swelling time on the swelling degree was investigated for 30, 60, and 90 min.

2.4 Entrapment studies with methoxy-poly (ethylene glycol) fluorescein (PEG-FITC)

The swelling effects was evaluated by entrapment studies with methoxy-PEG fluorescein. PEG-FITC is a coloured and fluorescent compounds which allow to monitor its spectral behaviour during entrapment processing. After swelling, the pH solutions containing the BC samples were adjusted to pH 8.0 by acetic acid addition. Then, BC samples were immersed in PEG-FITC solution (0.5 g/L) at room temperature for 120 min in an orbital agitator (50 rpm). Afterwards, the pH value of the solutions was adjusted to pH 4.0 by addition of acetic acid in order to close the BC structure and retain the highest amount of PEG entrapped. The monitoring was performed using UV-visible spectrophotometry in the wavelength range of 300 to 700 nm. The percentage of entrapped PEG-FITC was calculated according to the Eq. (1):

$$\%\ \text{PEG} - \text{FITC entrapped} = \frac{(A_i - A_f)}{A_i} \times 100 \qquad (1)$$

where A_i is the absorbance (420 nm) of the initial PEG-FITC solution and A_f is the residual absorbance of PEG-FITC solution in the supernatant after entrapment.

2.5 Laccase PEGylation

Laccase was PEGylated with two bifunctional PEGs: α, ω-Bis {2-[(3-carboxy-1-oxopropyl) amino] ethyl} polyethylene glycol and poly (ethylene glycol) bis (amine), according to the procedure described by Lomant et al. [19]. After PEGylation the enzymes were named along the article by: Lac-PEG-COOH and Lac-PEG-NH2, respectively.

2.6 Laccase immobilization

Laccase was immobilized on BC swelled samples after two pretreatments, namely drying for 2 h at 50°C and freeze-drying under vacuum for 24 h. Afterwards, BC was cut into small pieces (2 cm^2) and immersed in three different laccase solutions; (i) 10 mL of native laccase in acetate buffer (pH 4.0), (ii) 10 mL of Lac-PEG-COOH in acetate buffer (pH 4.0), and (iii) 10 mL of Lac-PEG-NH_2 in acetate buffer (pH 4.0); the immobilization proceed at two different temperatures, room temperature and 4°C for 12 h [11].

2.7 Laccase activity

The laccase activity was measured using ABTS (εABTS = 3600 M^{-1} cm^{-1}) as substrate. One unit (U) of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of used substrate per minute [18]. One milliliter of reaction mixture (enzyme diluted in acetate buffer, 0.5 mM) was added to 1mL of ABTS (0.5 mM) and incubated at 50°C and measurments are acquired every minute until 1o min of final incubation and analyzed at 420 nm.

The activity of laccase immobilized on BC was measured by incubating the BC-containing laccase samples with 3 mL of ABTS solution (0.5 mM) at 50°C for 60 min. Samples were taken every 10 min and the absorbance was measured by UV-visible spectrophotometry at 420 nm. The activity of immobilized laccase was calculated using the following Eq. (2).

$$\text{Activity of immobilized laccase}\left(U/g_{BC}\right) = \frac{k \times \text{Vtotal} \times 10^6}{M_{BC} \times \varepsilon} \qquad (2)$$

The pratical application was used to evaluate the activity of immobilized native laccase. For this, after immobilization of native laccase (as previously described), we proceed with the polymerization of a phenolic compound, catechol (20 mM) in acetate buffer pH = 5; 50°C, for 120 min; and evaluated the enzyme activity as spectral values (K/S) change quantification after 120 min of incubation.

2.8 In situ catechol oxidation by laccase immobilized on BC

Native laccase immobilized onto BC supports was used as catalyst for the oxidation of a phenolic compound, catechol. For this 2∗2 cm of BC sample containing native laccase immobilized at 25°C were incubated with 20 mM of catechol (5 mL) at 50°C for 120 min. Afterwards, the samples were washed with water until no polymer is detected in the washing water. The in situ oxidation of catechol was evaluated by means of spectral value measurement (K/S).

2.9 Characterization of bacterial cellulose

2.9.1 Determination of swelling degree

To evaluate the swelling behaviour of BC, a known weight of BC (1.05 ± 0.15 g) was immersed in the swelling solution described above in Section 2.4 using a liquor ratio of 1:10 (v/w) for different periods of time (30, 60, and 90 min) at room temperature. BC was then separated from the swelling solution and wiped with filter paper to remove the free water at the surface; subsequently, the wet mass was determined. The swelling mass (SM) of the BC was calculated as the difference between the mass of BC gels after swelling and the dry mass of BC [20,21].

2.9.2 Fourier Transform Infrared Spectroscopy–(FTIR)

The chemical structure of BC samples was analysed before and after swelling process and after PEG-FITC entrapment by FT-IR using a FT/IR-670 Plus spectrophotometer. Scans were completed between 4000 and 450 cm^{-1} at a resolution of 4 cm^{-1}. Baselines for each sample spectrum were normalized using spectrum software [22, 23].

2.9.3 Scanning electron microscopy (SEM)

The internal morphology of BC samples was examined by SEM. Prior to SEM observation the BC samples were freeze-dried under vacuum conditions. For SEM analysis, the samples were coated with an ultrathin layer of gold in an ion sputter and scanned at different points using an electron microscope model LEICA S360 at × 5000 and × 10 000 magnifications.

2.9.4 Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was carried out in a Perkin Elmer, TGA 4000 equipment. All the analyses were performed with a 5 mg sample in aluminium pans under a dynamic nitrogen atmosphere between 30 and 800°C. The experiments were run at a scanning rate of 20 K/min.

3 Results and discussion

3.1 Effect of ultrasound and swelling time

3.1.1 Bacterial cellulose structural analysis

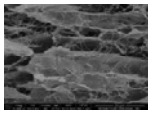
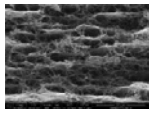
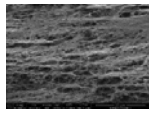
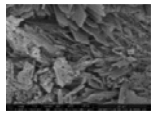
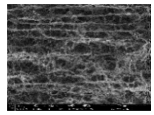
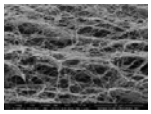
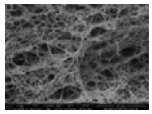
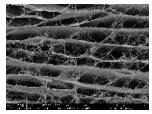
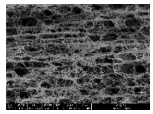
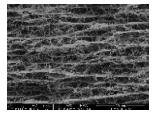
Cellulose biosynthesis is characterized by unidirectional growth and crystallization, where glucose molecules are linked by β(1-4)-glycosidic bonds. The union of glycosidic chains forms oriented micro fibrils with intramolecular hydrogen bonds. The growth mechanism of the cellulose polymer depends on the microorganism of cultivation and determines the morphology of the final cellulose [24].

The structural analysis of swelled samples cross section was analysed by SEM and the images evidence a well-organized three-dimensional network structure (Table 1). BC samples show a porous morphology in both states, oven dried and freeze-dried, evidencing a more organized structure in the oven dried state. The polyglycosidic chains of BC produced by bacteria are surrounded by layers of water molecules forming a soft gel which is stabilized by extensive hydrogen bonding. When this gel undergoes dehydration through water evaporation, it is converted into a "film" by irreversible formation of new hydrogen bonds between cellulose hydroxyl groups [25]. It would be expected therefore that freeze-dried samples present a differently organized structure with less interfibrilar spaces. The cross section analysis of swelled samples also demonstrated that longer time of swelling increases this disorganization (Table 1), increasing also the amount of swelling agent between pores, hindering posterior entrapment and immobilization procedures.

BC samples were also analysed by FTIR before and after swelling and after PEG-FITC entrapment. As shown in Fig. 1, the native BC presents the characteristic cellulose peaks at around 3300, 2880, 1640, and 1057 cm^{-1} [18, 25]. The broad and strong band at 3300 cm^{-1} [18] corresponds to the stretching vibration of the hydroxyl groups and N-H stretching vibrations [25, 26]. The peaks at 2880 and 1400 cm^{-1} are attributed to aliphatic C-H stretching vibration [26]. The spectrum shows also a strong band at around 1057 cm^{-1} [8], which corresponds to C-O-C and C-O-H of cellulose [8, 22, 23]. After BC swelling (8% NaOH for 30 min in the presence of ultrasound) FTIR spectra evidences significant modifications. The absorption intensity of the band at 3300 cm^{-1} assigned to O-H stretching vibration was sharply increased. It indicates an increase of the degree of intramolecular hydrogen bonding, resulting from increase of the hydroxyl groups. The bands at 1400, and 2880 cm^{-1} originated from the C-H stretching are enhanced due to the absorbed water and are described as related with the crystallinity of the BC [24]. The alkaline character of the swelling solutions promotes the hydrolysis of the cellulosic fibril which is detectable by an increase of the band intensity at ~1630 cm^{-1} band attributed to the presence of carboxylic groups and by another sharp peak appearing at 1400 cm^{-1} (C-H vibration).These results indicated that NaOH is the primary agent in cleaving the inter-and intra-hydrogen bonds in BC [8].

FTIR of swelled samples after PEG-FITC entrapment was also performed. After PEG-FITC entrapment, slight shift of the peak positions and relative intensities may be attributed to the amount of groups available on PEG-FITC [22]. Absorbance peaks at wave numbers 3300, and 2880 cm^{-1}, attributed to O–H and

Table 1. SEM images of BC samples after swelling under different conditions: Original BC; (A) swelling with 8% of NaOH solution for 30 min at room temperature in the presence of ultrasound; (B) swelling with 8% of NaOH solution for 30 min at room temperature using orbital agitation (50 rpm); (C) swelling with 8% of NaOH solution for 60 min at room temperature in the presence of ultrasound; (D) swelling 8% of NaOH solution for 90 min at room temperature in the presence of ultrasound (x5000 magnification)

	Original	A	B	C	D
Dry in oven					
Freeze-dry					

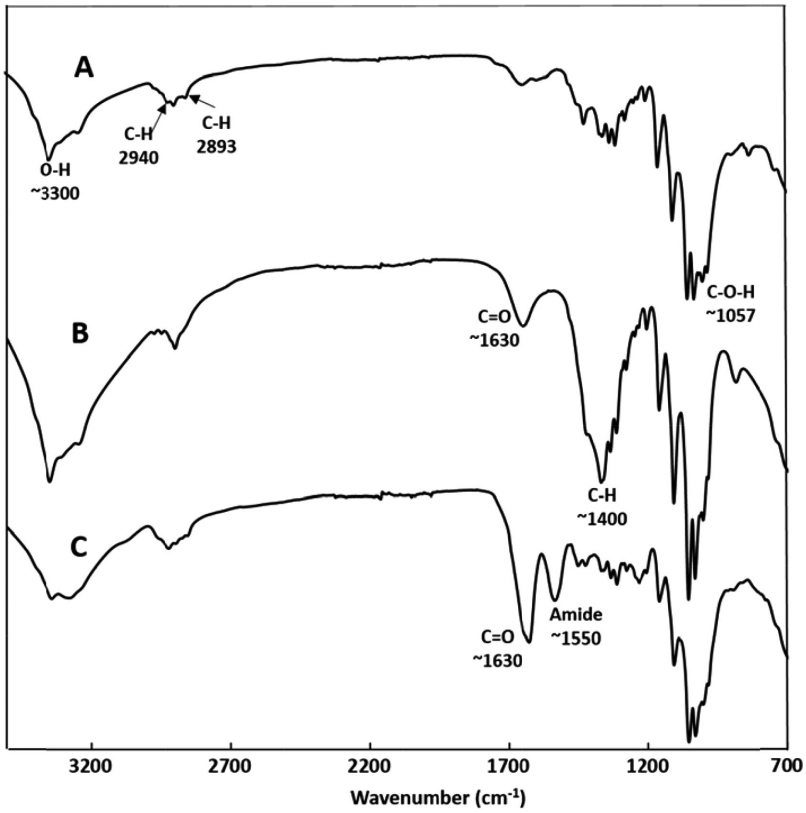


Figure 1. FTIR analysis of (A) Native BC samples; (B) BC after swelling (8% of NaOH for 30 min at room temperature in the presence of ultrasound) and (C) BC after PEG-FITC entrapment (0.5 mg/ml of PEG-FITC for 180 min at room temperature under orbital agitation).

C-H stretching vibrations respectively, became much broad. Absorbance peak at 1057 cm^{-1}, which was due to the presence of C–O–C stretching vibrations, present the same behaviour (Fig. 1). This is due to the increased intermolecular hydrogen bonding between BC and PEG, which possesses hydroxyl groups. An intensification of the band at around 1550 cm^{-1} corresponding to the amide linkages between PEG and FTIC confirm the presence of this conjugate in BC. The band intensification at 1630 cm^{-1} also indicates the presence of carboxylic and ketone groups from the aromatic nucleus of FITC. The frequency difference between the stretching bonds of BC and BC/PEG conjugate indicates that the intermolecular interaction occurred by hydrogen bonding through hydroxyl groups of BC and carbonyl or hydroxyl groups of PEG-FITC [22, 25].

Table 2. Swelling mass (SM) (g) and percentage of PEG entrapped of BC samples swelled in different conditions: (A) swelling with 8% of NaOH solution for 30 min at room temperature in the presence of ultrasound; (B) swelling with 8% of NaOH solution for 30 min at room temperature using orbital agitation (50 rpm); (C) swelling with 8% of NaOH solution for 60 min at room temperature in the presence of ultrasound; (D) swelling 8% of NaOH solution for 90 min at room temperature in the presence of ultrasound

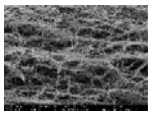
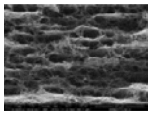
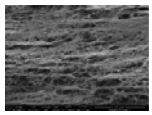
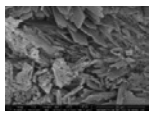
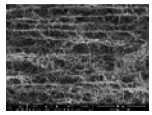
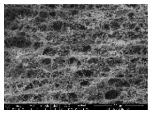
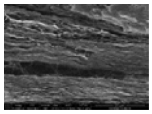
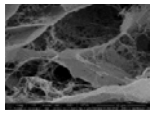
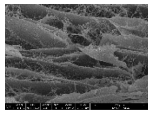
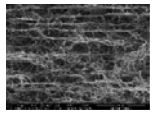
Samples	Swelling mass (SM) (g)	PEG entrapment (%)
A	0.264	49.74
B	0.179	22.66
C	0.25	41.44
D	0.06	4.66

3.1.2 Swelling degree and PEG-FITC entrapment

The effect of US on the swelling degree of BC samples was evaluated as the mass of water adsorbed by the samples, as shown in Table 2. The results demonstrate a swelling degree 1.5 fold higher for the samples swelled in the presence of US. Other researchers have already studied the influence of US on the properties of the swelled BC and observed that ultrasonication can result in significant changes in the hydrogen bonding network indicating changes in the physical properties of cellulose such as solubility and reactivity [27]. Even more, there were observed small variations among the crystallite sizes of samples. Ultrasonication resulted in the formation of a direct or closer contact between the cellulose fibrils and in the formation of strong intra- and interfibrillar hydrogen bonds [27]. Our results corroborate these assumptions, since high amount of water is adsorbed by the swollen BC samples in the presence of US (Table 2).

SEM microphotographs were taken after swelling in the presence and absence of US as shown in Table 3. As can be depicted native BC presents the characteristic original morphology revealing a porous fibrous structure. After swelling in the presence of ultrasound, a different morphology is observed. The porous-like structure gives rise to an uniform fibrillation revealing an ultrafine nanofiber network structure [26, 27]. On the other hand, swelled BC in the absence of US present a closed porous structure without evidenced nano fibrillation.

Table 3. SEM micrographs of BC after swelling and PEG entrapment at different conditions: original BC; (A) swelling with 8% of NaOH solution for 30 min at room temperature in the presence of ultrasound; (B) swelling with 8% of NaOH solution for 30 min at room temperature using orbital agitation (50 rpm); (C) swelling with 8% of NaOH solution for 60 min at room temperature in the presence of ultrasound; (D) swelling 8% of NaOH solution for 90 min at room temperature in the presence of ultrasound

	Original Non swelled	A	B	C	D
Before PEG-FTIC entrapment					
After PEG-FITC entrapment					

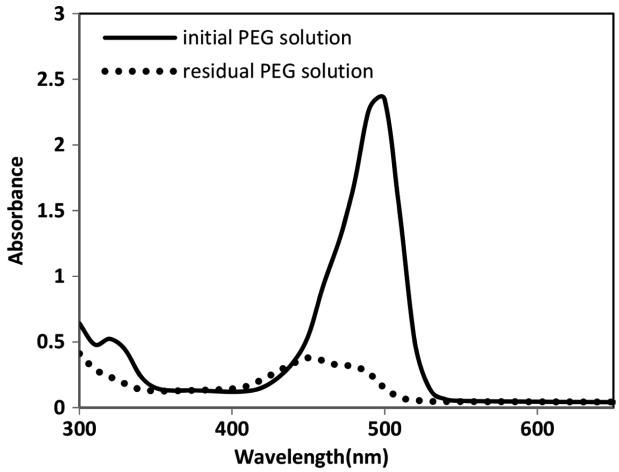


Figure 2. Visible spectra of solutions of PEG-FITC solutions (-) before and (...) after entrapment.

The percentage of PEG-FITC entrapment was calculated according to the absorbance of the entrapment solutions before and after entrapment. As shown in Fig. 2 the maximum absorption peak of initial PEG-FITC solution was observed at around 500 nm. After PEG-FITC entrapment, the maximum wavelength suffers a small shift to 450 nm and the intensity decrease significantly indicating a high amount of PEG transfer from solution to the inlayers of BC which remained entrapped after five washings. The nanofibrous network of BC provides enough nucleation sites as enabling the penetration of macromolecules like PEG into its large porous and surface area (Tables 3 and 4).

Data from Table 2 reveal a direct relation between swelling degree and the level of polymer entrapped. The highest entrapment was achieved on samples with the highest swelling degree. As expected, samples presenting lower swelling degree showed very low levels of entrapment. These results can be explained by the structural changes of BC samples during swelling (Table 3). After 30 min of swelling, BC became porous nano fibrillated structures. BC ribbon-shaped fibrils can assemble together forming porous structure with high aspect ratio [1, 4]. Contrarily, BC samples swelled for prolonged periods of time present aggregation of the fibrous structure as the time of swelling increase. These results corroborate the work reported by Wong et al. 2009 [28], where prolonged swelling resulted in the excessive reduction of cellulose crystallinity, leading to particles aggregation maybe due to the excessive formation of hydrogen bonds arising from the large number of hydroxyl groups [26]. Li et al. (2012) [29] also reported that short swelling times in the presence of US increased the possibility of the single cellulose fibres to react with the micro bubbles generated by the ultrasonication process. Moreover, high time of contact between the swelling agent and BC samples can be responsible for the fibrils degradation, destroying the inter- and intra-hydrogen bonds of cellulose molecules. This would contribute to the collapse of the pores hindering therefore the interaction of the hydroxyl groups of BC with PEG [24, 30].

After immersed in PEG-FITC solutions, BC fibrils are covered with PEG-FITC. The polymer fills the interfibrillar spaces forming a well-defined layer. A visual analysis to predict polymer entrapment was possible due to the use of fluorescein marked PEG. By photographs analysis depicted in Table 4 one can confirm the level of entrapment previously calculated (Table 2). The colour is much stronger on samples with higher PEG-FTIC entrapment.

Thermo-gravimetric analysis (TGA) measures the weight with the increase of temperature providing an understanding of the thermal samples decomposition behaviour (Fig. 3). We investigated the thermal stability and thermal decomposition of BC samples after swelling under the optimized conditions and after PEG-FITC entrapment. The TGA curves obtained by plotting percentage of weight loss against temperature. The results indicate that samples were stable up to a temperature of 250°C with the exception of samples with PEG entrapped. In this case, the samples presented two distinct decomposition events at the beginning of the cycle (30°C) and at 400°C. The percentage of weight loss was of 69, 35, 49, and 69% for original, swelled for 30, 60 and 90 min samples, respectively. The sample with PEG entrapped present a weight loss of 56%. The thermal degradation temperature is affected by the structural parameters like molecular weight, crystallinity and orientation. It is expectable a slight decrease of samples crystallinity after swelling and PEG-FITC entrapment. This theoretical assumption is corroborated by the TGA results. The swelled samples present lower decomposition temperature than original BC. Samples with PEG-FITC entrapped show a higher shift of the degradation temperature.

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Table 4. Photographs of BC samples after PEG-FITC entrapment and washing after swelling at different conditions: Original BC; (A) swelling with 8% of NaOH solution for 30 min at room temperature in the presence of ultrasound; (B) swelling with 8% of NaOH solution for 30 min at room temperature using orbital agitation (50 rpm); (C) swelling with 8% of NaOH solution for 60 min at room temperature in the presence of ultrasound; (D) swelling 8% of NaOH solution for 90 min at room temperature in the presence of ultrasound

	Non swelled	A	B	C	D
After swelling and PEG-FITC entrapment					
Washed samples after PEG-FITC entrapment					

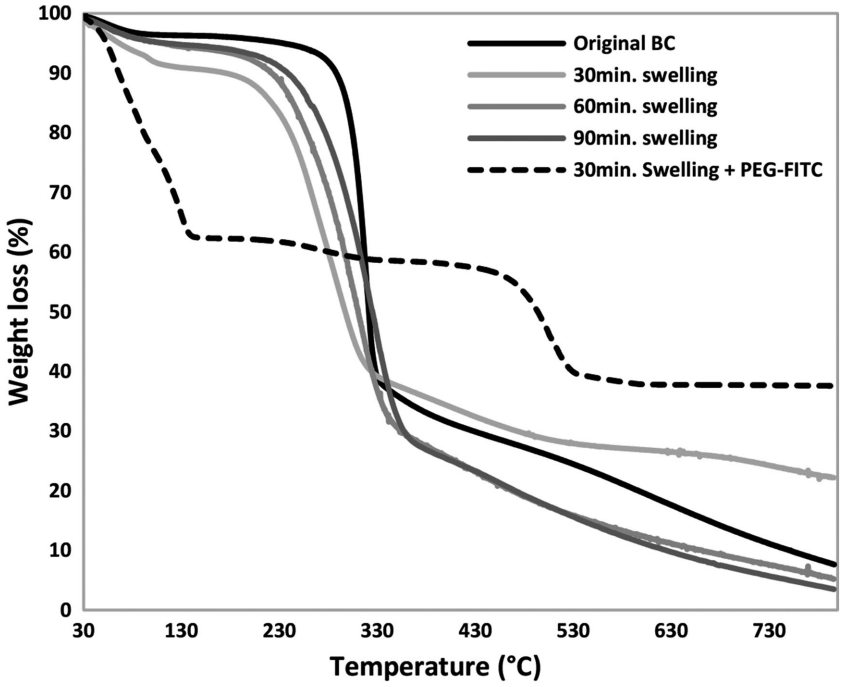

Figure 3. TGA spectra of original BC; BC after swelling with 8% NaOH in the presence of US and after PEG entrapment.

3.2 Immobilization of native and PEGylated laccase on swelled bacterial cellulose

Native (Activity: 634U/mL) and PEGylated laccase (40 U/mg) from *Myceliophthora thermophila* were immobilized on BC swelled samples (oven dried and freeze-dried) under different incubation conditions (25 and 4°C). It must be noteworthy that the activity of laccase decreases considerably after PEGylation. It has been observed an activity lost by laccase during the N-terminal PEGylation reaction which can reach more than 60%.

Table 5 shows the activity of enzymes immobilized on swelled and non swelled BC samples. The values of enzyme activity obtained for native laccase are comparable to some reported results in literature being, as predicted, more pronounced on swelled samples [31]. The data obtained for the activity of PEGYlated laccases after immobilization is still however different from other works published about laccase immobilization on BC supports using different methodologies. Chen et al. (2015) [10] used BC as support for laccase immobilization by adsorption-crosslinking and obtained a maximum retained activity of 69%. Sampaio, et al. (2016) reported that laccase immobilized on BNC by physical absorption was found to have almost the same specific activity of the free enzyme [11]. Contrarily to these authors, our results suggest a much lower activity for the PEGylated immobilized laccase. An higher enzyme loading to the most inner pores of the BC swelled structures promote a much lower affinity of the substrate for these immobilized enzymes (Table 5). Moreover, the presence of the PEG, contrarily to the expectations, drastically decreased the enzyme mobility when it is immobilized. Diffusional substrate limitations are more evident on the oven

Table 5. Activity of native and PEGylated laccase after immobilization (25°C and 4°C) on BC membranes at different state conditions

Enzyme/BC state	Activity (U/gBC)								Protein loading (mgprotein/gBC)							
	Oven dried; swelled		Freeze-dried; swelled		Oven dried; non-swelled		Freeze-dried; non-swelled		Oven dried; swelled		Freeze-dried; swelled		Oven dried; non-swelled		Freeze-dried; non-swelled	
	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C
NATIVE LACCASE	6.8	6.7	5.7	5.2	3.2	3.1	3.4	3.5	2.2	2.7	2.1	2.2	2.2	2.1	2.1	2.5
LACCASE-PEG-COOH	1.2	1.1	2.6	2.4	1.4	1.6	1.3	1.0	2.5	2.7	2.2	2.3	2.4	2.6	2.3	2.0
LACCASE-PEG-NH_2	1.2	1.2	2.1	2.0	1.2	1.5	1.3	1.4	2.5	2.6	2.4	2.3	2.4	2.6	1.7	1.6

Table 6. Spectral evaluation (Checksum *K/S*) of BC samples after in situ enzymatic polymerization of catechol (20 mM) at 50°C for 120 min

		Checksum K/S
BC without laccase + catechol (20mM)		148
BC with laccase immobilized + catechol (20mM)		319

dried BC swelled samples than in freeze-dried samples. In this case the enzyme is more available to react in the outer part of the BC samples.

Our data contribute to predict the design of the most favourable process of laccase immobilization. The temperature and the state of the matrix influence greatly the final catalytic behaviour. One can state that immobilization should be conducted at room temperature on freeze-dried samples. These matrices, despite providing less material entrapment, hold a higher nano and micro-fibrils surface area allowing higher adsorption of enzyme in the outer parts of BC. The catalyst is therefore more accessible to react with the ABTS [31–33] as well as with other substrates. To confirme this, the BC samples were used afterwards as support for the polymerization of a phenolic compound, catechol, after native laccase immobilization at 25°C. The results are presented as colour change (checksum K/S) after enzymatic-assisted polymerization. It is noteworth the high catalytic activity of immobilized native laccase when in contact with this substrate. The oxidation is confirmed by an increase of the colour from K/S = 148 to K/S = 319, as can be seen in Table 6.

4 Concluding remarks

In this study we demonstrate that swelling plays an important role on the entrapment and enzyme immobilization on BC samples. From all the conditions tested, we have established optimum swelling processing parameters: 8% NaOH for 30 min at room temperature in the presence of US.

These conditions conducted to high levels of PEG-FITC entrapment and enzyme immobilization yields. SEM characterization and entrapment data confirmed a more open porous structure when swelled is conducted under these conditions. The state of initial samples seems to influence not only the swelling processing but also the immobilization yield. Freeze-dried samples, present antagonist behaviour. Despite not allowing high levels of entrapment nor immobilization, the enzymes revealed to retain higher activities when immobilized on these samples. Swelled BC samples showed to be suitable supports for enzyme immobilization, e.g. polymerization of phenolics.

Practical application

Bacterial cellulose (BC) has been presented as a new functional material due to its excellent mechanical strength, tridimensional nanostructure and high purity and water absorption. This material can find applications on biomedical, environmental, agricultural, electronic, food, and industrial fields. The interest in using this material for the immobilization of microorganisms has been growing however still poorly explored. Few works describing the immobilization of microorganisms by chemical crosslinking have been reported as well as the physical

entrapment of enzymes on native BC. Our goal herein was to optimize the swelling process and increase the surface area available for molecules entrapment. An optimum swelling process was established: oven dried BC samples in 8% NaOH for 30 min, in the presence of ultrasound. A highly porous support was herein developed with great potentiality for enzyme immobilization applications like dyes decontamination, in situ polymerization, among others.

The authors would like to acknowledge Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684) and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte. The authors would like also to acknowledge the Basic Science Research Program through the National Research Foundation of Korea (NRF), which was funded by the Ministry of Education (2015R1D1A1A01060747). This work also received funding from the European Union Horizon 2020 research and innovation programme under grant agreement NMP-06-2015- 683356 FOLSMART.

The authors have declared no conflict of interest.

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