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Bacterial cellulose-based hydrogel for wound healing: characterization and *in vitro* evaluation

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ABSTRACT

Bacterial cellulose (BC) has been considered a promising biopolymer with applications in several areas of knowledge, including medicine, mainly due to its ability to assist in the treatment of dermal lesions. Many groups and companies have been making efforts to develop new BC-based materials in order to add new characteristics and therapeutic possibilities. Recently, Seven Indústria de Produtos Biotecnológicos Ltda company developed a BC-based hydrogel aiming to verify the interaction among the formulation components, its potential for wound healing and biocompatibility studies. BC-based hydrogel was characterized and compared with pristine BC film. Physicochemical characterization includes rheological measurements, thermal analyses, field emission gun - scanning electron microscopy (FE-SEM) and *in vitro* cell migration. BC-based hydrogel showed adequate interaction among the components of the formulation, which may positively influence its stability. In addition, the BC-based hydrogel accelerated the healing processes demonstrating its potential in dermal lesion treatment.

Introduction

Cellulose is the most abundant biopolymer on earth frequently obtained from plant sources. However, plant cellulose and BC show properties fairly different, which allow its applications in various fields such as physics, chemistry engineering and biological sciences. Additionally, BC production is an extremely pure process, which is entirely free of pectin, lignin and hemicelluloses, withoutcomponents from animal origin and without causing any allergic reaction leading to simpler purification process related to plant cellulose (1,2).

Currently, several microorganisms have been reported with the ability to produce BC, however Gram-negative bacteria of *Gluconacetobacter* genus have received great prominence in recent years due to their capacity to producecellulose in commercial quantities. During the biosynthesis, thesebacteria are able to synthesize cellulose in the form of membranesat the air/liquid interface of the static culture medium. These membranes present highly porous structures constituted of a random microfibrillar 3D-network of cellulose chains aligned in parallel with high permeability to fluids been favorable for adhesion and proliferation of cells (2-6).

BC membranes have shown to be a promising biomaterial for treatment of wounds healing, burns, tissue implants due to its unique properties such as high crystallinity, high mechanical strength, ultrafine fiber network structure andhigh water-uptake capability (water content > 90 %). BC provided a humid environment to the affected region promoting the exudate absorption and the wounds healing acceleration without any toxicity (1,2,4,7,8). In addition, randomly arranged cellulose nanofibers mimic some components of the extracellular matrix, such as collagen fibers, since they have similar diameter (near to 100 nm), which promotes a faster healing process (9).

BC membranes have been commercialized as an ideal wound dressing device due to its high *in vivo* biocompatibility and great efficacy when applied in cutaneous lesions promoting healing more efficiently than other products available for this purpose. In addition, BC membranes consists onphysical barriers that reduce pain and bacterial infections (6,10).

The mechanical treatment (defibrillation process) of BC membranes originates a dispersion of nanofibers which may to be incorporated into hydrogels, giving rise to a new therapeutic possibility for the treatment of burns, chronic ulcers, skin lesions and other lesions, protecting tissues formed around and over the wound (11). The possibility of treating deeper wounds, in which CB membranes are not capable of shaping the injured area, has been the subject of intense research by Seven Indústria de Produtos Biotecnológicos Ltda. (Brazil), since this characteristic can lead to successfully commercialization of BC-based hydrogel (e.g. Nexfill®).

Hydrogels are three-dimensional configurable polymers network with ability to absorb large amounts of water, saline and physiological solutions compared with general absorbent materials. They show excellent hydrophilic properties along with their high swelling ratio and biocompatibility, promoting their widely usage in biomedical, tissue engineering and drug delivery. Other characteristics of hydrogels are the long-term stability, facility of biochemical modification of the formed structures and the incorporation of several products inorder to combine the most important characteristics of each one (12-17).

To the best of our knowledge, few studies have investigated physicochemical characteristics and *in vitro* properties of BC-based hydrogel. Herein, we report the evaluation of the hydrogel containing BC as well as the interaction between the formulation components keeping the wound healing properties of the BC without toxicity effects.

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Materials and methods

Materials

BC-based hydrogel (Nexfill® Hydrogel) was provided by Seven Indústria de Produtos Biotecnológicos Ltda. (Ibiporã, PR, Brazil) for further characterization and *in vitro* evaluation. The BC-based hydrogel (Nexfill® Hydrogel) composition was obtained according to the PI 0601330-9 A2 patent.

Rheological properties

Rheological properties of the BC-based hydrogel (Nexfill® Hydrogel) were evaluated using an Anton Paar rheometer (MCR302), equipped with two parallel-plates (PP 25) sensor with a 25 mm, the gap between plates was 1.00 mm and temperature of 32 °C. Rheo Compass™ software was used to analyze the data.

The Flow curve was analyzed with shear rate range from 0 to 100 Pas^{-1} for the ascent ramp for 120 s and from 100 to 0 Pas^{-1} for the descent ramp for 120 s and it was applied "Power Law" model, according to Equation 1:

$\tau = K \times \gamma^n \tau = K \times \gamma^n$ (Equation 1)

Where τ is shear stress, γ is shear rate, K is consistency index and *n* is the flow rate. In this model, $n > 1$ represents a dilatant fluid, $n < 1$ represents a pseudoplastic fluid and $n = 1$ represents a Newtonian fluid (18).

The range of frequencies used was 0.1 to 500 rad s^{-1} at 50 % strain, which proved to be in the linear viscosity range.

The storage modulus (G'), loss modulus (G") and complex viscosity (η*) were performed as a function of angular frequency range of 0.1–500 rad s^{-1}.

Thermal analysis

Lyophilized BC-based hydrogel (Nexfill® Hydrogel) was evaluated by differential scanning calorimetry (DSC) technique using a DSC1 STARe System-Mettler Toledo. The sample of ± 5 mg were submitted to heating from 25to 200 °C at 10 °C/min under nitrogen atmosphere. Thermogravimetric analysis (TGA) and differential thermogravimetric analysis (DTG) of the lyophilized samplewas performed on TA Instruments (SDT-2960) (New Castle, DE, USA). Sample (5 mg) was accurately weighed in coated alumina pan and heated from 25 to 600 °C at 10 °C/min under nitrogen atmosphere. DSC and TGA from pristine BC films were similarly obtained and compared with hydrogel results.

Morphology of BC-based hydrogel

The surface morphology of the lyophilized BC-based hydrogel (Nexfill® Hydrogel) was investigated by scanning electron microscopy FE-SEM on a JEOL microscope (model JSM-7500F, Japan). The sample was frozen at -80 °C and lyophilized for 24 h. After that, lyophilized sample was attached to slab surface with double sided adhesive tape and coated with carbon as conductive material. The sample was examined using an accelerating voltage of 2 kV. FE-SEM from pristine BC films were also obtained as described by Machado et al. 2016 and compared with hydrogel results.

Fibroblast growing model

Fibroblast culture

Fibroblast cultures internally isolated by Invitrocell, of city of Paulínia, São Paulo (Brazil) for donation of explant human cells. Cells were cultivated T-75 cm^2 flasks containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), penicillin and streptomycin ((Sigma Aldrich®), USA), at 37 °C in humidified atmosphere containing 5 % CO_2. The medium was changed every dayuntil cells reach 80-90 % confluence, when fibroblasts were split with 0.05 % Trypsin/0.02 % EDTA.

Cell Migration (Wound healing assay)

The evaluation of fibroblast migration allows the evaluation of the ability of cells to repair an opening in the culture caused by injury. The test substance, BC-based hydrogel (Nexfill® Hydrogel) was evaluated at 3 different concentrations as defined in the cell viability assay (10, 100 and 1000 µg/mL). Cells were seeded at a density of 3 x 10^5 cells/well on 6-well plates. After 24 h, the cells were washed with PBS without calcium and it was created a "scratch" with a pipet tip. Cells with test substance were cultured in DMEM containing 10 % FBS and the evaluation was performed after 6 h of incubation with the treatment. The migration process was observed after 6 h of incubation and all images of the group were obtained under inverted microscope (200x) with camera coupled by photograph (increase 3x). The quantification of cell migration was done through image analysis by ImageJ (version 1.48v, National Institutes of Health, USA) and the quantification of wound extension was analyzed in relation the size obtained in the group of basal (untreated) cells. β-estradiol (0.1 mM) was used as inhibitory control of cell migration. The results are expressed as mean ± standard error of the mean calculated in Microsoft Excel software using t student test. Significant differences between the control and treated groups are indicated by ***p<0.001, **p<0.01 and *p<0.05 (19).

Results and discussion

Physicochemical studies

Rheology properties

The study of flow properties is related to the deformation of the formulation when subjected to a shear stress, providing information on stability and consistency of the final product (20,21) BC-based hydrogel (Nexfill® Hydrogel) as depicted in Fig. 1.

According to Fig. 1, the hydrogel behaves as a non -Newtonian system, as it does not present a linear relationship between shear stress and shear rate. In agreement with the Equation 1 applied in flow curve (Fig. 1), it was observed that the value obtained by *n* (flow index) is lower than 1 (Table I) indicating that the hydrogel presented pseudoplastic behavior.

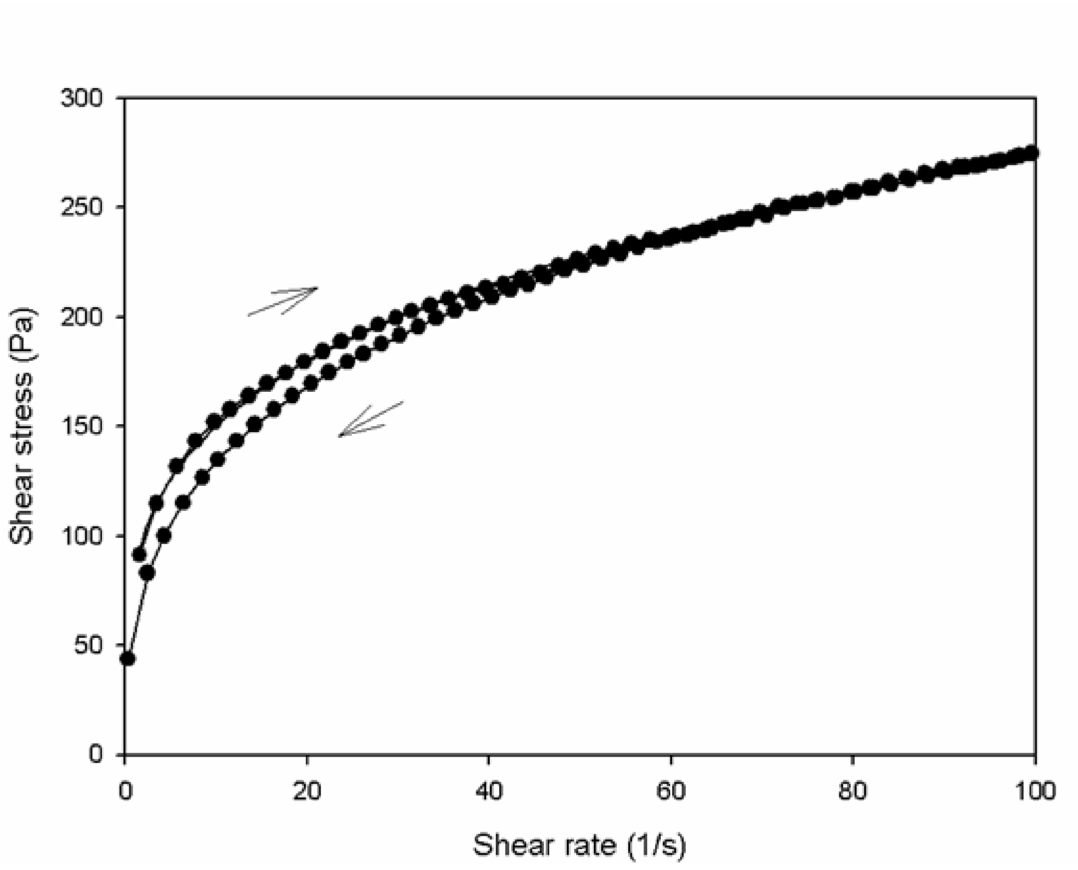

Figure 1. Tixotropic hysteresis loop of the BC-based hydrogel (Nexfill® Hydrogel).

Sample	Flow parameters		
	N	K	r^2
BC-based hydrogel (Nexfill® Hydrogel)	0.2579	83.0257	0.9987

Table I - Flow parameters of the BC-based hydrogel (Nexfill® Hydrogel) calculated by using the power law (Equation 1).

In the rheological behavior, the flow curve (Fig. 1) shows the pseudoplastic behavior that results from the alignment of the disrupted a three-dimensional network in the system in the direction of flow, providing the protective film formation characteristic that allows the skin surface to be covered, promoting a better protection (22). However, at flow curve (Fig. 1) there is hysteresis area and the hydrogel present rheological characteristics thixotropic.

Thixotropic products have the characteristic of deforming during application, becoming fluid, facilitating the scattering and recovering the initial viscosity at the time of application closure, avoiding the product to flow. Formulations with thixotropic characteristics tend to have greater self-life, because during storage it has a constant viscosity, making it difficult to separate the constituents of the formulation (23,24). Strain sweep test allows determining the amplitude in which the region of linear viscoelasticity is maintained for the sample and, through the identification of the strain values that the sample does not undergo deformation and thus other rheological tests, such as the frequency sweep.

The strain sweeps measurement was done to check for the linear-viscoelastic regime (LVE) limit and the curves are present in Fig. 2.

The hydrogel containing BC presented linear behavior until 10 %. So, a strain of 2 % was choose for the next steps, as frequency sweep.

BC-based hydrogel (Nexfill® Hydrogel) with 2 % of strain the sample did not suffer deformation and was used this value for test of frequency sweep. Frequency sweep was conducted to small amplitude oscillatory shear (SAOS) in the LVE. The ratio of the storage modulus (G ') and loss (G ") plotted by frequency, which provides important information about the structure of the gel (25). Important aspects of BC-based hydrogel (Nexfill® Hydrogel) structure, as well as, mechanical behavior was determinate the frequency-dependence of dynamic moduli (G' and G") and are present in Fig. 3.

Fig. 3 and it is observed that G' was higher than the corresponding to the G" over the entire frequency sweep range, and the complex viscosity decreases with increasing frequency. This behavior indicates hydrogels possess a solid-like gel structure (26).

The Frequency sweep (Fig. 3) provides information about the storage modulus (G') indicating the energy stored in the material and depends on the rearrangements that occur during the period of oscillation, which may characterize an elastic or solid character. On the other hand, the loss modulus (G") indicates the energy dissipated or lost during the period of oscillation, which may characterize a viscous or liquid behavior. Thus, when there is a predominance of the elastic modulus (G') on the viscous modulus (G") it is an indication that the analyzed system is more structured and there is a strong interaction between the components (27).

Similar results were observed (28) with gelatin hydro-

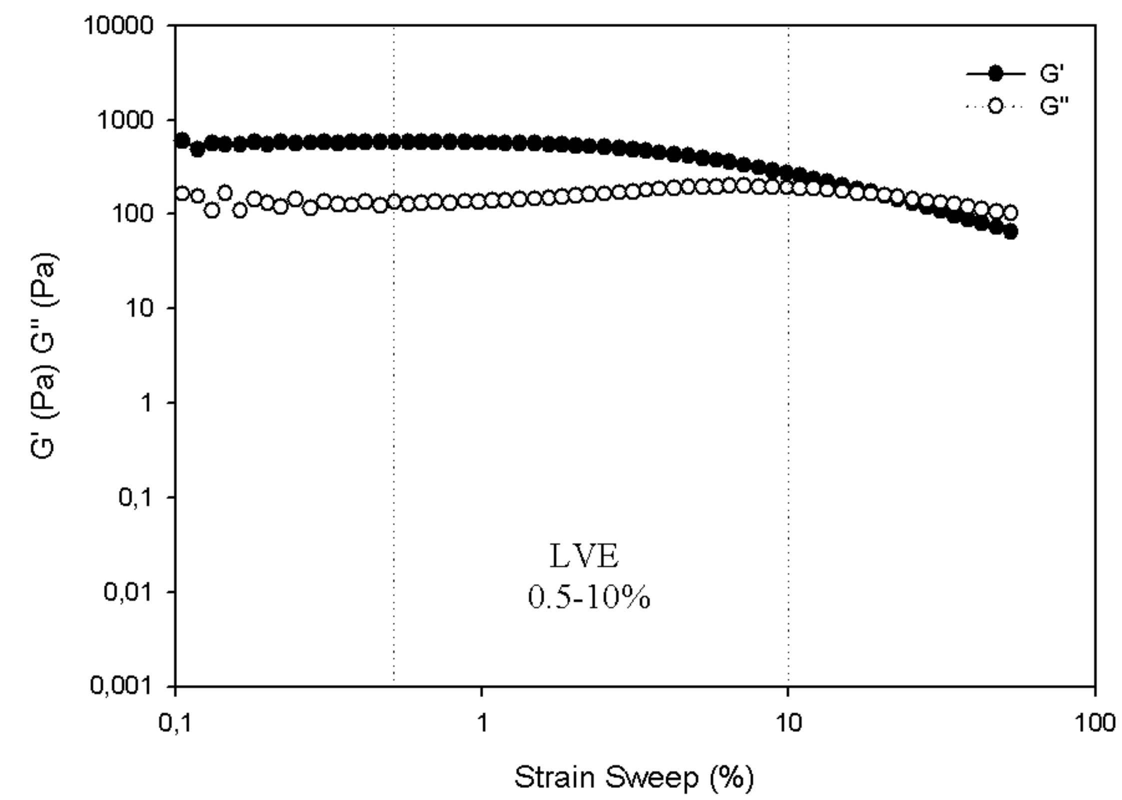

Figure 2 - Strain Sweep of the BC-based hydrogel (Nexfill® Hydrogel).

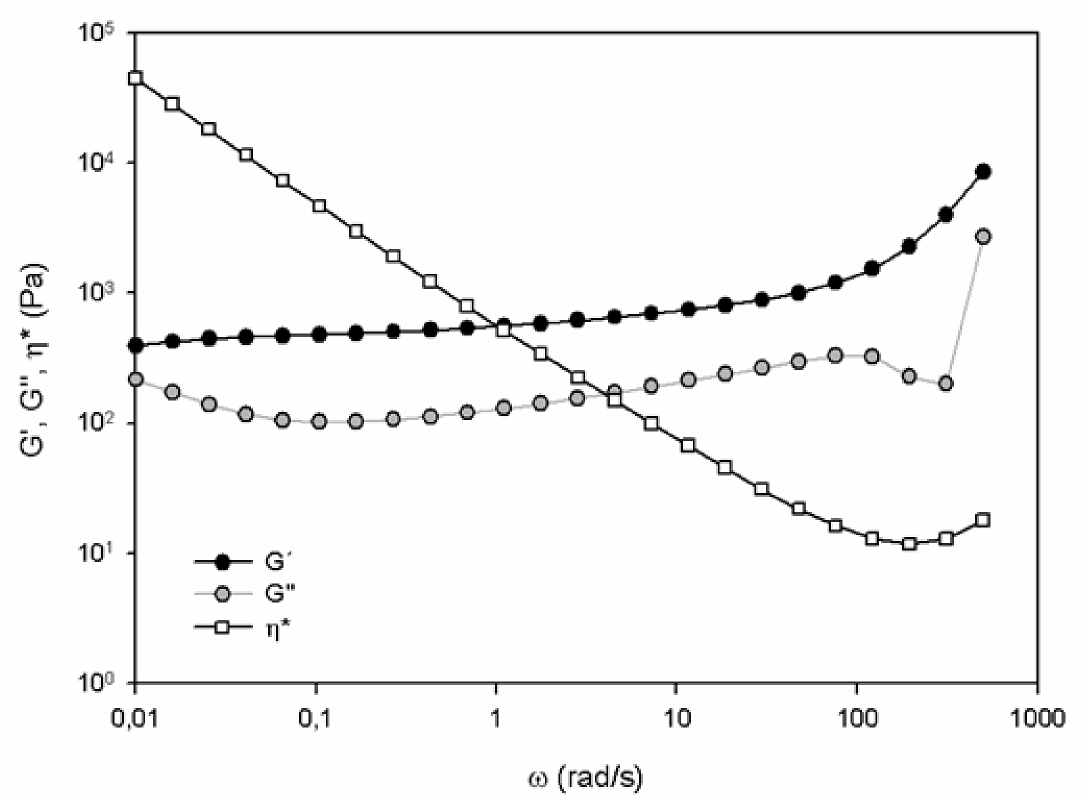

Figure 3 - Frequency Sweep of the BC-based hydrogel (Nexfill® Hydrogel).

gels reinforced with chitin of which the mechanical spectra, predominance elastic modulus (G') on the viscous modulus (G") and the G' values remained unchanged as the angular frequency (0.1–100 rad s^{-1}) indicating a strong and stiffness of the gelatin hydrogel.

Thermal behavior

Fig. 4 (A and B) shows TG/DTG results obtained from pristine BC films and BC-based hydrogel (Nexfill® Hydrogel), respectively.

For pristine BC films (Fig. 4A), two characteristic events were detected. First, the mass loss from 25 to 100 °C (3.51 %) was assigned to the water molecules. The second and main mass loss (75.46 %) starting from 250 to 450 °C (T_{onset} = 372 °C) was ascribed to the BC degradation process such as depolymerisation, dehydration and decomposition of glucose units (1,29). Four main mass loss events were observed for BC-based hydrogel (Fig. 3B). The first one, starting from low temperature to 110 °C (T_{onset} = 55 °C, 10.19 % of mass loss) corresponds to the solvents and water molecules (dehydration processes of the hydrogel) (1,29). The thermal degradation of the sample occurs in three subsequent steps (T_{onset} = 192, 322 and 377 °C). First step occurs from 110 to 250 °C (38.93 % of mass loss) attributed to the humectant compounds. The second (T_{onset} = 322 °C, 21.29 % of mass loss) and third (T_{onset} = 377 °C, 9.52 % of mass loss) steps were assigned to the degradation processes of BC and other polymers in

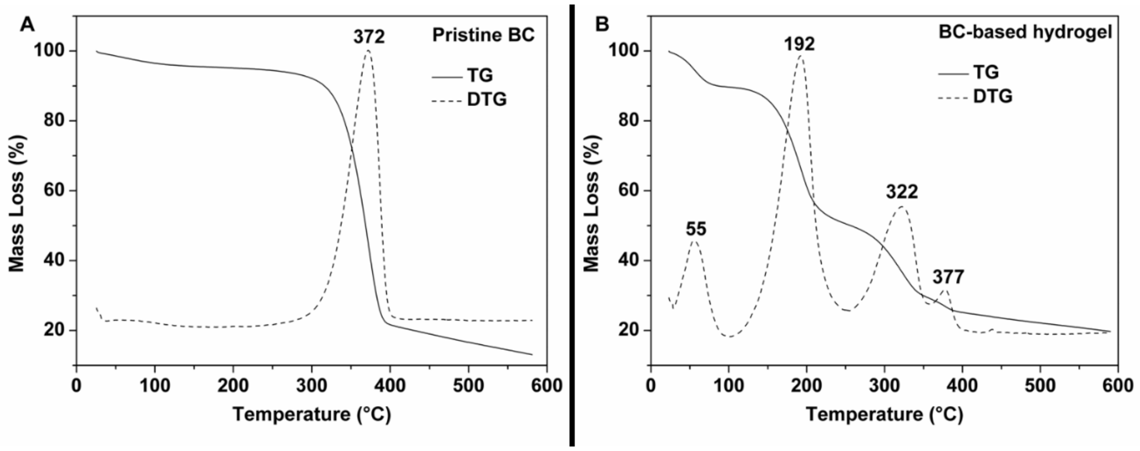

Figure 4 - Thermogravimetric (solid lines) and differential thermogravimetric (dashed lines) analysis of (A) pristine BC film and (B) BC-based hydrogel (Nexfill® Hydrogel).

agreement with pristine BC films (Fig. 4A) (1,29). Additionally, a residue of 19.70 % was detected for BC-based hydrogel (Nexfill® Hydrogel) and ascribed to inorganic salts and carbonaceous materials (carbon and carbon monoxide) (1,30).

The DSC curve of BC-based hydrogel (Fig. 5B) results showing an endothermic peak starting from room temperature to 110 °C as aforementioned in Fig. 4B, this event was assigned to solvents and water loss. It is worth noting that the hydrogel showed an endothermic event more pronounced and at similar temperature relating to pristine BC films (Fig. 5A). Although it was expected water loss from BC-based hydrogel (Nexfill® Hydrogel) at lower temperatures than for pristine BC film, the hydrogel shows similar behaviour to pristine BC film. These results suggest a strong interaction of the components seen in the hydrogel through the rheological measures to oppose to water loss.

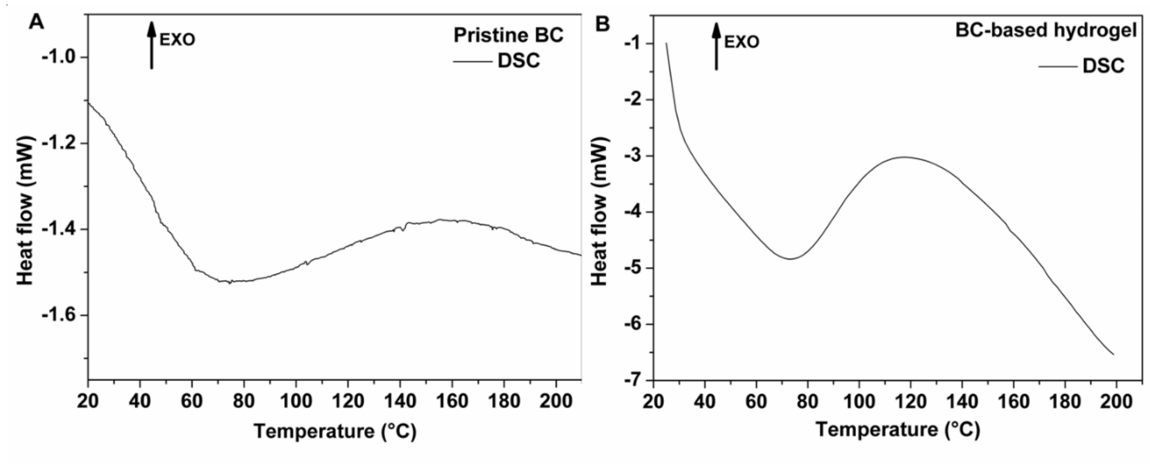

Figure 5 -. Differential scanning calorimetry curves of (A) pristine BC film and (B) BC-based hydrogel (Nexfill® Hydrogel).

Morphology analysis

The morphology of dried pristine BC film and lyophilized BC-based hydrogel (Nexfill® Hydrogel) were investigated by FE-SEM as shown in Fig. 6.

Fig. 6A displays pristine BC film composed by three-dimensional network porous structure containing randomly arranged cellulose nanofibers (31). Fig. 6 (B-E) exhibit BC-based hydrogel (Nexfill® Hydrogel) with BC nanofibers clearly and well dispersed on its surface. These results suggest that the hydrogel could keep the characteristic properties of pristine BC films in its composition (8,32).

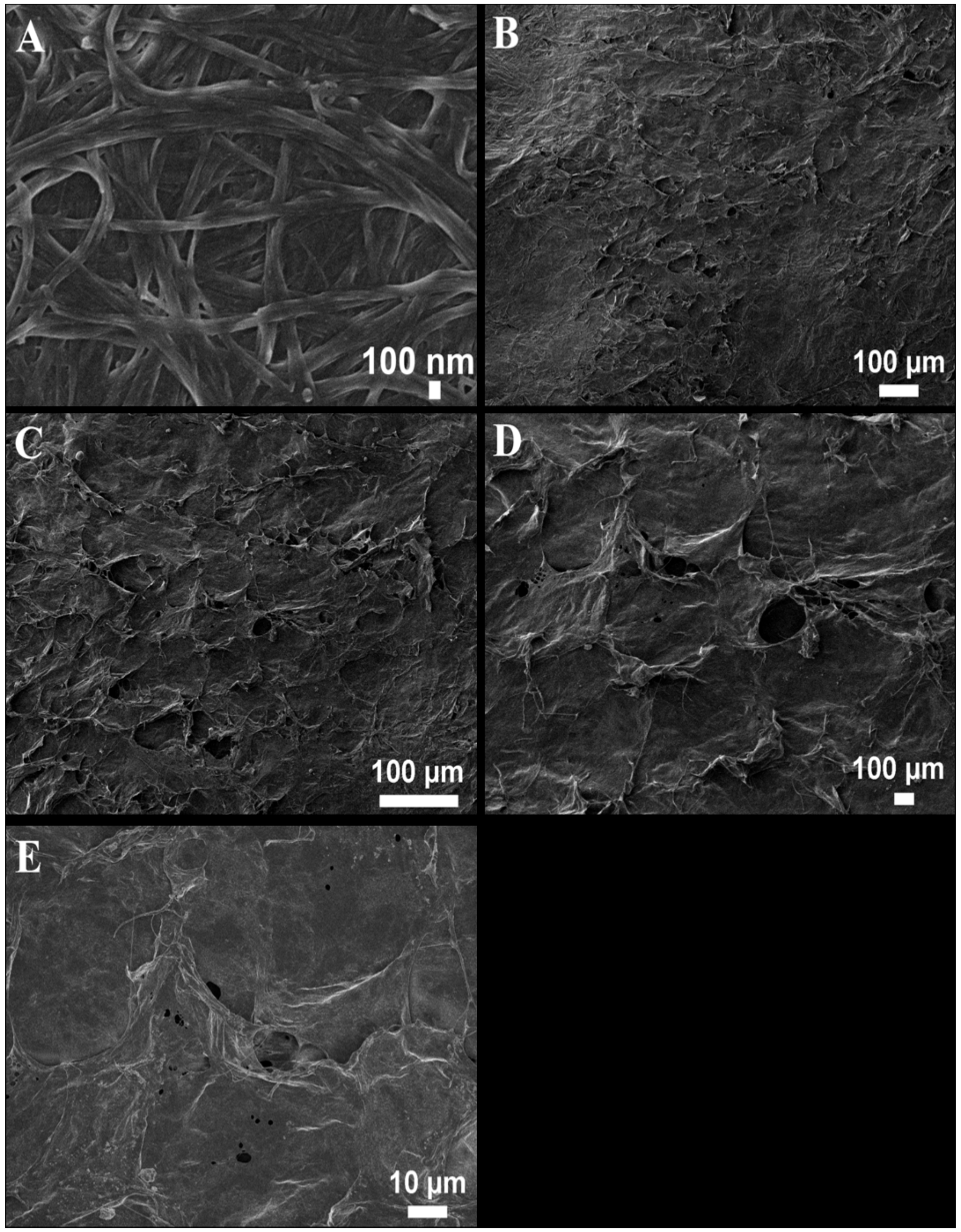

Figure 6 - FE-SEM of the pristine BC film (A), BC-based hydrogel (Nexfill® Hydrogel) (B) 100x, (C) 200x, (D) 500x and (E) 1000x.

In vitro studies

The fibroblast migration ability was also evaluated after opening a ''scratch'' in the middle of the semi-confluent culture of fibroblasts after 6 h of treatment with BC-based hydrogel (Fig. 7).

Fig. 7 illustrates healing activity of different concentrations (10, 100 and 1000mg/mL) of the BC-based hydrogel (Nexfill® Hydrogel) related to the basal control (untreated cells). The reference substance β-estradiol promotes delay in wound closure presents a statistically significant difference when compared to baseline control ($p<0.001$). BC-based hydrogel (Nexfill® Hydrogel) showed the highest significant when compared with basal control ($p<0.001$) in 1000 mg/mL, demonstrating that in this concentration the highest fibroblast migration toward the scratched area almost closing the wound while at 100 and 10 μg/mL a delay in fibroblast migration was observed relating to the basal control ($p<0.01$ and $p<0.05$, respectively).

Similar results were also observed (33), which evaluated chemically modified BC membranes and demonstrated good *in vitro* compatibility with fibroblasts, once the membrane helped in the healing process.

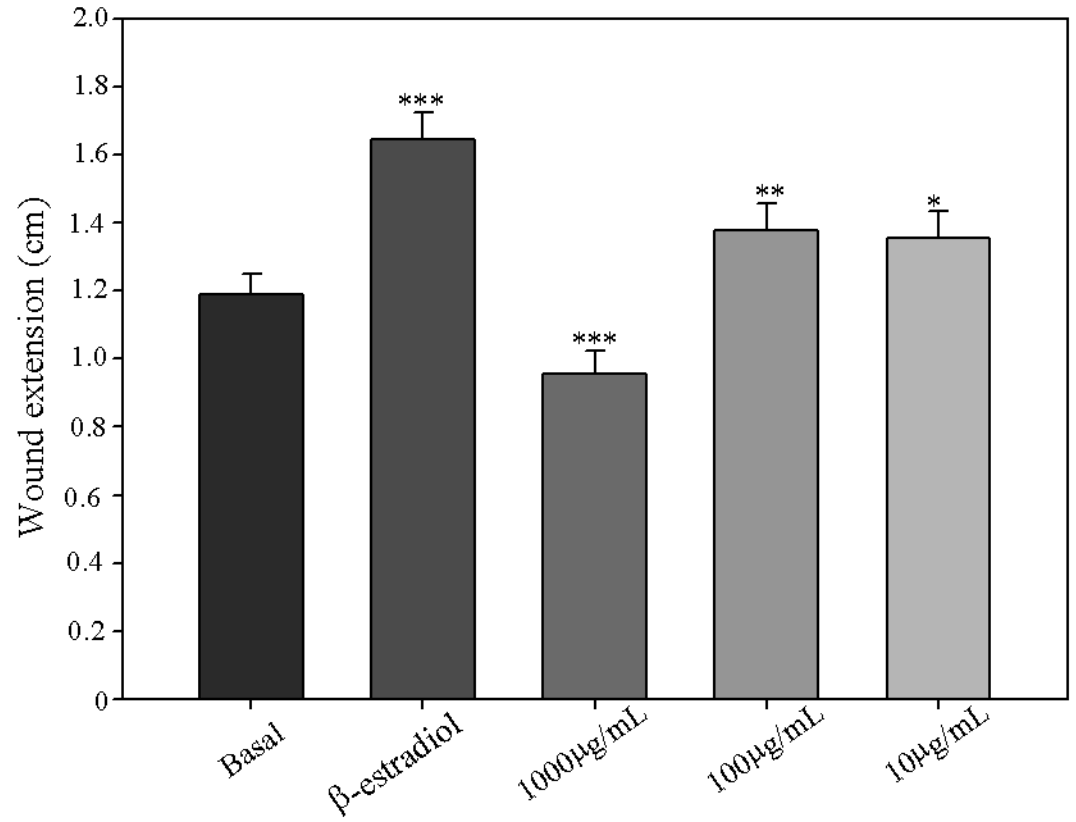

Figure 7 - Evaluation of the fibroblast migration capacity of the BC-based hydrogel (Nexfill® Hydrogel). The plot shows mean values ± Standard error of the mean obtained for each treatment. The values differ of the basal control at *** for when $p<0.001$, ** for when $p<0.01$ and * for when $p<0.05$ in the student t statistical test.

Conclusions

BC-based hydrogel (Nexfill® Hydrogel) were developed as a potential strategy for the treatment of chronic wounds in which membrane occlusion is not adequate, or even for those cases where the depth of the lesion promoted by tissue loss hinders the adaptation of the membranes in the wound bed. As expected, the BC nanofibers present in the hydrogel were responsible for the building of a strong and structured network which should lead to a high interaction pattern with the biological interfaces but allowing its adequate and comfortable spreadability on the wounds. The set of data suggest that the BC-based hydrogel consists on a suitable formulation for wound repair since fibroblasts represent the first defense line against injuries and the increase of fibroblast proliferation in wound bed is fundamental for lesion repair.

Conflict of interest

There are no conflicts to declare.

Acknowledgements

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