

Bacterial nanocellulose and fibroin: natural products to produce a structure membranes

Nanocelulose bacteriana e fibroína: produtos naturais para a produção de membranas estruturais

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ABSTRACT

Fibroin (FB) and bacterial nanocellulose (BC) are natural products, being used in biomedicine, electronics and food industries, and other areas. All of them show biocompatibility, able to be used for many different purposes. The blending of fibroin and bacterial nanocellulose was design to produce a biocompatible material to be applied with a medical device. For this reason, the objective of this work was to evaluate the structure properties of the blending of BC and FB. Thus, FB was extracted from *Bombyx mori* and BC was produced by fermentation process utilizing *Gluconacetobacter xylinus*. The membranes composed of BC-FB were produced by immersion contact for 24 hours, at 25°C, in 100 rpm, without crosslinking agent. After the production the membrane samples were dried and characterized by Fourier transform infrared spectroscopy (FTIR spectroscopy), mechanical proprieties, swelling efficiency, scanning electron microscopy (SEM) and computerized microtomography (μ Ct). Results indicate that the hydrogen-bonded porous membranes obtained displayed anisotropic, closed and interconnected porous morphology. The morphometric characteristics, which resemble a honeycomb and consist of a long structure with high connectivity and high total porosity, amplify the areas of BC-FB blend applications, with potential utilization with optoelectronic devices, in areas ranging from environmental to tissue engineering. Furthermore, the production by immersion contact will allow the upscale process and the development of green label material.

Keywords: Polymers conjugation; Membranes; Fibroin; Bacterial nanocellulose.

RESUMO

Fibroína (FB) e nanocelulose bacteriana (BC) são produtos naturais que podem ser usados na biomedicina, nas indústrias eletrônicas e de alimentos e outras áreas. Todos eles apresentam biocompatibilidade, podendo ser utilizados para diversos fins. A mistura de fibroína e nanocelulose bacteriana foi projetada para produzir um material biocompatível para ser aplicado com um dispositivo médico. Por esse motivo, o objetivo deste trabalho foi avaliar as propriedades estruturais da mistura de BC e FB. Assim, FB foi extraída do *Bombyx mori* e a BC foi produzida por processo de fermentação utilizando *Gluconacetobacter xylinus*. As membranas compostas de BC-FB foram produzidas por contato de imersão por 24 horas, a 25 °C, em 100 rpm, sem agen-

te reticulante. Após a produção, as amostras de membrana foram secas e caracterizadas por espectroscopia de Infravermelho com Transformada de Fourier (espectroscopia FTIR), propriedades mecânicas, eficiência de intumescimento, microscopia eletrônica de varredura (MEV) e microtomografia computadorizada (μ Ct). Os resultados indicam que as membranas porosas com ligações de hidrogênio obtidas apresentaram morfologia porosa anisotrópica, fechada e interconectada. As características morfométricas, que se assemelham a um favo de mel e consistem em uma estrutura longa com alta conectividade e alta porosidade total, amplificam as áreas de aplicações da mistura BC-FB, com potencial utilização com dispositivos optoeletrônicos, em áreas que variam desde ambiental à engenharia tecidual. Além disso, a produção por contato de imersão permitirá o processo de alta qualidade e o desenvolvimento de material de rótulo verde.

Palavras-chaves: Conjugação de polímeros; Membranas, Fibroína; Nanocelulose bacteriana.

1. INTRODUCTION

In bioengineering, the light fibroin (FB) chain found in the silk filaments of *Bombyx mori* has low immunogenicity and low risk for acute cellular rejection. FB can induce cell growth based on its structure, tissue biocompatibility and non-cytotoxicity. The material size, chains with molecular weight in the range of 200–350 kDa, does not allow the phagocytosis, and it does not cause inflammatory reactions [1-4]. Those are important characteristics in bioengineering. Nevertheless, to solubilize the FB and prevent its premature precipitation by self-assembly, the use of harsh solvents is needed. The advantage of silk fibroin is the possibility of biochemical manipulation of its solubility by artificial induction of β -sheet formation when polyaniline is incorporated [5]. The soluble FB can also be manipulated to acquire several shapes and thickness in film, fibres, nets, scaffold, mat, threads [4, 6].

Bacterial nanocellulose is a polysaccharide produced by various microorganisms including *Agrobacterium*, *Rhizobium*, *Pseudomonas*, *Sarcina* and *Acetobacter*. Its chemical composition is similar to vegetable cellulose, but its fibers are organized in nanometric dimensions and have unique properties such as high mechanical strength, chemical stability, hydrophilicity, crystallinity, and biocompatibility. [7-9]. Bacterial nanocellulose is a material of great industrial interest, with low cost and with a variety of application, as packing, biomedical devices [11-13]. A material composed only of fibroin does not show as good mechanical properties as one with reinforcing materials [2, 13]. With this in mind, fibroin films with bacterial nanocellulose were developed in order to improve the mechanical properties of the material and amplify the application areas. For this reason, this study aimed to evaluate the structural properties of the blend composed for fibroin and bacterial nanocellulose by immersion contact.

2. MATERIAL AND METHODOLOGY

2.1 Fibroin (FB) extraction

Fibroin was extracted utilizing our research group protocol [14] where 5g of *Bombyx mori* cocoons (Bratac – Londrina/PR-Brazil) were used. The cocoons were cut and immersed in sodium carbonate solution (P.A., Anidrol – Diadema/SP-Brazil) prepared with MilliQ water 4.54g/L. The solution was kept for 35 min at 80°C for sericin and other proteins withdrawal takes place since are soluble in heated sodium carbonate solution.

After the 35 minutes, the fibers were washed three times carried out for 30 min each with 1 L of purified water on a magnetic stirrer to remove the sodium carbonate. The remaining material was dried at 50°C for 24 h. In order to dissolve the fibers and to form a fibroin suspension, a ternary solution containing calcium chloride (P.A., Anidrol– Diadema/SP-Brazil), ethanol (95%, Anidrol– Diadema/SP-Brazil) and MilliQ water (1:2:8 molar ratio) was applied. The solution was then heated at 85°C in a water bath for 20 min. Once the fibers reached full dissolution, the mixture was removed from the bath and cooled to room temperature ($23 \pm 2^\circ\text{C}$).

The fibroin suspension (12 mL) was placed in dialysis using a cellulose membrane with a 33 mm MWCO 12.000 Da (Sigma Aldrich). Each membrane containing the solution was immersed in 1 L of purified water under stirring for 48 h, changing the water after 24 h. The membranes were withdrawn and the solution was centrifuged twice at 20,000 rpm for 30 minutes to remove possible remaining impurities. The proteins concentration in fibroin solution, pH 7, was 1 mg/mL, a value similar to that reported in the literature, which is 10% [14]. To avoid contamination, the material was autoclaved at 121°C for 15 min and then stored at 8°C.

2.2 Bacterial nanocellulose (BC) production

Bacterial nanocellulose (BC) was produced using *Gluconacetobacter xylinus* (ATCC 53582) in 20 mL of the

Hestrin & Schramm broth (20 g/L glucose, 5 g/L bacteriological peptone, 5 g/L yeast extract, 2.7 g/L anhydrous sodium phosphate, 1.5 g/L monohydrate citric acid) in an Erlenmeyer flask of 125 mL. The Erlenmeyers were kept for 4 days in static culture at 30°C, yielding 2 mm thick BC. After growth, they were washed in 2% sodium dodecyl sulfate (SDS) solution in a magnetic stirrer overnight. Afterwards, they were rinsed with distilled water until SDS removal, and immersed in 1 M NaOH solution with stirring (50 rpm) at 60°C for 1h30. After this period, membranes were washed again until reaching neutral pH. They were packaged and autoclaved at 121°C for 15 minutes in MilliQ water, and stored at 4°C [15, 16]

2.3 Bacterial nanocellulose and fibroin membranes (BC-FB) production

The BC-FB membranes were produced by immersion contact utilizing bacterial nanocellulose (with diameter of 150 mm and thickness of 2 mm) with 20 mL of the aqueous fibroin solution (1mg/mL) in a 125 mL Erlenmeyer's flask at 25°C, under agitation (100 rpm for 24h). After the immersion contact the BC-FB membranes were freeze for 24h in -80 °C and then the membranes were lyophilized.

2.4 Mechanical, physical-chemical and morphometric characterization

Fourier Transform Infrared Spectroscopy (ATR-FTIR)

FTIR technique (Shimadzu, IRAffinity-1, Kyoto, Japan) was used to collect FT-IR spectra via Labs Solutions Software v.2.10. The chemical functionalities of the samples were determined by an attenuated total reflectance (ATR-8200HA) cell on the FTIR spectrophotometer over the range between 4000 and 600 cm⁻¹, resolution of 4 cm⁻¹, interval of 0.5 cm⁻¹, averaging 128 scans. To evaluate the sample uniformity three samples were analysed. The membranes samples were carefully manipulated and put on the ATR support following the protocol reported by ARAUJO *et al.* [14].

Morphometry

The pore size, porosity (%), and the interconnectivity of the porous in the membranes were evaluated by microtomography (μCT) according to our research article [12]. The membranes pictures were captured by X-Ray microtomography (Bruckermicro CT - SkyScan 1174, Kontich, Belgium), scanner resolution of the 28mm pixel, and integration time at 1.7 s. The X-rays source was 35 keV and 795 mA. The projections were taken in a range of 180° with an angular level of 1° of circumrotating. A 3D virtual models, representative of various sections of scaffolds were built, and the data was mathematically managed by CT Analyzer software, v. 1.13.5.2.2.8 [15]

Swelling efficiency

The BC-FB membrane were kept at -80°C in an ultra-freezer for 24 hours, lyophilized for 72 hours and the weight was assessed. The lyophilized sample was immersed in phosphate buffer solution at room temperature (25oC) for different lengthly: 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 hours. The swelling ratio (SR) was calculated by the equation 1 [18]:

$$SE\% = \left(\frac{[BCFB]_{w} - [BCFB]_{d}}{[BCFB]_{d}} \right) \times 100 \quad (1)$$

in which BCFB_w and BCFB_d are respectively the weight of the wet and dry of BC+BF membrane.

To carry out the test, the membranes were cut to approximate in 20mm of diameter, weighed and then immersed in 3ml PBS at 37°C for up to 24 hours. At different times, they were removed, and two different measurements of their capacity to retain the PBS were made. The measurement was to assesses the ability of the membrane structure as a whole (the material itself together with the pore system) to absorb PBS. For this, at each time interval, the samples were removed from PBS, shaken gently, and then weighed without dripping (W). The scaffolds were then dried at 37°C until a constant weight was reached (W_d). The percentage of fluid uptake, in both cases, was calculated as shown (Eq. (2)):

$$\text{Fluid uptake of membrane} = \left(\frac{W - W_d}{W_d} \right) \times 100 \quad (2)$$

. Each sample was measured in triplicate.

Texturometer tests

Texture profile analysis (TPA) was performed to measure the mechanical properties of sample in compres-

sion mode for elasticity, resilience and relaxation according to ALVES *et al.* [17] in a Texture Analyzer (Stable Micro Systems - TA-XT Plus, Surrey, UK), with analytical Cylindric Probe P/2 and support HDP/FS-R. The travelling arm was outfitted with a load cell of 5 Kg and the force response of the sample to the deformation imposed on it was recorded. The samples with 150 mm diameter were clamp in a jaw probe constrained to move in a direction perpendicular to the axis of traction without rotating. The test speed was set to a rate of 2 mm.s^{-1} for perforation and 0.05 mm.s^{-1} for resilience and elastic modulus. The Elastic (Young's) modulus was obtained by compressed until densification of the sample, and it was calculated with a strain that ranged between 0 and 5%.

Scanning Electron Microscopy morphology

The microstructure of the materials and membranes were monitored by scanning electron microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) (JEOL, Model IT200). The BC, FB and BC-FB membranes were kept at -80°C in an ultra-freezer for 24 hours and lyophilized for 72 hours. The samples were previously fractured in liquid N_2 and fixed to the stub with double-sided adhesive tape. Microphotographs were taken using electron beams with energy and acceleration voltage of 15 kV. The samples were randomly scanned and photomicrographed at magnifications x5000

3. RESULTS AND DISCUSSION

The BC-FB membranes were visually similar to a BC sample. The BC immersed in FB solution has slightly changed the macroscopic appearance of the samples (Figure 1).



Figure 1: Photographs of bacterial nanocellulose (BC) membrane (A), BC-FB membrane.

The water swelling efficiency ratio (SR%) results for BC membranes showed the water absorption capacity around 850%, as shown in figure 2. When BC was added into FB solution, the water SR% was about 1700%, doubling the swelling capacity due to the FB hydrophilic characteristics [24]. Despite that BC can naturally hold a large amount of water and the SR is an important property involved in medical applications [27–32].

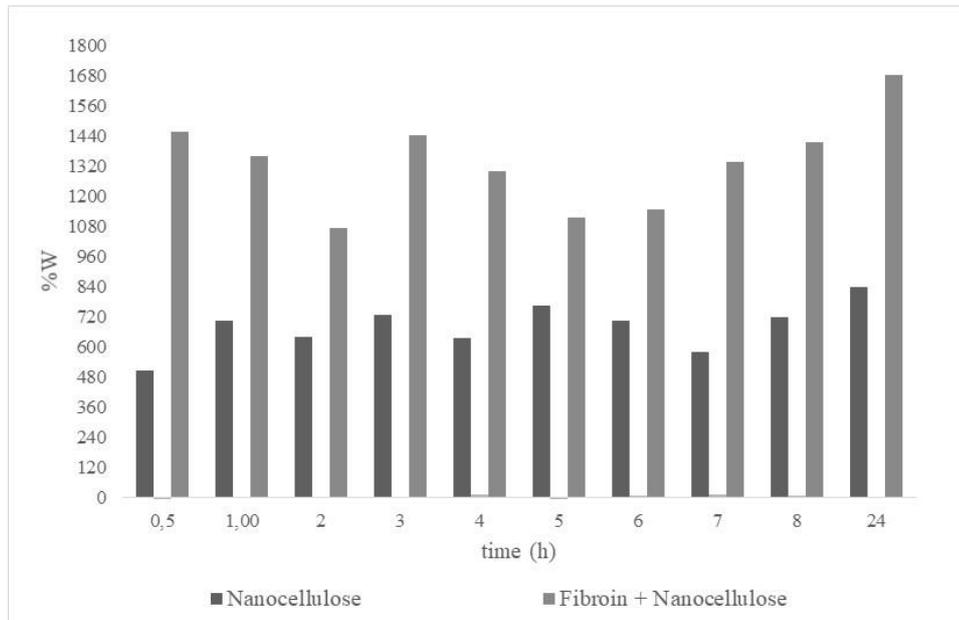


Figure 2: Swelling efficiency of BC (bacterial nanocellulose) and BC+FB (fibroin adsorbed on bacterial nanocellulose).

CHEN *et al.* [37] produced a material with fibroin aqueous solution (12%(w/v) and bacterial cellulose nanoribbon (BCNR). The materials were prepared in ratios of 0:20, 1:20, 2:20, 3:20, and 4:20 via a multi-staged freeze-drying method. They observed that water swelling efficiency ratio (SR%) increased when the material reached a ratio of 2:20. The authors have concluded the material intercalated network improved the SR% capacity.

MEFTAHI *et al.* [28] studied BC production in static culture medium and its properties after purification. BC simple dried membranes showed the water absorption capacity at around 450% after 196 hours immersion. The water absorption capacity was around two-times less than that obtained here for BC. We believe the reason for this difference in behaviour is that although the BC have the same production, the drying process was different.

FTIR results analyses (Figure 3) show that the chemical interaction during immersion of BC membrane into FB colloidal dispersion occur by simple σ -type polymer conjugation as there are no important band shifts after fibroin impregnation in the bacterial nanocellulose.

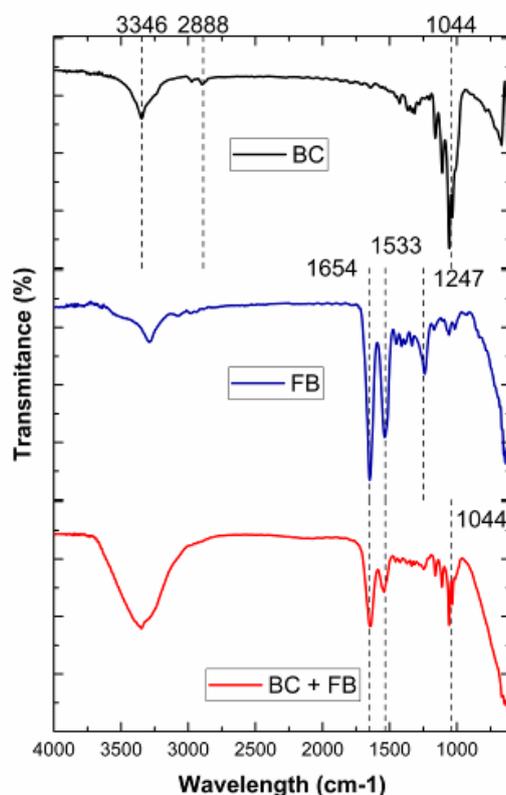


Figure 3: Fourier transform infrared spectroscopy spectra: BC (bacterial nanocellulose), FB (fibroin), BC+FB (fibroin adsorbed on bacterial nanocellulose).

The results confirm the found in FTIR spectra (Figure 3), which showed that the BC-FB interaction by hydrogen-bonded polymer conjugation. A broad band at 3500 cm^{-1} is associated with OH stretching. In addition, the positions of these bands indicate, respectively, the conformations of the protein materials (amide I, amide II, and amide III) [21, 22]. The obtained spectra of amide I (1654 cm^{-1}) is related to α -helix, β sheets, β turns and irregulars. Amide II spectra (1533 cm^{-1}) represents the angular deformation in the plane of the N-H group (60%) and by the C-N stretch (40%). The Amide III peak is a results of the combination of stretching vibrations of N - C and flexion of group C = O, and is related to the formation of β -leaf [14, 23].

The main spectra associated with BC are 3346 cm^{-1} (stretch O-H); 2888 cm^{-1} (C-H stretch), 1653 cm^{-1} (OH-deformation) and 1044 cm^{-1} (CO-deformation) [19, 24-27]. However, a peak correspondent the β -glucoside linkages between the glucose units at $\sim 890\text{ cm}^{-1}$ and C-O symmetric bridge stretching of primary alcohol (1040 cm^{-1}) and C-O-C antisymmetric bridge stretching (1168 cm^{-1}). The C-H deformation for CH_3 and OH in plane bending is observed at 1340 cm^{-1} , and the band centred at 1400 cm^{-1} is related to CH_2 bending and OH plane blending. The peak at $2888 - 2895\text{ cm}^{-1}$ corresponds to the C-H stretching vibrations of aliphatic hydrocarbons. For FB was indicated by shifts of transmittance peaks characteristic vibrational bands at 1654 cm^{-1} (amide I: C = O stretching), 1533 cm^{-1} (amide II: secondary NH bending), $1230\text{-}1270\text{ cm}^{-1}$ (amida III: C - N stretching, and C -O flexing).

For fibroin grafting on bacterial nanocellulose (BC + FB) by immersion method, the nanocellulose bands in the region $1000 - 1300\text{ cm}^{-1}$ are observed at the same positions although less relative intensities than BC spectra. It is possible to see amide I and amide II related of fibroin but less intense than the standard. The peaks at $2888\text{-}2895\text{ cm}^{-1}$ referent at C-H stretching vibrations are absent in BC + FB.

After physico-chemical analysis, the mechanical properties for BC and BC-FB membranes are explored and the results are shown in Table 1.

Table 1: Mechanical properties for BC and BC-FB membranes

| PARAMETER | BC | BC-FB |
|-----------------------|-------------------|-------------------|
| Resilience (MPa) | 0.71 ± 0.16 | 2.59 ± 0.22 |
| Relaxation (MPa) | 1.49 ± 0.05 | 1.86 ± 0.33 |
| Young's modulus (MPa) | 0.131 ± 0.007 | 0.113 ± 0.006 |

The presence of fibroin increased the membrane capacity to absorb energy when deformed (resilience) by compression. The relaxation reflects the induced stress as functions of time for BC and for the BC-FB polymeric membrane, and it helps to understand solvent transport as kinetics hydration, water permeation and pervaporation behaviours. The relaxation mechanism is essential to effectively design the biomedical membrane, especially for dressing. The stress generated on BC-FB membranes was less than on BC membranes, due to stickiness of the membrane imposed by fibroin chain. For BC-FB membranes, a lower value in Young's modulus has been observed due to the presence of fibroin, that it is related with random coil conformation (Fibroin I), which derives from processing transition in the secondary structural from β -sheet to random coil molecular conformation state [31, 32].

It is possible to improve mechanical properties of fibroin as showed by LONG *et al.* [33] when studied silk fibroin and collagen-based membrane to repair corneal tissues and observed the improvement of optical and mechanical properties of biomaterial when fibroin was added to the samples.

WANG *et al.*, [34] in order to prepared materials suitable for tissue engineering concluded when FB films were also prepared with polyethylene glycol, the samples presented greater flexibility compared to films composed of pure fibroin.

The mechanical properties of biomaterials are used as a guide to select its application. Highly resilient materials have high yield strength and low Young's modulus and this ability makes them ideal for bone tissue material [32, 34]. NOISHIKI *et al.* [35] studied films composed of FB and cellulose fibers and the results showed improved mechanical resistance. In our study, we observed the same material behaviour when FB was added to BC as the authors above [19, 24, 32, 34, 35], which means the mechanical properties were improved.

Besides the improvement of the mechanical properties, another remarkable fact is the structure with randomly distributed pores that is formed when BC and FB are mixed (Figure 4).

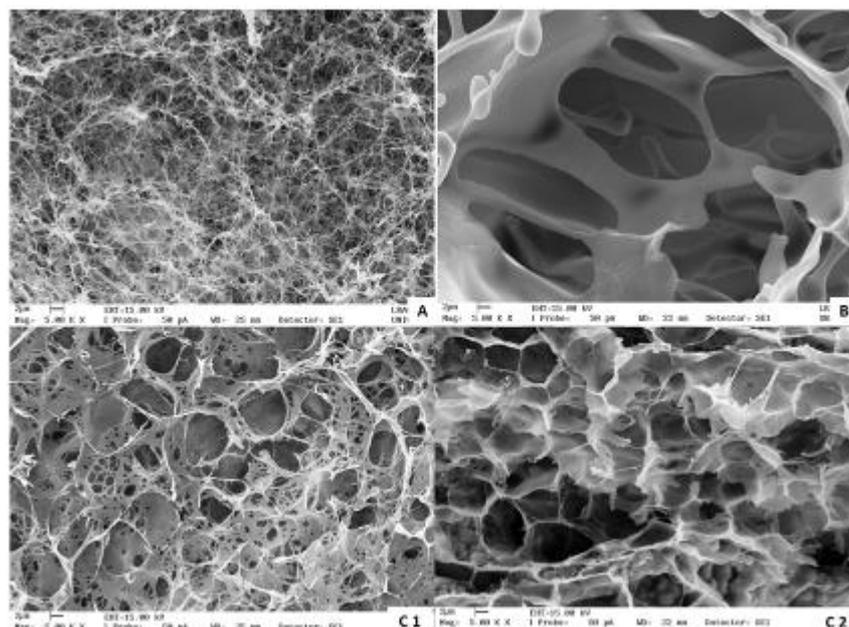


Figure 4: Scanning Electron Microscopy (SEM) microphotographs. In A, Bacterial Nanocellulose (BC); in B, Fibroin (FB) and in C1 and C2, BC-FB membranes frontal and lateral. SEM images were obtained using an accelerating voltage of 15 kV Magnification 5.00 KX and scale of $2\mu\text{m}$.

The structure resembles a honeycomb, and a similar structure was observed by CHEN *et al.* [32]. However, the authors prepared silk FB and BC nanoribbon composite scaffolds utilizing a multi-staged freeze-drying method.

Therefore, the incorporation of FB makes the membrane more anisotropic, generating a different elastoplastic behaviour and allowing the application in medical and pharmaceutical areas, tissue engineering and electronics [37, 38]. The quantitative results of the organized structure were confirmed by X-Ray microtomography assay, presented in Table II. The pores were interconnected and distributed throughout the sample and open porosity ranged from 79.2 to 88.5%, as shown in Table II. The BC structure has a lower degree of anisotropy (DA) than the BC-FB membrane. When FB was added to BC, a 49.5% increase in DA was obtained. The parameters were analysed by 3D virtual models, representative of various sections of scaffolds were built, and the data was mathematically managed by CT Analyzer software, v. 1.13.5.2.2.8.

Table 2: X-ray microtomography parameters for samples of Bacterial nanocellulose (BC) and BC and Fibroin (BC-FB) membrane

| Parameter | BC-FB | BC |
|--|--------------|---------------|
| Connectivity | 58841 ± 2942 | 22421 ± 1121 |
| Degree of anisotropy | 0.794 ± 0,04 | 0.692 ± 0,035 |
| Total porosity (%) | 88.52 ± 4.43 | 79.15±3.96 |
| Open porosity (%) | 88.51± 4.43 | 79.15±3.96 |
| Volume of open pore space (cm ³) | 489.49±24.47 | 237.47±11.87 |

*standard deviation SD ±5% ** data was mathematically managed by CT Analyzer software, v. 1.13.5.2.2.8

Although the BC already has a porous structure, when FB was added the volume was altered. Probably, because it was created a membrane with more elongated structure. This can be explained through the Structure Model Index (SMI), parameter indicates the presence of elongated or plate-like structures, data was mathematically managed by CT Analyzer software, v. 1.13.5.2.2.8. A plate-only structure has an SMI of 0 while if the structure is elongated, rod-like, the SMI value is 3 [35]. The BC-FB membrane showed high SMI value compared to the BC sample, indicating a membrane with more elongated structure. Moreover, the FB loaded in the BC structure showed an increase in the Fragmentation index, confirming the results of high connectivity between the structures. Connectivity is a geometric property that provides information about the structure of empty space [38, 39].

4. CONCLUSION

The results indicated the work reach the target of the developing a BC-FB membrane. Blending of fibroin and bacterial nanocellulose led to membranes with improved mechanical and chemical properties, making them a viable option for different applications, as scaffolds, biomaterial or packaging.

The images observed showed the structure resembling a honeycomb, with a long structure, high connectivity and high total porosity. Since the structure is essential for materials, the BC-FB membrane could be exploited in other applications such as filter membranes or bone regeneration.

Additionally, the membrane production by immersion did not use hazardous substances, promoting an environmental-friendly material and reducing costs.

5. ACKNOWLEDGMENTS

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