Research Article

Basic treatment of bacterial cellulose for use in regenerative medicine

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Abstract

Objective: In this work, samples of bacterial cellulose tried with sodium hydroxide solution at 30% in water or isopropyl alcohol was characterized. **Material and methods:** The treated sample was analyzed using Thermogravimetric Analysis, Differential Scanning Calorimetry, X-ray Powder Diffraction studies, FTIR spectroscopy and Scanning Electron Microscopy. **Results and conclusion:** The results showed an increment in the solubility of the cellulose. The yield was superior to 85%. Thermogravimetric analysis showed that the treated sample with sodium hydroxide solution at 30% in isopropyl alcohol is more stable. The X-ray diffraction analysis showed a decrease in the intensities of the curves for both samples compared with untreated cellulose. The crystallinity index diminishes lightly and changes in the morphology of the cellulose after treated were observed. In conclusion, the results showed that the predominant region is crystalline after the treatment with sodium hydroxide.

Keywords: Bacterial cellulose, crystallinity index, Infrared spectroscopy, X-ray diffraction, Hydrogen Bands Intensity, Lateral Order Index.

Introduction

Tissue engineering is a recent field that creates functioning artificial tissues and organs. Major considerations in tissue engineering include both the type of cell and the substrate (scaffold) to be used. As alternative biomaterials, the ceramic-based materials were highlighted because bioceramics have no local or systemic toxicity, absence of response to a foreign body or inflammation and appears to have the ability to integrate with the host tissue (LeGeros, 2002; Filho et al., 2013). Other biomaterials are biopolymers based on chitosan, polyurethane (PU), poly(lactic-co-glycolic acid) (PLGA) and among these, bacterial cellulose produced by *Acetobacter xylinum* (Olyveira et al., 2013).

Bacterial cellulose is a natural polymer. It possesses high crystallinity, purity (free from lignin and hemicelluloses), a large capacity for water absorption and excellent mechanical properties. The cellulose synthesized by bacterial has a fribillar nanostructure which determines its physical and mechanical properties. Due to these properties the bacterial cellulose are necessary for modern medicine and biomedical research (Czaja et al., 2007; Gathenholm and Klemm, 2010; Oliveira et al., 2015).

The structural features of the microbial cellulose and its properties can be changed by modifying its culture medium, surface modification by physical methods and modification by chemical methods to obtain a biomaterial with better properties and less rejection with cellular contact and blood contact cell interaction, which facilitates its use in tissue engineering (Olyveira et al., 2015a; Olyveira et al., 2014; Olyveira et al., 2015b).

The cellulose is renewable, biodegradable and biocompatible and can be derivatized to yield various useful products. Yet, cellulose has poor solubility due to the high amount of hydrogen bonds having the molecule (Elidrissi et al., 2012). This phenomenon affects the extensive used of this material in the development of biomaterials for medical use. This handicap is

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conventionally overcome by chemical modification of cellulose. Previous studies have evaluated the modification of cellulose by acid treatment (Rodriguez-Chanfrau et al., 2016). In this scope, treatment with alkaline solutions is a process used for cellulose activation. Dissolution of cellulose in sodium hydroxide solution depends on molecular weight, crystalline form and degree of crystallinity (Liebert et al., 2008; Northolt et al., 2001; Roy et al., 2003). In this work, the modification of the crystallinity of bacterial cellulose by basic treatment was studied.

Materials and methods

Bacterial cellulose membranes were supplied by Innovatec's – Product Biotechnology LTDA, São Carlos – São Paulo, Brazil. The acetic fermentation process was achieved by using glucose as a carbohydrate source. Results of this process are vinegar and a nanobiocellulose biomass. Bacterial cellulose (BC) is produced by Gram-negative bacteria *Gluconacetobacter xylinus,* which can be obtained from the culture medium in the pure 3-D structure, consisting of an ultra-fine network of cellulose nanofibers (Olyveira et al., 2015a; Olyveira et al., 2014; Olyveira et al., 2015b).

To achieve the modification of cellulose, sodium hydroxide solution at 30% in water (W) or isopropyl alcohol (IA) was used (Heydarzadeh et al., 2009). Sodium hydroxide were supplied by Merck. The cellulose fibers were hydrolyzed in medium basic at room temperature (32 ± 2ºC) under constant stirring for 4 h. In both case, the hydrolyzed pulp was thoroughly washed with distilled water until pH 7.0, and was wetted with ethanol and dried in an oven at 37°C until constant mass. The yield and solubility was determined according to Ioelovich (2012).

Thermogravimetric Analysis

Thermal stability of the cellulose extract was determined using a SDT-2960 Simultaneous DTA/DTG de TA Instruments (USA). Analysis was performed on samples of 10 – 15 mg in a nitrogen atmosphere from 30ºC to 800ºC at a heating rate of 5°C/minute.

X-ray powder diffraction studies

The XRD spectra were recorded at room temperature (25ºC) with a SIEMENS D5000, DIFFRAC PLUS XRD diffractometer (Germany) with BRAGG-Brentano geometry, Cu Kα radiation (λ=0.154 nm), Flicker detector and graphite monochromator. The scattering angle range from 4º to 80º with 2θ step interval of 0.02º was used. Cellulose samples were cut into small pieces and laid on the glass sample holder, analyzed under plateau conditions. An operating voltage of 40 kV and current of 30 mA was utilized, and the intensities were measured in the range of 5° < 2θ < 30°. Peak separations were carried out using Gaussian deconvolution. The d-spacings were calculated using the Bragg equation.

The surface method estimates the crystallinity index of the cellulose samples was carried out according to Ciolacu et al. (2011). The apparent crystallite size (L) using the Scherrer equation and the surface chains occupy a layer approximately 0.57 nm thick so the proportion of crystallite interior chains (X) was calculated according to Poletto et al. (2013). While Z-discriminant function was calculated according to Wada and Okano (2001).

FTIR spectroscopy

FTIR spectra of the cellulosic samples were measured on a FTIR - VERTEX 70 / BRUKER spectrometer. A total of 64 cumulative scans were taken, with a resolution of 4 cm^{-1}, in the frequency range of 4000 to 400 cm^{-1}, in transmission mode. The HBI (Hydrogen Bands Intensity), LOI (Lateral Order Index) and cell I/cell II ration was determined (Poletto et al., 2013; Wada and Okano, 2001).

Scanning Electron Microscopy

Scanning electron microscopy (SEM) imaging of crystalline cellulose was carried out using a FEG-MEV; JEOL 7500F scanning electron microscope. The equipment was operated at an acceleration voltage of 2 kV. For each sample, different parts of the grid were used to determine both average shape and size distributions. The samples were coated with a carbon layer with a thickness of 15 nm.

Results

The effect of sodium hydroxide concentration on structure and properties the cellulose were studied. Table 1 show that the solubility of the initial sample increased approximately 8%, not observed differences between the treatments used. In both cases, the yield was superior to 85%. These results showed that the treatment used not affect the yield in this working condition.

Table 1. Determination of crystallinity index, yield and solubility samples treated with sodium hydroxide solution at 30% in water or isopropyl alcohol

Sample	CI (%)	Yield (%)	Solubility (%)
NaOH 30% (W)	70.5	85.6	8.68
NaOH 30% (IA)	71.9	86.8	8.38

Thermogravimetric Analysis and Differential Scanning Calorimetric

The TG curves for all the samples show two stages of mass loss within the temperature range 25 – 600°C (Figure 1 a, b and c). Around 51°C the first degradation for all samples

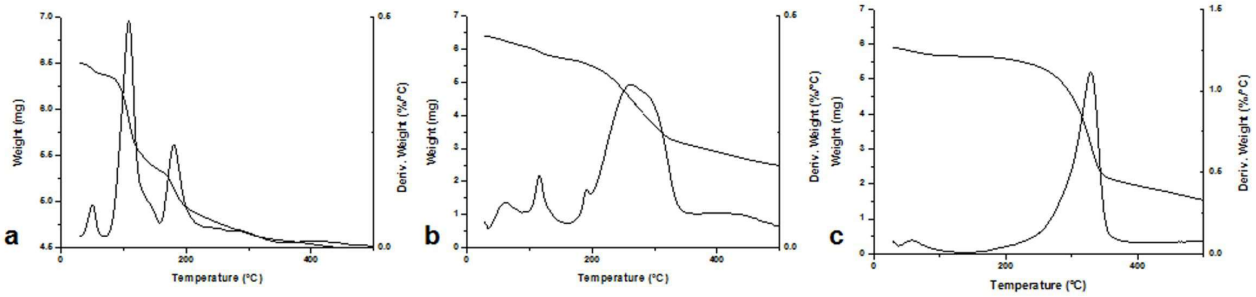

Figure 1. Results of thermal analysis. a: TG analysis of Bacterial cellulose; b: TG bacterial cellulose treated with sodium hydroxide solution at 30% in water and c: TG bacterial cellulose treated with sodium hydroxide solution at 30% in isopropyl

occurs, due to water loss (Nelson and O`Connor, 1964; Wada et al., 2010: Barud et al., 2010; Cabrales and Abidi, 2010).

For bacterial cellulose sample, the second degradation stage between 100 °C and 350°C with a mass loss was observed. In this range, two peaks of degradation were observed (108°C and 180°C), which as reports in the literature, the first corresponds to the intermolecularly H-bonded water which evaporates about 120°C (Zohuriaan and Shokrofahi, 2004). This result suggests that the peak observed at 108 °C may be related to the loss of intermolecular water of the sample evaluated. While, the peak observed at 180.7°C it is linked to thermal degradation of cellulose (Figure 1 a).

In the case of sample treated with sodium hydroxide solution at 30% in water, the TG curve showed decrease in the degradation peaks corresponding to 108 °C compared to the untreated sample (Figure 1 b). While, in the range between 200 °C and 350 °C main mass loss was observed.

In the case of sample treated with sodium hydroxide solution at 30% in isopropyl alcohol, the TG curve show only the peak corresponding to degradation process of cellulose to a temperature higher than those shown in the previous samples (range between 250°C and 350°C) (Figure 1c).

In general line the behavior was similar for both samples treated with sodium hydroxide solution at 30%, but the results indicate that the treated sample with sodium hydroxide solution at 30% in isopropyl alcohol is more stable.

X-ray diffraction studies

The XRD pattern of different samples is shown in figure 2. The XRD of bacterial cellulose shows three diffraction peaks at 2θ = 16.6; 22.7 and 35.3; which are supposed to represent the typical cellulose-I crystalline structure (Olyveira et al., 2015b; Elidrissi et al., 2012; Richmond, 1991). The cellulose crystals exhibit characteristic assignments of 110, 200 and 004 planes, respectively (Olyveira et al., 2015b). A decrease is observed in the intensities of the curves for both samples compared with untreated cellulose.

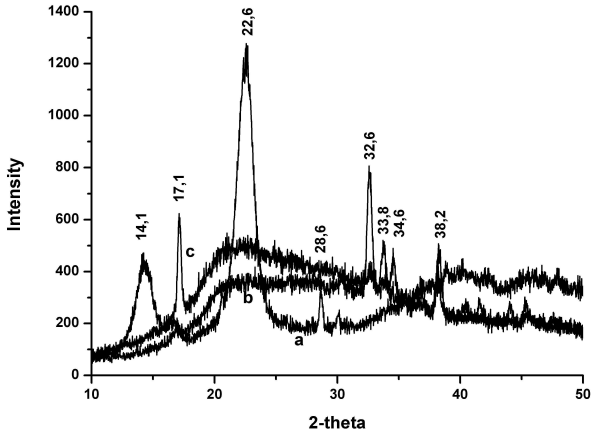

Figure 2. X-ray diffractogram. a: Rx analysis of Bacterial cellulose, b: Rx analysis of Bacterial cellulose treated with sodium hydroxide solution at 30% in water and c: Rx analysis of Bacterial cellulose treated with sodium hydroxide solution at 30% in isopropyl

The band position (2θ values) and d-spacings of the celluloses calculated from X-ray diffractograms profiles are depicted in table 2. Values of band position and d-spacings were similar.

Table 2. Band position (2θ) and d-spacings of crystalline and amorphous cellulose regions for the samples studied

Samples	(1¯10)		(110)		Amorphous	(200)	
	2θ	d (nm)	2θ	d (nm)	2θ	2θ	d (nm)
CB	14.2	0.6216	16.6	0.5359	20.6	22.7	0.3999
NaOH 30% (W)	14.4	0.6143	16.8	0.5242	20.4	21.8	0.4328
NaOH 30% (IA)	14.7	0.6068	17.1	0.5167	20.9	22.0	0.4027

The crystallinity index of untreated cellulose was 73.9%. The calculated crystallinity indexes of the different samples are given in Table 1. A very small variation of this parameter in the treated samples (values of around 4% and 2%, for the samples treated sodium hydroxide solution at 30% in water or isopropyl alcohol, respectively) was observed.

The proportion of crystallite interior chains shows

slight differences between untreated samples and treated simple with sodium hydroxide solution at 30% in isopropyl alcohol. On the other hand, the Z-values for treated samples indicate that the cellulose samples belong to the Iα dominant type (Z>0) similar to untreated sample (Table 3).

Table 3. Parameters obtained from the XRD analysis of the samples studied

Samples	L 200 (nm)	X	Z
CB	2.99	0.3828	19.99
NaOH 30% (W)	3.04	0.3906	18.19
NaOH 30% (IA)	2.35	0.2651	12.25

FTIR spectroscopy

Figure 3 shows FTIR spectra of untreated and treated bacterial cellulose. The bands at 3341 (O-H stretching intra and intermolecular H-bonds for cellulose I), 2892 (C-H stretching), 1635 (associated to the bending mode of the naturally absorbed water), 1430, 1323, 1163, 1036, and 894 cm^{-1} are associated with bacterial cellulose (Ciolacu et al., 2011; Poletto et al., 2013; Wada and Okano, 2001; Nelson and O`Connor, 1964).

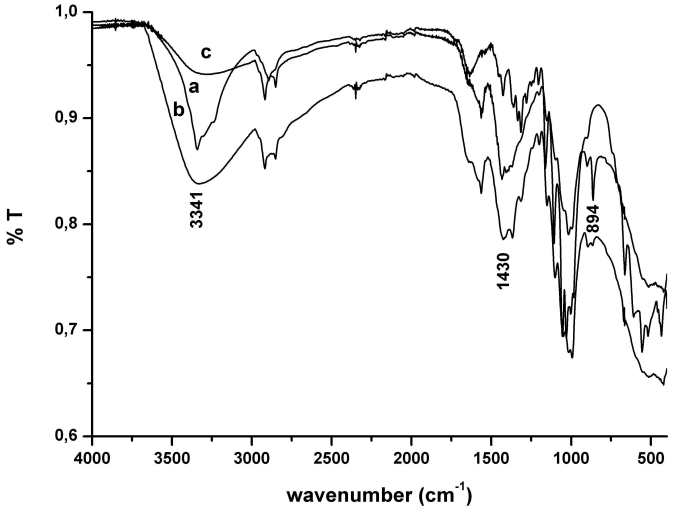

Figure 3. FTIR spectra of original and treated celluloses. a: FITR analysis of Bacterial cellulose, b: FITR analysis of Bacterial cellulose treated with sodium hydroxide solution at 30% in water and c: FITR analysis of Bacterial cellulose treated with sodium hydroxide solution at 30% in isopropyl

After treatment with sodium hydroxide solution at 30% the band at 3341 cm^{-1} (corresponding to O-H stretching intra and intermolecular H-bonds) is shifted to lower wave numbers such as 3334 cm^{-1} and 3306 cm^{-1} for treated samples with sodium hydroxide solution at 30% in water or isopropyl alcohol, respectively. In both cases a broad band was observed, being less intense band corresponding to the treated sample with sodium hydroxide solution at 30% in isopropyl alcohol. Also it observed with the band at 2892 cm^{-1} (C-H stretching) which is shifted to 2849 cm^{-1}.

The bands at 1430 cm^{-1} and 894 cm^{-1} are sensitive to the amount of crystalline versus amorphous structure in the cellulose (Oh et al., 2005). In this study, a broad band at 1430 cm^{-1} was observed which was less intense in the treated sample with sodium hydroxide solution at 30% in isopropyl alcohol. On the other hand, a sharper and intense band at 894 cm^{-1} associated with amorphous cellulose was observed. Differences between the treated samples and the untreated samples for the determination of HBI, LOI and cell I/cell II ration were not observed (Table 4).

Table 4. FTIR analysis parameters for calculated HBI, LOI and Cell I/Cell II ration

Samples	HBI	LOI	cell I/cell II
CB	0.98	0.96	0.83
NaOH 30% (W)	1.02	0.99	0.90
NaOH 30% (IA)	0.99	0.97	0.95

Scanning Electron Microscopy

Figure 4 shows SEM micrographs of the cellulose and cellulose treated with sodium hydroxide solution at 30% in water or isopropyl alcohol. Changes in the morphology of the cellulose were observed after treated with sodium hydroxide solution at 30%.

Discussion

The reason why is poorly soluble the cellulose in aqueous solution is the existence of large quantities of hydrogen bonds which group cellulose chains together to form a network (Wang, 2008). One of the main processes used in cellulose technology for activate the hydroxyl groups for the modification and/or dissolution is the treatment in strong alkali. In this the process the cellulose in the strong alkali changes the crystalline structure from cellulose I to cellulose II. Is known that the cellulose is partly soluble in sodium hydroxide solution and the amount of cellulose that is soluble depends on degree of polymerization and also mode of crystallinity (Poletto et al., 2013). Several investigations have reported that the solubility of cellulose occurs at high concentration of sodium hydroxide solution, it needs to be cooled well below room temperature (Poletto et al., 2013; Kamide et al., 1984; Wang, 2008).

In this study, high concentration of sodium hydroxide solution was used, but the reaction process was conducted at room temperature (32 ± 2°C). This can explain the solubility results obtained.

Furthermore, due to the interaction with sodium

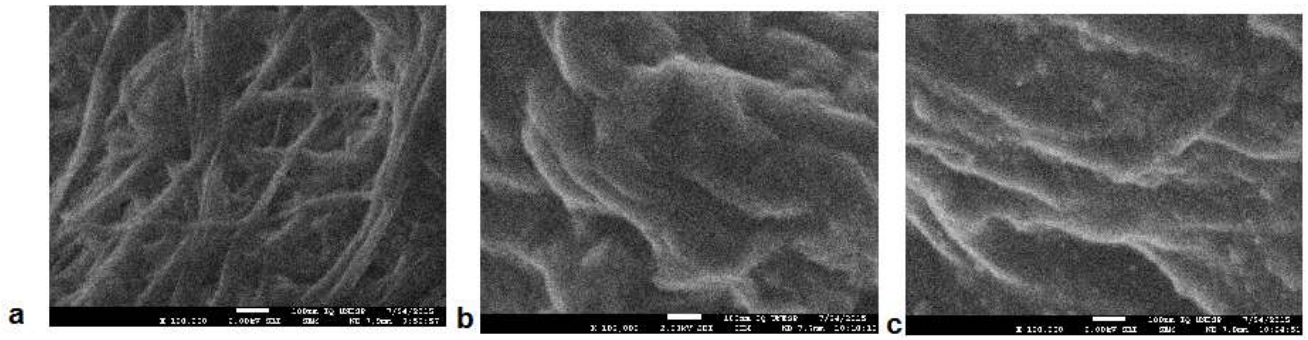

Figure 4. Results of analysis by scanning electron microscopy. a: Bacterial cellulose; b: Bacterial cellulose treated with sodium hydroxide solution at 30% in water and c: Bacterial cellulose treated with sodium hydroxide solution at 30% in isopropyl

hydroxide solution, the cellulose chains in amorphous region are rearranged while crystalline regions are hardly affected at this time (Wang, 2008). The degree of cellulose crystallinity is one of the most important crystalline structure parameters. The rigidity of cellulose fibers increase and their flexibility decrease with increasing ratios of crystalline and amorphous regions (Poletto et al., 2013). In this study, the values of crystallinity index were similar between treated samples and the untreated sample, suggesting that during the process the amorphous region undergoes rearrangement.

The main decomposition step occurs in the range of 250ºC to 350ºC. According to literature in this stage occurs the cleavage of the glycosidic linkages of cellulose, reducing the polymerization degree leading to the formation of CO_2, H_2O and a variety of hydrocarbon derivative (Bourbigot et al., 2002).

The cellulose treated with sodium hydroxide solution at 30% in isopropyl alcohol initiate a more pronounced degradation process at around 250ºC. While for cellulose treated with sodium hydroxide solution at 30% in water a more pronounced degradation process at around 200ºC. According to results, differences in the decomposition profile of the two cellulose samples indicate slight thermal stability differences for the samples.

Values of X represent the proportion of crystallite interior chain (Poletto et al., 2013). In this study lower values of X for the treated sample with sodium hydroxide solution at 30% in isopropyl alcohol was observed. However, in general the results of values of X suggests that the treated samples contain cellulose chains in a highly organized form in the interior of the cellulose crystallite.

On the other hand, the hydrogen bond intensity (HBI) of cellulose is closely related to the crystal system and the degree of intermolecular regularity, that is, crystallinity, as well as the amount of bound water, while that the lateral order index (LOI) is correlated to the overall degree of order in the cellulose and can be used to interpret qualitative changes in cellulose crystallinity and is based on the ratio of absorbance bands at specific wavenumbers. Generally, when this index decreases, crystallinity also decreases. The values of hydrogen bond intensity (HBI) and the lateral order index (LOI) were similar between treated samples and the untreated sample, indicating that the cellulose treated with sodium hydroxide solution in the proposed conditions of work, not change the crystal structure.

The FTIR analysis showed that a broad band at 1430 cm^{-1} was observed which was less intense in the treated sample with sodium hydroxide solution at 30% in isopropyl alcohol. On the other hand, a sharper and intense band at 894 cm^{-1} associated with amorphous cellulose also was observed. However, the values of crystallinity index confirms the high percentage of crystallinity, present in samples.

On the other hand, the values of cell I/cell II ration were slightly higher for treated samples. Known is that typically, cellulose I is the most abundant phase and the most sought after due to its optimal elastic properties. The structures of conventional amorphous cellulose samples are unstable in the presence of water or moisture; they usually form partially crystalline cellulose II (Poletto et al., 2013). This results indicate that the hydrolysis with sodium hydroxide solution at 30% does not modify the cellulose I type in cellulose II type, under the conditions used in this study.

Finally, although the results between both treatments are similar, a slight difference is observed. In the treated sample with sodium hydroxide solution at 30% in isopropyl alcohol the results obtained in the different evaluated parameters by X-ray and FTIR were lower than those obtained in the analysis of the treated sample with sodium hydroxide solution at 30% in water. These results indicate that the treated sample with sodium hydroxide solution at 30% in water contain more cellulose chain in a highly organized form, maybe due to higher hydrogen bond

intensity amoung neighboring cellulose chain resulting in a more packed cellulose structure.

Is known that when cellulose is treated with sodium hydroxide solution in the presence of a inert solvent (ethanol or isopropanol), the solvent acts as a swelling agent and as a dilutant which facilitates good penetration to the crystalline structure of cellulose, which facilitates the conversion of the crystalline region to amorphous region (Tasaso, 2015). However, in working conditions used in this study, results showed that the predominant region is crystalline.

Conclusions

The methods used in the characterization of the cellulose treated with sodium hydroxide solution (in water or isopropyl alcohol) showed a predominant crystalline region after the treatment. There was not significant changes in the crystallinity index of bacterial cellulose.

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