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Bacterial cellulose and hyaluronic acid hybrid membranes: Production and characterization

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ABSTRACT

In this study, the effect of the addition of hyaluronic acid (HA) on bacterial cellulose (BC) production, under static conditions was evaluated in terms of the properties of the resulting BC hybrid membranes. HA was added to the fermentation process in three distinct time points: first day (BC-T0), third day (BC-T3) and sixth day (BC-T6). Analyses of FT-IR and CP/MAS ^{13}C NMR confirmed the presence of HA in bacterial cellulose membranes. The crystal structure, crystallinity index (I_c) surface roughness, thermal stability and hybrophobic/hydrophilic character changed. Membranes with higher roughness were produced with HA added on the first and third day of fermentation process. The surface energy of BC/HA membranes was calculated and more hydrophilic membranes were produced by the addition of HA on the third and sixth day, also resulting in more thermally stable materials. The results demonstrate that bacterial cellulose/hyaluronic acid hybrid membranes can be produced in situ and suggest that HA interacts with the sub-elementary bacterial cellulose fibrils, changing the properties of the membranes. The study and understanding of the factors that affect those properties are of utmost importance for the safe and efficient use of BC as biomaterials in numerous applications, specifically in the biological field.

1. Introduction

One of the main requirements of any biomedical material is that it must be biocompatible, which is the ability to remain in contact with living tissue without causing any toxic or allergic side effects. Because of its unique properties, bacterial cellulose (BC) or microbial cellulose (MC) has been shown to be a highly effective wound dressing material. In fact, the results of various studies indicate that topical applications of BC membranes improve the healing process of burns and chronic wounds [1].

The first use explored of bacterial cellulose was as new skin substitute and membranes of bacterial cellulose commercialized and produced in Brazil as Biofill®, Bionext® and Membracel®, proved to be a very successful wound covering for skin problems such as burns and chronic ulcers [2–6]. Another product called XCell, which is manufactured by Xylos Corporation was very effective in (a) promoting autolytic debridement, (b) reducing pain, and (c) accelerating granulation, all of which are important for proper wound healing [7]. Several research groups in different places of world (United States, Canada, China, Brazil, Portugal, Japan, Sweden, France, Spain, Italy and others) develop researches with this biotechnological product and because of its biofabrication mode, great potential for use in novel materials have emerged.

The first studies with bacterial cellulose started with static fermentation (films production), after BC spheres [8,9] or fiber bundles were produced from agitated conditions (shaking the reactor), hollow tubes were prepared not directly in the reactor vessel but on a glass or silicon cylinder as a matrix/template is placed in the culture medium inside of the reactor [10]. The small and short tubes are useful as blood vessel substitutes in microsurgery; the larger represent novel types of cardiovascular implants [11].

BC hydrogel have recently been produced using porous wax particles in the fermentation broth, leading to macroporous BC materials that can be used to support human smooth muscle cell migration, proliferation, and differentiation [12]. These BC materials are very attractive for use as a scaffold for regeneration of cartilage, bone, urethra, and bladder [11]. Scaffolds are mechanical substrates that may interact with the cells and the surrounding tissue [13]; they can be classified depending on their morphology as hydrogels, fibrous constructs and porous scaffolds [14].

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Different additives were added to *Acetobacter xylinum*, nowadays *Gluconoacetobacter xylinum*, growth media for study their influence on yield, morphology and crystalline constituents of BC, including agar [15], sodium alginate [16], carboxymethylcellulose (CMC) [17,18], pectin [16], carbon nanotubes [19], polyacrylamide [20], xylan [21], xyloglucan [22], acetyl glucomannan [23], lignosulfonate [24] and microcrystalline cellulose [25].

The fabrication of scaffolds from natural materials, such as hyaluronic acid (HA), can impart intrinsic signals within the structure that can enhance tissue formation. HA is a linear polysaccharide formed from disaccharide units containing *N*-acetyl-D-glucosamine and glucuronic acid and is a common component of synovial fluid (SF) and extracellular matrix (ECM). Industry has turned to microbial fermentation processes for the production of HA; *Streptococcus epizooticus* and *Streptococcus equi* strains are commercially used in HA synthesis [26]. *Bacillus subtilis*, carrying the *hasA* gene from *Streptococcus equisimilis* encoding the enzyme HA synthase is one of the promising potential candidates for production on a large scale [27].

Ocular surgery, visco supplementation for osteoarthritis, in cosmetics, in ophthalmology, in aesthetic medicine, in surgery, topical drug delivery, wound healing and in tissue engineering are some of HA uses [28–30]. However, the poor mechanical properties, rapid degradation and clearance in vivo of uncrosslinked soluble HA limit many direct clinical applications.

Therefore, the objectives of this study were to observe if HA can be introduced in the bacterial cellulose membranes during fermentation process and analyze the interference with in situ BC crystallization, morphology, thermal stability and surface properties.

Investigations have reported the synthesis of novel biomimetic hydrogels based on crosslinking cellulose derivatives with hyaluronic acid, between hydroxyethylcellulose (HEC) and carboxymethylcellulose (CMCNa) through the difunctional crosslinker divinyl sulfone [31] or with a water-soluble carbodiimide [32]. These methods are important and show potential for development of biomimetic products but they are complex, expensive and the biocompatibility of the crosslinking agent used is particularly important.

In our work we evaluated the production of hybrid membranes through a simple, fast and low-cost method, without addition of any component biologically incompatible, seeking the formation of hybrid membranes with different crystallinity, morphology, roughness of surface and with distinct surface properties, which can make them useful as future scaffold for tissue regeneration.

2. Materials and methods

2.1. Materials

The bacterial strain used in this study was *A. xylinum* ATCC 23769 (reclassified as the genus *Gluconacetobacter*) obtained from Foundation André Tosello from Campinas, São Paulo which was grown in a glucose medium based on the Hestrin–Schramm's medium culture [33], this glucose medium is composed of 4 g L^{-1} glucose, 5 g L^{-1} yeast extract, 5 g L^{-1} peptone, 2.7 g L^{-1} Na_2HPO_4, and 1.15 g L^{-1} citric acid. All media were autoclaved at 121 °C and 1.02 atm for 20 min. The hyaluronic acid (sodium hyaluronate) from *S. equi* (53747) (CAS number 9067-32-7) was added in the fermentation process.

2.2. Methods

2.2.1. Storage of bacteria and growth conditions

The cell suspensions of *G. xylinum* were stored at −80 °C in glycerol solution. One milliliter of cell suspension stored at −80 °C was added into flask with 40 mL of Hestrin–Schramm's medium. The pre-inoculum was prepared by growing *G. xylinum* at 28 °C using a rotary shaker (180 rpm) for two days.

2.2.2. Fermentation process

During growth in shake flasks the culture appeared as a fine suspension of cells and irregular clumps of different sizes. The volume of inoculum was 10% of the total volume, to the static process. After two days of growth the ratio of clumps to cell suspension was transferred to a 160 mL flask containing Hestrin–Schramm's medium. The static fermentation process was realized at 28 °C during 10 days. At the end of this period the bacterial cellulose membranes were removed from static culture and purified by immersion in an aqueous solution of 0.1 mol L^{-1} NaOH for one day. The BC pellicles were then washed with deionized water several times to completely remove the alkali. The membranes were dried in stove at 40 °C.

2.2.3. Bacterial cellulose/hyaluronic acid membranes production

For the production of hybrid membranes, hyaluronic acid (sodium hyaluronate, $M_W = 1.1–1.7 \times 10^6$ Da, predispersed in water 1%) was added to the fermentation process at different time points: first day of fermentation (T0), which corresponds to the moment of *G. xylinum* inoculation, third day (T3) and sixth day (T6) after the start of fermentation. The modified membranes were identified, respectively as BC-T0, BC-T3 and BC-T6. To eliminate impurities such as bacteria and other interfering substances, cellulose membrane floating on the surface of the culture medium was collected and immersed in an aqueous solution of 0.1 mol L^{-1} NaOH for one day. The BC pellicles were then washed with deionized water several times to completely remove the alkali, and afterwards dried in stove at 40 °C (same procedure described above).

2.2.4. Characterization of membranes native and modified

2.2.4.1. X-ray diffraction—XRD. X-ray diffraction spectra of the bacterial cellulose membranes were obtained using a Panalytical X'Pert PRO MPD diffractometer (The Netherlands), using *K*α copper radiation (λ = 1.5418 Å), at 40 kV and 30 mA. All assays were performed with ramping at 1° min^{-1}, analyzing the range of 5–40° (2θ).

The degree of crystallinity was taken as $CrI = (I_{200} - I_{am})/I_{200}$, where I_{200} is the overall intensity of the peak at 2θ (about 22.9°) and I_{am} is the intensity of the baseline at 2θ (about 18°) [34].

2.2.4.2. Fourier transform infrared spectroscopy analysis–FT-IR. FT-IR spectra of dried membranes were recorded on a FT-IR Bommen MB-100 in transmission mode in the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1}.

2.2.4.3. Solid-state CP-MAS ^{13}C NMR. NMR experiments were performed at 24 °C using a ADVANCE 400 (Bruker Spectrometer) operated at a ^{13}C frequency of 100.6 MHz, using the technique of cross-polarization (CP) at the magic angle (MAS) from finely comminuted or particulate samples and using glycine as external standard.

2.2.4.4. Scanning electron microscopy—SEM. The qualitative assessment of the morphology of the membranes was performed using a FEI Quanta 200 microscope (Oregon, USA). Membranes pieces were mounted on the bronze stubs using double-sided tape and then coated with a layer of gold (40–50 nm), allowing surface visualization. The average width of the microfibrils was estimated from measures performed on 10 microfibrils, using the software ImageJ.

2.2.4.5. Atomic force microscopy—AFM. Tapping mode AFM images were recorded in air using a Agilent 550 microscope (Agilent