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Enhancement of fibroblast growing on the mannosylated surface of cellulose membranes

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ABSTRACT

Bacterial cellulose membrane is a biomaterial with high value in the biomedical field. Many groups have been making efforts to promote chemical modifications of its structure and, consequently, add new characteristics. Recently, our group has developed a methodology to insert monoester succinic acid in bacterial cellulose membrane without disrupting the microfibril network and bind a protein on it. Considering the role of carbohydrates in the molecular recognition process in biological events, we continued these studies by inserting covalently multiples copies of aryl monosaccharide to bacterial cellulose succinylated and to study the in vitro tissue compatibility using fibroblasts. The mix of synthetical chemistry and material modification was performed to prepare aminoaryl mannoside and conjugate it, via amide bond using ultrasonic irradiation, to succinic group of bacterial cellulose. This material was characterized chemically (IR, UV–vis, ^{13}C NMR CP-MAS) and physically (TGA and AFM). Mannosylated cellulose showed good in vitro compatibility with fibroblasts demonstrating its potential in the tissue engineering field which could provide a tissue compatible scaffold.

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1. Introduction

Bacterial cellulose (BC) produced by *Gluconacetobacter xylinus* is a biopolymer constructed by union of units of anhydroglucose linked by β—(1–4) bonds. It has the same chemical structure of vegetal cellulose, in which there are many hydroxyls groups [1], but shows different polymerization degrees [2] and higher crystallinity [3]. This special material with many commercial applications, from electronics to biomedical field has his characteristics explained by the intrinsic structure characteristics, which results in macroscopic attributes, like strength and biocompatibility [4–7]. In biomedical field, bacterial cellulose has been used as skin bandage, coating cardiac stents, covering of chirurgical material, drug release, etc. [5,8–13] The compatibility with tissues and the inefficiency of human body to degrade it due to lack of β-glucosidases among other factors, explains the successful of the great range of applications of bacterial cellulose in this field.

In the material science field, many research groups have been making efforts to study the structure of BC and to modify it. In general, chemical composition modification, especially those in which bioactive molecules are introduced, might add new biological functions to any material, which becomes a biomaterial. Some strategies have been applied to achieve new derivatives of cellulose (bacterial or vegetal), including composites formation and attaching material by covalent bond [14–22,13]. The latter represents an elegant methodology due to the finesse of the modification at molecular level forming a better know structure material.

Regarding the biological context, the molecular recognition process is a very attractive event to be mimicked by these new design materials and the introduction of different bioactive or biomolecules has been bonded with different host materials [15]. Carbohydrates and proteins have been a constant target addressed to be attached in new materials due to their role in nature [23]. Besides the role and the importance of enzymes, the carbohydrate-protein interactions also justify the attachment of both classes of molecules. Many biological events take place after their mutual recognition, thus selecting the appropriated chemical motif in a material can bring together a desired biological activity.

Covalent attachment of carbohydrates on the surface of different materials has been described as a proof of concept to investigate whether their protein counterpart can recognize these materials. Modification

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of graphene surface by dendronized mannosides conferred better water solubility and recognition by concanavalin A [24]. In a similar way, mannose dendronized cellulose was able to recognize the same lectin and be used as biosensor, after some chemical steps modifications [25]. Therefore, the insertion of carbohydrates in a multivalent presentation, in this particular case over a surface of a material, is an important step in the development of potential new biomaterials.

In the case of bacterial cellulose, the research aimed to bring to it new characteristics besides the native ones is an attractive field of work. Our group has been pursuing chemical methodologies to modify and bond biological relevant molecules to the surface of bacterial cellulose in parallel to understand the structural changes that it could bring. As a sequence of the methodology presented by Ribeiro-Viana [26], we have used a mix of homogeneous and heterogeneous chemical synthesis to insert an aryl mannoside over a surface of a succinylated bacterial cellulose membrane and fully chemically (NMR, IR and UV–vis) and topologically (TGA and AFM) characterize it. Besides, this new material showed good compatibility with growing of fibroblast, demonstrating its potential in the tissue engineer field.

2. Material and methods

Reagents were purchased from Sigma-Aldrich and Fluka, and were used without purification. Pyridine and dimethylformamide were dried over molecular sieves (3 Å) for 48 h.

2.1. Bacterial cellulose production

The *Acetobacter xylinum* ATCC 23769 (*Gluconacetobacter xylinus* ATCC 23769) bacterial strain was grown in a glucose medium based on the Hestrin–Schramm culture medium [27]. The cellulose was produced as described by Goelzer et al. [28] The bacterial cellulose pellicle was purified by immersion into an aqueous solution of 0.1 mol.L^{-1} sodium hydroxide for one day. The films were repeatedly washed with deionized water, until it reached pH 7.

2.2. Analytical methods

2.2.1. Liquid-state NMR analysis

^{1}H and ^{13}C spectra were recorded on a Bruker Avance III 400 MHz spectrometer operated at a frequency of 400.6 MHz for ^{1}H, and at 100 MHz for ^{13}C using a 5 mm inverse probe, BBI, at 25 °C. Chemical shifts (δ) for ^{1}H spectra are expressed in ppm and calibrated according to the residual solvent signal ($CHCl_3$; δ = 7.26 ppm; HOD: δ = 4,76 ppm). Chemical shifts (δ) for ^{13}C spectrum are expressed in ppm and calibrated according to the solvent signal ($CDCl_3$; δ = 77.00 ppm).

2.2.2. Solid-state ^{13}C CP-MAS

NMR analysis NMR experiments were performed at 25 °C using a Bruker AVANCE III 400 MHz spectrometer operated at the ^{13}C frequency of 100 MHz, using the technique of cross-polarization (CP) at the magic angle (MAS) from finely comminuted or particulate samples which were sandwiched between CaO powder to fill homogeneously the 4 mm rotor, and using glycine as an external standard. The ^{13}C spectra were obtained using Bruker standard pulse program (CP) with SI of 32 K and contact time of 2 ms (p15). The spectra were processed by manually correcting the phase and automatically smoothing by the software (ACD NMR Processor Academic Edition—polynomial 5th, number of points = 7).

2.2.3. Fourier transform infrared (FT-IR) spectroscopy analysis

Infrared spectra were recorded on a Shimadzu FT-IR PRESTIGE-spectrometer directly from the film in the range of 4000–400 cm^{-1}. 16 scans were taken for each sample with a resolution of 1 cm^{-1} in the transmission mode.

2.2.4. UV–Vis analysis

UV–vis analyses of the membranes were carried out in a Shimadzu UV-2600 spectrophotometer equipped with a film holder (P/N 204–58909) directly in the film, in the 200–800 nm range.

2.2.5. Atomic force microscopy

AFM micrographs were recorded with a NanoSurf FlexAFM (AG, Switzerland) instrument, using silicon AFM probes. The topography and surface roughness of films were analyzed in air. For some films, the average of pore size and roughness was estimated from line scans performed on about 3 films using ImageJ software.

2.2.6. Thermogravimetric analysis (TGA)

Thermal analyses were performed on a Shimadzu TGA-50. The scans were run from room temperature to 450 °C at a rate of 10 °C per minute under nitrogen flow.

2.3. Chemical synthesis

2.3.1. Succinylation of cellulose membrane

Bacterial cellulose membranes were prepared as described previously [26]. Briefly, before the reactions, the membranes were prepared by solvent exchange in order to eliminate the excess water. First, they were immersed in MeOH and soaked for five minutes. The solvent was discarded and the same procedure was repeated twice. Then, the membranes were immersed in CH_2Cl_2 and gently soaked for five minutes. The solvent was discarded and the same procedure was repeated twice.

The succinylated cellulose (BC-Sc) was prepared by immersing cellulose film into 10 mL of CH_2Cl_2, 174 μL of pyridine and 216 mg of succinic anhydride and performed in a static system. The reaction was performed in 16 h under reflux. After that time, the reaction was quenched by adding 2 mL of MeOH. Then, the membrane was washed three times with water, then with EtOH and finally dried at room temperature.

2.3.2. Mannosyl derivatives

2.3.2.1. Synthesis of p-nitrophenyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (2). In a 50 mL flask, per-*O*-acetyl mannose (1) [29] (0.5 g, 1.28 mmol), dry CH_2Cl_2 (5 mL) and *p*-nitrophenol (0.53 g, 3.84 mmol) were added under inert atmosphere. $BF_3.Et_2O$ (0.78 mL, 6.4 mmol) was added dropwise under ice-bath for 20 min. The solution was stirred under Ar atmosphere and at room temperature for 24 h. The reaction medium was washed with NaOH (0.1 mol.L^{-1}) until the pH 10, then with saturated NaCl solution until reach pH 7. The organic layer was dried over Na_2SO_4, filtered and then the solvent evaporated. The residue was recrystallized from AcOEt/hexane, furnishing a light yellow solid (0.183 g, 30%).

^{1}H NMR (400 MHz, $CDCl_3$), δ 8.22 (d, J = 9.28 Hz, 2H, Ph), 7.20 (d, J = 9.25 Hz, 2H, Ph), 5.62 (d, $J_{1,2}$ = 1.70 Hz, 1H, H_1), 5.53 (dd, $J_{3,2}$ = 3.4, $J_{3,4}$ = 10.0 Hz, 1H, H_3), 5.46 (dd, $J_{2,1}$ = 1.7, $J_{2,3}$ = 3.4 Hz, 1H, H_2), 5.38 (t, $J_{4,3}$ = $J_{4,5}$ = 10.0 Hz, 1H, H_4), 4.26 (dd, $J_{6,5}$ = 5.5, $J_{6,6'}$ = 12.4, 1H, H_6), 4.07 (dd, $J_{6',5}$ = 2.3, $J_{6',6}$ = 12.4 Hz, 1H, $H_{6'}$), 4.01 (ddd, $J_{5,6'}$ = 2.3, $J_{5,6}$ = 5.5, $J_{5,4}$ = 10 Hz, 1H, H_5), 2.21 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.02 (s, 3H, OAc).

2.3.2.2. Synthesis of p-nitrophenyl α-D-mannopyranoside (3). In a 25 mL flask, mannoside 2 (261 mg, 0.55 mmol) was dissolved in anhydrous MeOH (2.60 mL) and 1 mol.L^{-1} MeONa (0.2 mL) was added under ice bath. After 10 min stirring, the ice bath was removed and the solution was stirred at room temperature for 40 min. Amberlite IRA- 120 was added until pH 6, then the solution was filtered and solvent evaporated (0.132 g, 78%).

^{1}H NMR (400 MHz, D_2O) δ 8.26 (m, J = 9.25, 2H, Ph), 7.29 (m, J = 9.31 Hz, 2H, Ph), 5.77 (d, $J_{1,2}$ = 1.8 Hz, 1H, H_1), 4.20 (dd, $J_{2,1}$ = 1.8,

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