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Bacterial cellulose as a support for the growth of retinal pigment epithelium

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Bacterial cellulose as a support for the growth of retinal pigment epithelium

Abstract
The feasibility of bacterial cellulose (BC) as a novel substrate for retinal pigment epithelium (RPE) culture was evaluated. Thin (41.6 ± 2.2 μm of average thickness) and heat-dried BC substrates were surface-modified via acetylation and polysaccharide adsorption, using chitosan and carboxymethyl cellulose. All substrates were characterized according to their surface chemistry, wettability, energy, topography, and also regarding their permeability, dimensional stability, mechanical properties, and endotoxin content. Then, their ability to promote RPE cell adhesion and proliferation in vitro was assessed. All surface-modified BC substrates presented similar permeation coefficients with solutes of up to 300 kDa. Acetylation of BC decreased it's swelling and the amount of endotoxins. Surface modification of BC greatly enhanced the adhesion and proliferation of RPE cells. All samples showed similar stress-strain behavior; BC and acetylated BC showed the highest elastic modulus, but the latter exhibited a slightly smaller tensile strength and elongation at break as compared to pristine BC. Although similar proliferation rates were observed among the modified substrates, the acetylated ones showed higher initial cell adhesion. This difference may be mainly due to the moderately hydrophilic surface obtained after acetylation.

Keywords
growth, cellulose, bacterial, support, retinal, epithelium, pigment

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Bacterial Cellulose As A Support For The Growth
Of Retinal Pigment Epithelium

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KEYWORDS: Bacterial Cellulose; Surface Modification; Retinal Pigment Epithelium; Macular
Degeneration.
ABSTRACT: The feasibility of bacterial cellulose (BC) as a novel substrate for retinal pigment epithelium (RPE) culture was evaluated. Thin (41.6 ± 2.2 µm of average thickness) and heat-dried BC substrates were surface modified via acetylation and polysaccharide adsorption using chitosan and carboxymethyl cellulose. The BC substrates were characterized according to surface chemistry, wettability, energy, topography, permeability, dimensional stability, mechanical properties and level of endotoxins present. Then, the ability to promote RPE cell adhesion and proliferation in vitro was assessed. BC substrates were porous and permeable to solutes up to 300 kDa. The acetylation decreased substrate swelling and the amount of endotoxins present. Surface modification greatly enhanced the adhesion and proliferation of RPE cells in BC. Although similar proliferation rates were observed between the modified substrates, the acetylated ones showed higher initial cell adhesion. This difference may be mainly due to the moderately hydrophilic surface obtained after acetylation.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness among the elderly in industrialized countries, and its cure is of increasing socioeconomic interest within the progressive demographic right-shift.1-4 This retinal degenerative disease affects the retinal pigment epithelium (RPE), the Bruch’s membrane (BM), and the choroid, consequently leading to the damage of the photoreceptors.1,2 The RPE constitutes the outer blood-retina barrier, preventing the transport of molecules larger than 300 kDa into and out of the retina.5,6 Apically facing the photoreceptors and basolaterally the BM, RPE is a cell monolayer responsible for several complex functions essential for the visual function.5 Located between the RPE and the
fenestrated choriocapillaries, the BM is an extracellular matrix structure composed of elastin and collagen that partly regulates the reciprocal exchange of biomolecules, nutrients, oxygen, fluids and metabolic waste products between the retina and the general circulation. When the RPE cells are lost and the BM is compromised in retinal degenerative diseases, treatment approaches are limited to regenerative strategies, visual prostheses (either electrical or biologic), or standard vision rehabilitation (facilitate a closer reading distance or magnify a distant image). Human retinal transplantation has followed many years of experimental research showing that transplanted RPE cells have the potential to rescue photoreceptors. Therefore, healthy stem cell derived RPE-like cells, delivered as an integer epithelial sheet on a BM prosthetic substrate, represents a promising therapeutic approach in AMD. A BM prosthetic substrate should be able to perform the BM’s primary functions: regulation of (bio-) molecules passive diffusion between choroid and RPE; provide physical support for RPE adhesion, migration and perhaps differentiation; and act as a division barrier for cell migration. In addition, these substrates should be biocompatible, able to maintain the RPE phenotype, and exhibit favorable surgical properties (handling stability and resistance to tear). Recent works in the development of biocompatible substrates to patch or replace diseased BM for the delivery and long-term survival of RPE transplants include: polyethylene terephthalate and poly(L-lactide-co-e-caprolactone) films and electrospun substrates; montmorillonite clay based polyurethane substrates; methacrylate-based copolymer electrospun fibrous scaffold; ultrathin and biofunctionalized polyimide membranes; and ultrathin collagen membranes.

Gluconacetobacter xylinus bacteria synthesize bacterial cellulose (BC) in a complex process that results in a three-dimensional gelatinous structure formed on the surface of a liquid medium. The biosynthetic process involves the polymerization of single glucose residues into linear β-
1,4-glucan chains, the extracellular secretion of these linear chains, and the assembly and crystallization of the glucan chains into hierarchically composed ribbons. The combination of BC unique structural and mechanical properties, with biocompatibility, moldability in situ, permeability for gas and fluid exchange, high hydrophilicity, transparency and non-toxicity make it an attractive candidate for biomedical applications. BC has proven to be a versatile biomaterial, and particularly interesting for tissue-engineered products towards both wound care and regeneration of damaged or diseased organs. Its unique properties have sustained the elevator pitch of several BC applications, especially in the biomedical field, where temporary skin substitutes and artificial blood vessels appear as patented products (such as Biofill and BASYC). Recent studies on the potential use of BC as a biomaterial include artificial skin, vascular grafts, conduits in urinary reconstruction and diversion, cartilage replacement, bone regeneration, artificial cornea, tissue engineering hydrogels and scaffolds. Also BC is not biodegradable in the human body, which can be beneficial since substrates developed with degradable materials and biological tissues can be difficult to handle and may induce retinal degeneration due to material degradation.

In this work, we evaluated the ability of RPE cells to adhere and grow on BC-based substrates. The ultimate goal of this research is to evaluate the potential of BC as a novel substrate for RPE transplantation in retinal degenerative diseases. Since surface properties play an important role in cell adhesion and proliferation, the BC surface was surface modified to obtain different surface profiles that could differently affect RPE cell response. Two surface modification approaches were performed, namely acetylation and polysaccharide adsorption using chitosan or carboxymethyl cellulose. The first modification decreases the surface hydrophilicity of BC, while the second increases protein adsorption by the incorporation of amine or carboxymethyl
groups. BC substrates were characterized according to surface chemistry, topography, free energy, wettability, permeability, dimensional stability (handling stability and swelling), mechanical properties and the amount of endotoxins present. RPE cultures were performed to evaluate cell viability and proliferation on the BC substrates.

MATERIAL AND METHODS

**Bacterial Cellulose (BC) Production.** BC substrates were produced in static cultures of *G. xylinus* (ATCC ® 53582™) as reported elsewhere. Cultures were performed in 1 L Erlenmeyer flasks with 200 mL of Hestrin–Schramm culture medium for 1 month. The resulting BC sheet was washed with abundant tap water and placed for 24 h in a 1.0 N NaOH solution to remove residual medium and bacteria. The alkaline residues were removed by washing thoroughly with distilled water. Afterwards, the BC sheets were sliced into thin pellicles, cut into the desired geometric forms, dried in an oven at 50 °C for 8 h, autoclaved (120 °C, 1 bar, 20 min) and maintained in distilled water. Only dried BC was used in this study to reduce its water-holding capacity, and consequently minimize undesired swelling. Thickness measurements were performed using a digital micrometer (No. 293-5, Mitutoyo, Japan) in each sample to normalize the swelling estimation data and for the stress-strain assays. The average thickness used for all experiments was 41.6 ± 2.2 µm.

**Surface Modification.** Dried BC samples were surface modified by two different approaches: (1) acetylation (Figure 1A) to introduce acetate functional groups; and (2) polysaccharide adsorption using either chitosan (Figure 1B) or carboxymethyl cellulose (CMC) (Figure 1C) to
introduce amine and carboxymethyl groups, respectively. The surface area per reaction volume ratio was approximately 1 mL.cm\(^{-2}\) of BC samples, for both modification approaches.

**Figure 1.** Scheme of bacterial cellulose (BC) acetylation reaction (A), and molecular structures of chitosan (B) and carboxymethyl cellulose (C).

For the surface acetylation process, dehydrated samples were first added to the reaction mixture of 40 mL acetic acid (Fisher), 50 mL toluene (Fisher) and 0.2 mL perchloric acid (Panreac). Afterwards, 8 mL of anhydride acetic (Merck) was added to the reaction mixture with BC samples, and stirred for 15 min at room temperature.\(^{38}\) In the end, acetylated substrates (ABC) were washed, sterilized in ethanol and maintained in sterile distilled water until use.

Chitosan (85% deacetylated, Sigma) and CMC (British Drug Houses) were adsorbed onto BC substrates as described in the literature.\(^{38}\) Polysaccharides were first dissolved at 1 mg.mL\(^{-1}\) in an aqueous acidic solution of 50 mM sodium acetate buffer (SAB) containing 50 mM NaCl (pH 5.0). BC samples were added to either CMtabelC or chitosan solutions, and allowed to adsorb for
18 h at room temperature, under constant stirring. BC samples with adsorbed chitosan (CBC) and CMC (CMBC) were washed three times with SAB solution (1 h each time) to remove non-adsorbed polysaccharides, autoclaved in SAB solution and maintained in sterile PBS solution.

**Surface Chemistry.** Attenuated Total Reflectance–Fourier Transform Infrared (ATR-FTIR) spectroscopy was used to identify surface functional groups, using a Perkin-Elmer Spotlight 300 FTIR microscope with Spectrum 100 FTIR spectrometer. All spectra correspond to the average of 100 scans measured at a resolution of 4 cm$^{-1}$. The vibration transition frequencies of each spectrum were baseline corrected and the absorbance was normalized between 0 and 1.

**Surface Energy and Wettability.** Static contact angle measurements were performed using the sessile drop method to assess the degree of surface hydrophobicity and surface free energy (SFE, $\gamma$). However, this method requires a flat and smooth solid surface. Since the surface of BC substrates may present some roughness, the contact angles that will be measured are the apparent contact angles, as referred to in the literature for rough surfaces.$^{39}$ The apparent contact angles will therefore be used to evaluate surface wettability and estimate surface free energy. Static contact angles were measured at room temperature, using a Dataphysics OCA-20 drop shape analysis system (DataPhysics Instruments GmbH, Filderstadt, Germany) controlled by SCA20 software (droplet size 3 µL). The water contact angle indicates directly the degree of hydrophobicity. For SFE estimation, the van Oss-Chaudhury-Good (vOCG) method was selected since it provides more information regarding the interfacial acid-base interactions.$^{40,41}$ Through this method, the forces involved in a solid SFE can be divided into two components: Lifshitz van der Walls or dispersive ($\gamma^{\text{LW}}$) and acid-base or polar ($\gamma^{\text{AB}} = 2\sqrt{\gamma^+\gamma^-}$), where the latter can be
further subdivided in basic ($\gamma^-$) and acidic ($\gamma^+$) constituents.\textsuperscript{40,41} The equation that describes the interaction of these forces in the vOCG method is the following:

$$\gamma_L(1 + \cos \theta) = 2\sqrt{\gamma_S^{\text{LW}}\gamma_L^{\text{LW}}} + 2\sqrt{\gamma_S^+\gamma_L^-} + 2\sqrt{\gamma_S^-\gamma_L^+}$$ \hspace{1cm} (1)

Since there are three unknowns, $\gamma_S^{\text{LW}}$, $\gamma_S^+$, and $\gamma_S^-$, the solution of a system of three independent linear equations is needed to determine these quantities. Therefore, three reference liquids, with known energy components (see Table 1), were used, one non-polar (bromonaphthalene) and two bipolar liquids (water and formamide).\textsuperscript{41-43}

**Table 1.** Surface energy components of probe liquids used in contact angle measurements\textsuperscript{35,36}

<table>
<thead>
<tr>
<th></th>
<th>mJ/m$^2$</th>
<th>$\gamma \equiv \gamma^{\text{LW}} + \gamma^{\text{AB}}$</th>
<th>$\gamma^{\text{LW}}$</th>
<th>$\gamma^{\text{AB}} = 2\sqrt{\gamma^+\gamma^-}$</th>
<th>$\gamma^+$</th>
<th>$\gamma^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>72.80</td>
<td>21.80</td>
<td>51.00</td>
<td>25.50</td>
<td>25.50</td>
<td></td>
</tr>
<tr>
<td>Formamide</td>
<td>58.00</td>
<td>39.00</td>
<td>19.00</td>
<td>2.28</td>
<td>39.60</td>
<td></td>
</tr>
<tr>
<td>Bromonaphthalene</td>
<td>44.40</td>
<td>44.40</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Permeation.** To assess substrate permeation, diffusion experiments were performed based in previous works.\textsuperscript{7,44} The three systems used were composed of two chambers, each with a capacity of 1.2 mL, connected with a circular opening with 10 mm diameter. Since the diffusion setup was equivalent to the one performed by Julien et al.\textsuperscript{7}, the same model (Equation 2) was used to estimate the permeation coefficient (P):

$$P \cdot t = -\log\left(\frac{(C_0 - 2C)}{C_0}\right) \cdot 2303(\frac{L \cdot V}{2S})$$ \hspace{1cm} (2)

where $C$ is the concentration (g.cm$^{-3}$) in the acceptor chamber at a given time point $t$ (s); $C_0$ is the concentration in the donor chamber at $t = 0$; $P$ is the permeability constant (cm$^2$.s$^{-1}$); $L$ is the
membrane thickness (cm); V is the volume of the solutions in the chambers (cm$^3$); and S is the membrane surface area (cm$^2$). The permeability constant can be obtained from the slope, when plotting the right side of the equation as a function of time. The donor chamber was filled with a 4 % w/v solution of polyethylene glycol (PEG), and the acceptor chamber with distilled water. Two PEGs with different molecular weights were used, 35 kDa (Sigma) and 300 kDa (Acros). The BC substrates were placed between the two chambers, and PEG diffused passively across the substrate from the donor chamber to the acceptor chamber. Samples were taken from the acceptor chamber at several time points and the concentration was determined by refraction index detection (six replicates per time point). Additionally, samples of cellulose acetate (0.45 µm pore size, Whatman) were used as a permeable substrate of reference, under the same experimental conditions.

**Dimensional Stability.** The *in vitro* stability of BC substrates was evaluated by incubating samples in phosphate buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.4), at room temperature for 12 weeks and analyzing them for handling stability by manipulating the membranes with tweezers. To assess the occurrence of substrate swelling, these same samples were weighted at different time points (1, 4 and 12 weeks). Twelve specimens were measured for each substrate. Since the samples presented variable thicknesses, swelling data taken individually were normalized with their respective initial volume using the following equation:

$$\text{Swelling/Volume} = \frac{\text{Weight}_{\\text{wet}} - \text{Weight}_{\\text{dry}}}{\text{Weight}_{\\text{dry}} \times \text{Volume}}$$  \hspace{1cm} (3)

where $\text{Weight}_{\\text{dry}}$ was the initial weight in dry state, and $\text{Weight}_{\\text{wet}}^t$ was the weight measured in wet state at each time point, $t$.  


**Mechanical Properties.** Stress-strain assays were performed to evaluate the mechanical properties of the developed substrates, using a Shimadzu Universal Testing Machine (AG-IS with a 50 N load cell) in tensile mode, at a strain rate of 0.5 mm.min\(^{-1}\) and at room temperature. The samples were immersed for approximately 5 min in distilled water at room temperature, immediately prior to the tensile tests. From the stress-strain data, the modulus of elasticity (\(E\)) was calculated in the linear zone of elasticity, between 0 and 1% of strain, for all the samples. The maximum stress (\(\sigma_{\text{max}}\)) and the strain-to-failure (\(\varepsilon_{\text{break}}\)) were also determined. Measurements were performed on five specimens of each type and the mean values and standard deviation were calculated.

**Endotoxin Analysis.** BC substrates were analysed for the presence of endotoxin using the limulus amebocyte lysate test (Pierce ® LAL Chromogenic Endotoxin Quantitation kit, Thermo Scientific). Substrate (BC, ABC, CBC and CMBC) samples were autoclaved in distilled water. Each substrate (approximately 0.88 mm\(^3\) of volume) was immersed in 40 mL pyrogen-free water. The assay was performed according to the kit supplier instructions. The reaction was stopped with 25 % acetic acid and the absorbance was measured at 405 nm.

**Retinal Pigment Epithelium Culture.** Human RPE cells (hTERT-RPE1, ATCC CRL-4000), immortalized by the transfection with human telomerase gene (hTERT) were previously described as having a normal RPE phenotype.\(^{45}\) In this work, these cells were used between 200 and 250 population doublings and were grown in Dulbecco’s MEM/Ham’s F12 (1:1 mixture) culture media supplemented with 10 % v/v fetal bovine serum (FBS), 100 U.mL\(^{-1}\) penicillin and 100 \(\mu\)g.mL\(^{-1}\) streptomycin (DMEM/F12 complete medium) at 37 °C in a humidified atmosphere of 95 % air and 5 % CO2. All cell culture reagents were purchased from Biochrom, unless stated otherwise. At confluence, hTERT-RPE cells were harvested with 0.05% (w/v) trypsin-EDTA
and then were subcultured in the same medium. PBS was used for all washing steps. For the viability assays, the exposure media (EM) used consisted of DMEM (without phenol red) supplemented with 100 U.mL\(^{-1}\) penicillin and 100 µg.mL\(^{-1}\) streptomycin. Cells were seeded on the substrates and on tissue culture polystyrene (TCP; used as a control surface) at a density of 40,000 cells.cm\(^{-2}\).

**Cell Viability and Proliferation Studies.** Viability studies were performed at three time points (3, 7 and 14 days) to assess cell proliferation. Three independent cultures per condition were performed. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega) and the LIVE/DEAD Viability/Cytotoxicity kit for mammalian cells (Invitrogen). The MTS assay measures the metabolic activity of viable cells via its dehydrogenase activity. Cells were washed, incubated for 2 h with 20 µL MTS solution reagent in 100 µL EM, and then the absorbance was recorded at 490 nm with a 96-well plate reader. The LIVE/DEAD kit provides a two-colour fluorescence cell viability assay, based on the simultaneous determination of live (green) and dead (red) cells with two probes, Calcein AM and Ethidium Homodimer (EthD-1), that measure intracellular esterase activity and plasma membrane integrity, respectively. Cell cultures were incubated for 30 min in 200 µL EM with 20 µL LIVE/DEAD working solution (2 µM EthD-1 and 4 µM Calcein AM in PBS pH 7.4). Afterwards, the substrates were observed in a fluorescence microscope (Leica DMIRE2, with a DFC350FX camera) and pictures were taken from six random fields for each substrate condition.

**Scanning Electron Microscopy.** For surface topography analysis, the substrates were dried, sputter coated with gold, and transferred to the microscopic carrier. The cells adhered to the substrates, after 14 days of cell culture, were fixed with 2.5 % glutaraldehyde in PBS solution for
1 h, washed with distilled water, dehydrated through six changes of ethanol (55, 70, 80, 90, 95 and 100 %, 30 min each), and finally sputtered with gold. Images were obtained using a Leica Cambridge S360 scanning electron microscope (SEM) with electron beam energy of 15 KV.

**Statistics.** All data is presented as the mean ± standard error mean. Six independent replicas were analysed for each condition, unless stated otherwise. Statistical analysis of variance (ANOVA) and the Tukey multiple comparison test were used to compare results within and between each time point for each substrate condition. Statistical differences were assigned to groups with a p-value less than 0.05.

**RESULTS AND DISCUSSION**

**Substrate characterization.** Cell adhesion to substrates is affected by surface properties such as wettability, topography, surface charge and chemical functionalities.\textsuperscript{46,47} ATR-FTIR spectroscopy was used to confirm the BC surface modification via acetylation and polysaccharide adsorption using chitosan and CMC (Figure 2). After surface acetylation (Figure 2A), BC substrates show a decrease in the alcohol O–H stretch (3200 – 3400 cm\(^{-1}\)) and in the alkane C–H stretch peaks (2875 cm\(^{-1}\)). On the other hand, there is a significant increase in the ester C=O stretch (1750 cm\(^{-1}\)), in the alkane –C–H bending (1355 cm\(^{-1}\)), and in the two peaks relative to the ester C–O stretch (1210, 1025 cm\(^{-1}\)). This suggests the successful substitution in BC surface of hydroxyl and alkane groups by the acetate related functional groups. According to the spectra of chitosyl and BC samples exposed to chitosan (Figure 2B), an increase in the chitosan characteristic bands was found in the exposed BC samples: carboxyl -COOH (1425 cm\(^{-1}\)), amino groups (1070 cm\(^{-1}\)), amide I (1650 cm\(^{-1}\)) and amide II (1570 cm\(^{-1}\)). This is in
accordance with previous works on chitosan-cellulose substrates.\textsuperscript{38,48} CMC original spectra indicates a significant peak at 1590 cm\textsuperscript{-1} that corresponds to carboxylic acid C=O stretch; this peak can also be observed, although in a much lower intensity, in the BC substrate exposed to CMC (Figure 2C). Additionally, CMC showed other peaks that were almost undetectable on CMBC substrates: the carboxylic acid O–H bend (1415 and 1020 cm\textsuperscript{-1}) and the carboxylic acid C–O stretch (1320 cm\textsuperscript{-1}). Although the carboxylic acid peak is weak, its presence indicates the incorporation of the carboxymethyl groups in BC.
Figure 2. ATR-FTIR spectra of substrates in the following conditions: A, unmodified bacterial cellulose (BC) and acetylated BC (ABC); B, unmodified BC, chitosan adsorbed BC (CBC) and chitosan; C, unmodified BC, carboxymethyl cellulose (CMC) CMC adsorbed BC (CMBC).

Therefore, surfaces with different functional groups were obtained after modification (hydroxyl for unmodified BC, carboxyl for ABC, amine for CBC and carboxymethyl groups for CMBC), in accordance with the literature. The incorporation of different surface chemical functionalities may affect surface wettability, charge, roughness and porosity, and in turn lead to different cell responses. Surface wettability influences protein and cellular adhesion, where highly hydrophilic materials do not allow stable adsorption of cell adhesion-mediating proteins or they are bound very weakly. Anionic-hydrophilic surfaces bearing relatively weak base functional groups resist protein adsorption by hydrogen-bonding to water so strongly that protein cannot displace interphase water and enter the adsorbed state. On the other hand, optimal cell adhesion and the adsorption of cell adhesion-mediating molecules or proteins are promoted in moderately hydrophilic substrates, which show water contact angles in the range of 40-70 °. Since the substrates may present some roughness, the Young equilibrium or ideal contact angles, which include the roughness factor, could not be reported. Instead, the apparent contact angles were reported and were considered equally valid in the literature to assess surface hydrophobicity and free energy. The measured apparent contact angles, as well as free energy and charge information, are presented in Table 2.

Table 2. Measured apparent contact angles of each probe liquid and the estimated surface free energy components
<table>
<thead>
<tr>
<th></th>
<th>BC</th>
<th>ABC ***</th>
<th>CBC</th>
<th>CMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WCA / °</strong></td>
<td>22.8 ± 1.3</td>
<td>64.8 ± 1.9</td>
<td>25.3 ± 1.0</td>
<td>21.8 ± 2.0</td>
</tr>
<tr>
<td><strong>FCA / °</strong></td>
<td>22.8 ± 2.2</td>
<td>43.7 ± 1.4</td>
<td>18.2 ± 1.1</td>
<td>17.9 ± 1.4</td>
</tr>
<tr>
<td><strong>BCA / °</strong></td>
<td>33.0 ± 2.2</td>
<td>16.5 ± 0.6</td>
<td>26.6 ± 1.6</td>
<td>30.8 ± 1.2</td>
</tr>
<tr>
<td>$\gamma^+ / \text{mN.m}^{-1}$</td>
<td>1.1</td>
<td>0.3</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>$\gamma^- / \text{mN.m}^{-1}$</td>
<td>50.7</td>
<td>13.4</td>
<td>46.2</td>
<td>49.1</td>
</tr>
<tr>
<td>$\gamma^{AB} / \text{mN.m}^{-1}$</td>
<td>15.2</td>
<td>4.3</td>
<td>14.8</td>
<td>16.3</td>
</tr>
<tr>
<td>$\gamma^{LW} / \text{mN.m}^{-1}$</td>
<td>37.5</td>
<td>42.6</td>
<td>39.8</td>
<td>38.4</td>
</tr>
</tbody>
</table>

Abbreviations: WCA, water contact angle; FCA, formamide contact angle; BCA, bromonaphthalene contact angle; $\gamma^+$, acid parameter; $\gamma^-$, base parameter; $\gamma^{AB}$, acid-base component; $\gamma^{LW}$, Lifshitz van der Waals component; BC, bacterial cellulose; ABC, acetylated BC; CBC, chitosan adsorbed BC; CMBC, carboxymethyl cellulose adsorbed BC.

Statistics: Contact angle results presented as mean ± standard error mean, with two measurements in each substrate and six replicas per condition. *** Only the contact angles measured for ABC are statistically different from the other substrates.

Surface acetylation increases water contact angle to approximately 65°, while polysaccharide adsorption did not change the high hydrophilic degree of BC surface (approximately 23°). Similarly to water, the formamide and bromonaphthalene contact angles did not change significantly after chitosan and CMC adsorption. Therefore, the calculated surface free energy components differed only for the ABC substrates, where $\gamma^{LW}$ energy component showed a slight increase from 37.5 to 42.6 mN.m$^{-1}$ and $\gamma^{AB}$ energy component is much lower than $\gamma^{LW}$, decreasing after acetylation from 15 to 4 mN.m$^{-1}$. The change in the basic constituent was the
most substantial, from 50.7 mN.m$^{-1}$ in unmodified BC to 13.4 mN.m$^{-1}$ in acetylated BC. The BC substrates had only a residual positive charge ($\gamma^+$, Table 2), with acid base forces resulting from negative charges. The decrease in the polar forces after acetylation is coherent with the decreased surface hydrophilicity observed in the ABC substrates. Also, amine, hydroxyl and carboxyl functional groups confer a relatively weak acid-base strength to the surface, in contrast with ion-exchange functionalities such as carboxymethyl groups that confer a strong acid-base strength.$^{54}$ However CMBC substrates did not show an increased acid-base energy; in addition, a very weak carboxylic acid peak was observed in ATR-FTIR spectra (Figure 2C), which may indicate a low incorporation of carboxymethyl groups in the CMC adsorption process. In a cell adhesion study with fibroblasts, the presence of amine and carboxyl groups led to a strong cell interaction with the surfaces when compared to those with hydroxyl groups, which was associated with an enhanced activity of integrins.$^{56}$ Therefore, modified BC substrates were expected to induce stronger interaction with cells than the unmodified BC ones, specially the ABC ones due to its moderate hydrophilicity.

In addition to good surface interaction with biological entities, these substrates must not constitute an obstacle to the diffusion of nutrients and by-products. The permeation coefficients obtained in the control samples were $4.2 \times 10^{-6}$ and $3.8 \times 10^{-6}$ cm$^2$.s$^{-1}$, for PEG 35 kDa and PEG 300 kDa, respectively. All BC substrates presented similar permeation coefficients that were $2.9 \pm 0.2 \times 10^{-6}$ and $1.8 \pm 0.2 \times 10^{-6}$ cm$^2$.s$^{-1}$, for PEG 35 and PEG 300 kDa, respectively. Therefore, BC substrates show similar permeation coefficients relatively to the control, indicating that they allow fluid transport and are permeable to macromolecules up to a molecular weight of 300 kDa, which is the maximum that is transported by RPE cells.$^6$
Since permeation coefficients did not differ between BC conditions, it was expected the presence of porous structures in all BC substrates, which was confirmed through SEM analysis of BC, ABC, CBC and CMBC substrates (Figure 3).

Figure 3. Scanning electron micrographs of the surface topography for the different bacterial cellulose substrates: bacterial cellulose (BC), acetylated BC (ABC), chitosan adsorbed BC (CBC) and carboxymethyl cellulose adsorbed BC (CMBC).
BC’s porosity is an advantageous feature when comparing to other non-biodegradable biomaterials that have been proposed such as polydimethylsiloxane,\textsuperscript{57} polypropylene,\textsuperscript{58} and the already patented for RPE transplantation parylene (patent publication number: US20120009159 A1; publication date: 12 Jan 2012). All the referred biomaterials are non-porous, and a non-biodegradable substrate has to meet the passive diffusion requirements to be a BM substitute, which BC’s was shown herein to possess. However, the biomaterial parylene C has been described to be semipermeable to macromolecules up to 250 kDa when the thickness does not surpass the 0.3 µm.\textsuperscript{59}

Additionally, slight differences in topography are observed between substrates. It was possible to observe the presence of fine fibrils in the CBC surface, while the other BC samples showed a more cohesive surface structure. This slight differences may also lead to a different biological response, since biomaterial surface topography influences protein adsorption and subsequent biological responses.\textsuperscript{60} In the case of substrate acetylation, it was not expected a significant change in surface topography since it has been reported that the microfibrillar morphology of BC is maintained in the acetylation process.\textsuperscript{37}

In RPE transplantation, biomaterial manipulation is required and it has to conform to the movements and available space of the surrounding tissue after transplantation. Therefore, structural stability (easy manipulation, no swelling nor degradation) and mechanical integrity (resistance to tear) are relevant features.\textsuperscript{61,62} After 12 weeks, all substrates showed no signs of degradation and were easily manipulated with tweezers. However, acetylated substrates were easier to manipulate due to a stiffer behaviour, when compared to pristine and polysaccharide-adsorbed BC substrates that easily wrinkled. In this sense, ABC substrates seem to be the most appropriate for this application, where the biomaterial manipulation for a correct positioning of
ultrathin substrates in the subretinal space is vital for the success of the transplant procedure.\textsuperscript{7,14} However some signs of swelling were observed and quantified (Figure 4). After one week, BC, CBC and CMBC samples presented similar swelling profiles, between 3 and 4 times its dry weight in water, while ABC substrates showed at least a third of the swelling effect shown by the other substrates. Swelling stabilized after 4 weeks in PBS solution in 5, 2, 6 and 8 times its dry weight in water for BC, ABC, CBC and CMBC substrates, respectively. ABC samples showed the lowest swelling, with a two-fold increase in its dry weight. Therefore, BC acetylation led to a less swelling effect, which is beneficial for the envisaged application, in opposition to the adsorption of polysaccharides that increased swelling.

![Swelling Profiles](image)

**Figure 4.** Swelling of unmodified bacterial cellulose (BC), acetylated BC (ABC), chitosan adsorbed BC (CBC) and carboxymethyl cellulose adsorbed BC (CMBC) substrates in PBS pH 7.4. Twelve replicas were performed per condition. All swelling profiles are statistically different for the exception of BC with CBC.

Tensile strength ($\sigma_{\text{max}}$), elongation-at-break ($\varepsilon_{\text{break}}$) and elastic modulus ($E$) were obtained from the stress-strain assays performed in tensile mode (Table 3, Figure 5). All samples showed similar stress-strain behaviour, with stress increasing with strain increment until sample collapse

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(Figure 5). Furthermore, BC and ABC samples showed the highest elastic modulus, but the latter had slightly smaller tensile strength and elongation at break when compared to pristine BC samples. This is correlated with a stiffer behaviour, when manipulated, shown by the ABC substrates. Moreover, it was observed a three-fold and five-fold decrease in the elastic modulus, respectively, for similar tensile at break, and an increase in the strain at break from 8 % for neat BC to approximately 25 and 30 % for CBC and CMBC substrates (Table 3). This decrease in elasticity modulus after polysaccharide adsorption may indicate an altered porous morphology. On the other hand, the porous morphology was not significantly altered since similar permeation coefficients were obtained between the BC substrates.

**Table 3.** Elastic modulus (E), maximum stress (σ\(_{\text{max}}\)) and elongation-at-break (ɛ\(_{\text{break}}\)) results

<table>
<thead>
<tr>
<th>Mechanical Property</th>
<th>BC</th>
<th>ABC</th>
<th>CBC</th>
<th>CMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>E / MPa</td>
<td>643.3 ± 35.9</td>
<td>677.5 ± 43.5</td>
<td>225.0 ± 51.7</td>
<td>127.6 ± 31.5</td>
</tr>
<tr>
<td>σ(_{\text{max}}) / MPa</td>
<td>54.9 ± 8.4</td>
<td>37.6 ± 4.4</td>
<td>55.0 ± 3.5</td>
<td>51.0 ± 6.1</td>
</tr>
<tr>
<td>ɛ(_{\text{break}}) / %</td>
<td>8.1 ± 0.5</td>
<td>4.8 ± 0.5</td>
<td>24.9 ± 3.3</td>
<td>29.5 ± 7.1</td>
</tr>
</tbody>
</table>

Abbreviations: unmodified bacterial cellulose (BC), acetylated BC (ABC), chitosan adsorbed BC (CBC) and carboxymethyl cellulose adsorbed BC (CMBC). Five replicas were used for each substrate.
Figure 5. Stress-strain measurements obtained for unmodified bacterial cellulose (BC), acetylated BC (ABC), chitosan adsorbed BC (CBC) and carboxymethyl cellulose adsorbed BC (CMBC). Five replicas were used.

Nonetheless, the mechanical results obtained for all BC substrates are in accordance with others reported in the literature, where nanofibrous membranes of poly(lactic-co-glycolic acid) (PLGA) and collagen type I were likewise evaluated as potential BM substitutes\(^6\). The values obtained in the PLGA nanofibrous membrane were 1.5 ± 0.4 MPa, 28.8 ± 4.9 % and 131.9 ± 13.3 MPa, for maximum tensile strength, tensile strain, and elastic modulus, respectively. Also, higher values were obtained in the collagen nanofibrous membrane, with 10.8 ± 0.7 MPa, 70.0 ± 4.6 % and 217.9 ± 15.3 MPa for maximum tensile strength, tensile strain, and elastic modulus, respectively. In the current work, the values obtained for the developed BC substrates are in the same range or even higher.

Since BC is a biomaterial produced by a gram-negative bacterium, the presence of endotoxins may constitute a problem. The endotoxin levels measured in the water incubated with BC substrates were 0.85 ± 0.19, 0.11 ± 0.01, 0.79 ± 0.07 and 0.67 ± 0.18 EU.L\(^-1\) for BC, ABC, CBC and CMBC substrates, respectively. These values are much lower than the limit of 500 EU.L\(^-1\) for general medical devices and of 60 EU.L\(^-1\) for devices that contact cerebrospinal fluid.\(^6\)}
These results show that the endotoxins were successfully removed during the washing procedure. In the literature\textsuperscript{25}, the BC treatment with 1N NaOH solution, followed by rinsing with deionized water, was found to be an effective purification/depyrogenation method for thin BC scaffolds. On the other hand, the acetylation process showed an 8-fold decrease in the amount of endotoxins present. This indicates a further removal of residual endotoxins from the BC matrix in the acetylation modification procedure. Bodin et al.\textsuperscript{25} obtained similar endotoxin results for their BC scaffolds (produced by G. xylinus, ATCC ® 700198\textsuperscript{TM}). Therefore these substrates can be classified as non-pyrogenic and should not induce complications \textit{in vivo}, with ABC substrates showing the lowest value (0.11 ± 0.01 EU.L\textsuperscript{-1}).

**Cell Viability and Proliferation.** Cells were observed after 14 days of hTERT-RPE cell culture in the different BC substrates using SEM (Figure 6). Pristine BC substrates showed only residual cells adhered to the surface, in contrast to the modified BC substrates. The low cell adhesion and lack of cell proliferation in unmodified BC is in accordance with previous works of our research group, where BC surface functionalization with proteins and nitrogen plasma modification were required to improve the cell interaction with the substrate.\textsuperscript{20-21} The CBC and CMBC substrates presented cell monolayers growing towards each other, while ABC showed a surface nearly fully covered by a cell monolayer. Therefore, CBC and CMBC required a longer period of time to obtain a cell monolayer covering the entire surface.
Figure 6. Scanning electron micrographs of hTERT-RPE1 cells grown on unmodified bacterial cellulose (BC), acetylated BC (ABC), chitosan adsorbed BC (CBC) and carboxymethyl cellulose adsorbed BC (CMBC) substrates after 14 days of cell culture.

The extent and the strength of cell adhesion play a decisive role in regulating the subsequent cell proliferation activity. Therefore, fluorescence microscopy and MTS assays were performed to assess cell viability and proliferation on the BC substrates, and on tissue culture polystyrene (TCP) used as a control surface. Metabolic activity was measured after 3, 7 and 14 days (Figure 7) with MTS assay.
When observing the metabolic activity after 3 days in culture, a difference in the initial cell attachment between the BC surfaces and the TCP control was observed (Figure 7). In TCP surface, cells appear to show growth arrest after 7 days, since the same metabolic activity is obtained after 14 days, indicating the presence of a confluent cell monolayer. After 3 days, cells adhered in the modified BC substrates were less than those in TCP, but they were able to proliferate by the increasing metabolic activity shown with prolonged cell culture of 7 and 14 days. After 14 days, the ABC substrates presented the closest metabolic values to the cells cultured in TCP after 7 days. BC unmodified substrates showed only residual metabolic activity, indicating the reduced presence of adhered cells. The metabolic data is in accordance with the SEM pictures of cells adhered in the different BC substrates after 14 days of culture (Figure 6).

To observe the cell viability on the different BC substrates, LIVE/DEAD fluorescence images were taken (Figure 8). Surface area occupation with viable and dead cells was also quantified to
compare with the metabolic activity data (Figure 9), using the open source platform FIJI for LIVE/DEAD image analysis.

**Figure 8.** Representative live/dead fluorescence micrographs of HTERT-RPE1 cells cultured for 3, 7, and 14 days on unmodified bacterial cellulose (BC), acetylated BC (ABC), chitosan adsorbed BC (CBC), carboxymethyl cellulose adsorbed BC (CMBC) and tissue culture polystyrene (TCP, control surface).
Figure 9. Data inferred from the live/dead micrographs regarding the surface area occupied by cells per total surface area. The total area occupied with live cells is represented in the left side of the figure and the total area corresponding to dead cells in the right side. Six representative fields were used for each condition. The same letters are given to data not different statistically.

The LIVE/DEAD fluorescence micrographs (Figure 8) were coherent with SEM visual observations. The LIVE/DEAD related cell quantification with FIJI (Figure 9) also corroborated the metabolic data. Residual cell death was additionally indicated in the quantified dead cells (below 3 %) on the BC substrates. The highest value of cell death was obtained in the control at 14 days of culture, which is explained by a potential cell overgrowth that led to a decreased availability to nutrients and consequently cell death. In TCP surface after 7 days and in ABC after 14 days almost 100 % of the surface area was occupied by viable cells, indicating a surface nearly fully covered with viable cells. Acetylated substrates presented higher initial cell adhesion and similar proliferation rate when compared to the other modified BC substrates as suggested by the results obtained in the viability (Figure 8 and 9) and metabolic activity (Figure 7) assays.
When comparing the cell response to surface properties, it is possible to observe that for similar contact angles different cell responses were obtained, from very low adhesion and no proliferation on BC unmodified surface to moderate adhesion and proliferation on BC surface with chitosan and CMC. This confirms that in addition to surface wettability and free energy, other properties have affected RPE cell adhesion to the substrates (such as material elasticity and surface topography). Additionally, RPE cells interacted better with the moderate hydrophilic surface of the ABC substrates. The similar wettability found for TCP (water contact angle of 20° reported elsewhere\textsuperscript{58}) and the unmodified BC substrate indicates that, according to the results, this was not a decisive parameter for cell adhesion. The major difference between the two surfaces is the presence or absence of roughness in the case of BC and TCP, respectively. The effect of flat plasma polymer (PP) films with different chemical functionalities (amine, carboxyl, hydroxyl and hydrocarbon) on RPE cell adhesion and growth was evaluated in the literature.\textsuperscript{58} The cell culture results after 10 days with the ARPE-19 immortalized cell line and the primary human RPE cells indicated no statistical difference between different surfaces, except for the hydrophobic surface with the hydrocarbon chemical functionality, which had a significantly lower number of cells adhered comparing to the unmodified tissue culture polystyrene (TCP) and the other modified PP surfaces. In our study, all substrates were hydrophilic; however different cell responses were obtained. This further confirms the strong influence of additional biomaterial properties in cell response, besides surface chemical groups and wettability. In fact, a previous study has reported that RPE cells preferred smooth rather than rough surfaces.\textsuperscript{46}

Also, the RPE cell adhesion obtained in the modified BC substrates was still lower than other substrates developed for the same purpose, where cells have reached confluence at the same time as the control surface (TCP or glass)\textsuperscript{12,14}, due to a higher initial cell adhesion. Therefore, BC
may require further or alternative substrate improvement approaches to achieve a better cell response. It was found that protein adsorption modified the surface roughness by smoothing its irregularities.\textsuperscript{46} Hence, surface coating with proteins relevant to cell adhesion and that simulate the RPE natural environment, may further improve cell response to the BC substrates herein developed. A similar strategy has been reported by other authors, namely through the adsorption of glycosaminoglycans on a polymerized allylamine/octadiene surface leading to an increased ARPE-19 proliferation.\textsuperscript{65} Although BC may present a rough surface, we showed cell adhesion and proliferation in the modified BC substrates, along with other appropriate physico-chemical characteristics, showing the potential of this biomaterial for the ultimate application of RPE transplantation.

CONCLUSIONS

The interesting properties of BC for biomedical applications such as permeability to gas and fluid transport and stability make it a potential substrate for the successful RPE transplantation. The results gathered in the current study suggest the feasibility of this substrate for this application. The data on surface wettability, energy, charge, swelling, stability, mechanical properties, amount of endotoxins detected, cell adhesion and proliferation obtained for all BC modified substrates studied indicate that the acetylated BC substrate presented the most interesting features. The bulk properties were found to be similar among the substrates, which were porous, permeable up to 300 kDa, and dimensionally stable (particularly the ABC ones). Surface modification approaches performed were able to address the low cell adhesion and lack of proliferation in unmodified BC, with ABC showing increased initial cell adhesion and similar proliferation when compared to the other modified BC substrates. However, ABC substrates
required 14 days to obtain a surface completely covered with cells while in TCP control this was achieved after 7 days. To validate BC substrates as viable RPE cell carriers in retinal degenerative diseases, the evaluation of RPE function, morphology and tissue reaction is required, in particular for the ABC substrates that seem the most promising of the modified BC substrates studied in this work. Additionally, other improvements of the surface characteristics may be studied towards a better cell response, such as the adsorption of proteins that promote cell adhesion prior to cell culture. The level of surface roughness should also be assessed in a future study to better understand the cell behaviour when exposed to these substrates with the comparing of this parameter with the literature.

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