



Acetylated bacterial cellulose coated with urinary bladder matrix as a substrate for retinal pigment epithelium



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ABSTRACT

This work evaluated the effect of acetylated bacterial cellulose (ABC) substrates coated with urinary bladder matrix (UBM) on the behavior of retinal pigment epithelium (RPE), as assessed by cell adhesion, proliferation and development of cell polarity exhibiting transepithelial resistance and polygonal shaped-cells with microvilli. Acetylation of bacterial cellulose (BC) generated a moderate hydrophobic surface (around 65°) while the adsorption of UBM onto these acetylated substrates did not affect significantly the surface hydrophobicity. The ABC substrates coated with UBM enabled the development of a cell phenotype closer to that of native RPE cells. These cells were able to express proteins essential for their cytoskeletal organization and metabolic function (ZO-1 and RPE65), while showing a polygonal shaped morphology with microvilli and a monolayer configuration. The coated ABC substrates were also characterized, exhibiting low swelling effect (between 1.5–2.0 swelling/mm³), high mechanical strength (2048 MPa) and non-pyrogenicity (2.12 EU/L). Therefore, the ABC substrates coated with UBM exhibit interesting features as potential cell carriers in RPE transplantation that ought to be further explored.

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1. Introduction

The retinal pigment epithelium (RPE) is a highly polarized monolayer of polygonal-shaped and pigmented epithelial cells with apical microvilli and basolateral infoldings [1]. This monolayer is maintained and stabilized by the cytoskeleton of individual cells and their interactions at the basolateral junctional complexes (or tight junctions) [2–4]. These complexes are composed of zonular occludens-1 (ZO-1), occludin, claudins 1, 2, 5, 12 and AL [1]. RPE performs several complex functions essential for visual function, namely light energy absorption, photoreceptor outer segments phagocytosis, outer blood–retina barrier, secretion of immunosuppressive and growth factors [1,5]. As an outer blood–retina barrier, the RPE prevents the transport of molecules larger than 300 kDa

via intracellular mediation [6]. To be able to perform its complex functions, RPE cells have unique morphological and functional polarity properties, with an expression and polarized distribution of receptors, transporters, channels and enzymes (many of these are markers of differentiated RPE) [5].

With age, the RPE may be affected, compromising retinal integrity and leading ultimately to retinal degenerative diseases such as age-related macular degeneration (AMD)—the most common cause of blindness worldwide [7]. A promising therapeutic approach for AMD involves the replacement of diseased RPE by healthy stem cell-derived RPE-like cells, transplanted as an integer epithelial sheet on a carrier substrate [8,9]. An optimal cell scaffold for RPE transplantation should simulate the RPE natural microenvironment; allow nutrient flow and/or biodegrade rapidly; maintain the RPE phenotype; exhibit favorable surgical properties and biocompatibility [9]. Since the Bruch's membrane (BM) is also affected in AMD, the success of RPE transplants requires the replacement of this tissue, as these RPE cells are transplanted in a BM prosthetic substrate. Recent studies on the development of BM prosthetic substrates include the use of polyethylene terephthalate and poly(L-lactide-co-ε-

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caprolactone); parylene (already patented for stem cell therapy to treat diseased or damaged ocular tissue); montmorillonite clay-based polyurethane nanocomposite; copolymers of methyl methacrylate and poly(ethylene glycol) methacrylate; and gelatin-biofunctionalized polyimide membranes [10–14]. A prosthetic BM substrate should be able to perform the BM's primary functions, namely to provide physical support for RPE; maintain the RPE phenotype; and act as a division barrier for cell migration. A BM substitute should also be thin (BM is 2–4 μm thick) to prevent distortions of the retina after implantation; minimize the resistance to nutrient diffusion from the choriocapillaris to the RPE cells; and limit the mass of material implanted [6].

Bacterial cellulose (BC) holds unique structural and mechanical properties, such as permeability to gas and fluid exchange, biocompatibility and non-toxicity [8,12,15,16]. In fact, BC has shown to be an interesting and versatile biomaterial, particularly in tissue-engineered applications (tissue scaffolds and drug delivery systems) for the regeneration of damaged or diseased tissues and organs [17–19]. In our previous work, BC was surface-modified using three different approaches, namely *via* acetylation and polysaccharide adsorption, using either chitosan or carboxymethyl cellulose. The ability of these surface-modified BC substrates to promote RPE cell adhesion and proliferation *in vitro* was assessed [20]. Briefly, acetylation of BC (ABC) was found to decrease the swelling and the amount of endotoxins in the final substrate. Also, ABC substrates showed the highest elastic modulus. All surface modifications of the BC substrate enhanced the adhesion and proliferation of RPE cells. Although similar proliferation rates were observed among the modified substrates, ABC showed a higher initial cell adhesion. This difference was proposed to be mainly due to the moderately hydrophobic character of the ABC substrate. Indeed, optimal cell adhesion has been described to occur in moderately hydrophobic surfaces (water contact angles in the 40–70° range) [21].

In this work, the ability of ABC substrates coated with an extracellular matrix (ECM) to support the phenotype of RPE cells was studied for the first time. An ECM extracted from porcine urinary bladder–theurinary bladder matrix (UBM), was used.

UBM has been widely studied in several pre-clinical studies as a biologic scaffold for the reconstruction of damaged tissues [22–28]. Therefore, ABC substrates coated and non-coated with UBM were first characterized according to their surface properties (hydrophobicity, free energy, topography, chemical composition), dimensional stability (swelling and handling stability), mechanical properties and endotoxins content. Cell viability and cytotoxicity assays were performed to evaluate the ability of RPE cells to adhere and proliferate in the modified substrates. This work was further expanded by evaluating the expression of specific RPE markers and the development of cell polarity exhibiting transepithelial resistance and polygonal shaped-cells with microvilli, these being indicators of an adequate RPE cell morphology. The markers here studied were the junctional ZO-1 protein and the retinal pigment epithelium-65 (RPE65), which is a RPE specific protein involved in the process of the visual cycle of retinal [1,4]. To the authors knowledge this is the first time such a specific study was conducted using BC as a substrate for RPE cells adhesion and proliferation.

2. Experimental

2.1. Reagents

Analytical grade acetic anhydride, acetic acid, toluene and perchloric acid were purchased from Merck (United States of America, USA), Fisher Chemical (USA), Fisher Chemical and Panreac (Spain), respectively. Rat-tail collagen type-I was purchased from BD Bio-

sciences (USA). Pepsin from porcine gastric mucosa was purchased from Sigma–Aldrich (USA). Cell culture reagents were purchased from Biochrom (Germany). The Pierce® BCA Protein Assay Kit, the Pierce® limulus amebocyte lysate (LAL) chromogenic endotoxin quantitation kit, the Invitrogen™ LIVE/DEAD Viability/Cytotoxicity kit for mammalian cells, Whatman™ polyethersulfone (PES) filters and Sigma Mowiol® mounting solution were purchased from Thermo Fisher (USA). The [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (USA). The primary antibodies rabbit polyclonal anti-ZO1 immunoglobulin type G (IgG, 61-7300) and mouse monoclonal anti-RPE65 IgG (Ab13826) were purchased from Life Technologies and Abcam, respectively. The secondary antibodies were purchased from Life Technologies, namely goat anti-rabbit IgG (Alexa Fluor® 488, A11034) and goat anti-mouse IgG (Alexa Fluor® 488, A11001). The phosphate buffer saline (PBS) solution was prepared with 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , and 2 mM KH_2PO_4 (pH 7.4).

2.2. Acetylated bacterial cellulose (ABC) substrates preparation

BC was produced by static culture of *Gluconacetobacter xylinus* (ATCC® 53582™) as previously described in Ref. [20,29–31]. Briefly, fermentation was performed in 1 L Erlenmeyer flask with 200 mL of Hestrin–Schramm medium for 1 month. The resulting BC sheet was washed at room temperature (RT) with abundant tap water to remove residual medium, and placed for 24 h in a 1.0 M NaOH solution to remove bacteria and pyrogens. Alkaline residues were removed by washing thoroughly with distilled water until the final pH of the supernatant became that of distilled water. Then, the washed BC sheets were sliced into thin pellicles, dried in an oven at 50 °C for 8 h and cut into the desired geometric shapes, all with an average thickness of $61.5 \pm 4.8 \mu\text{m}$ (and with 5 and 9 mm diameter circles for 96 and 48 well plates, respectively, and 4.5 mm per 20 mm rectangles for all other assays).

BC acetylation and characterization has been published elsewhere [20]. Briefly, the acetylation reaction mixture was composed of 40 mL acetic acid, 50 mL toluene and 0.2 mL perchloric acid. Oven-dried BC samples were added at a ratio of 1 cm^2 of substrate surface area per 1 mL reaction mixture. A volume of 8 mL of acetic anhydride and 15 min reaction time were used to generate a moderate hydrophobic surface (water contact angle: $64.8 \pm 1.9^\circ$). The ABC substrates were then sterilized in 100% ethanol and maintained in sterile PBS solution until use. ABC surface was coated with UBM, as further described below. For the physicochemical and mechanical characterization (Section 2.4) of both coated and non-coated (control) substrates, these were again dried until constant mass. For the cell culture assays, hydrated ABC substrates (as maintained in sterile PBS solution) were used.

2.3. Urinary bladder matrix (UBM) preparation, characterization and coating

UBM extraction was performed according to the method reported by Freytes et al. [23] and consists in separating the basement membrane of the urothelial cells and subjacent lamina propria from porcine bladders (kindly provided by a local slaughterhouse). The urothelial cells were removed by soaking the tissue in 1.0 M saline solution. After being washed with PBS solution and sterile water, UBM sheets were lyophilized, triturated, and kept at –20 °C until use. UBM was enzymatically digested immediately before being applied onto the ABC substrates at 37 °C in 0.01 M HCl solution (pH 3–4). Two digestion conditions were studied, namely 10 g/L UBM and 1 g/L pepsin (as described by Freytes et al. [23]) and 20 g/L UBM and 3 g/L pepsin. These conditions were studied for up to 48 h of digestion to evaluate the effect of these parameters

on the amount and molecular mass of the solubilized protein. The digested UBM solution was neutralized and centrifuged (20 min, $9000 \times g$) to remove insolubilized particles and then, sterilized by passing the solution through $0.22 \mu\text{m}$ PES filters. The protein content of the digested UBM solution was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the bicinchoninic acid (BCA) quantitation method. UBM and collagen type-I were electrophoresed on 10% polyacrylamide gels under reducing conditions (5% 2-mercaptoethanol) and stained with Coomassie Blue for proper visualization. The BCA Protein Assay Kit was used in accordance with the manufacturer guidelines, using bovine serum albumin as standard. Protein contents were corrected according to the pepsin concentration used. Pepsin inactivation occurred after increasing the pH to 7.4 with $90 \mu\text{L}$ of NaOH 1 M and 1.01 mL of $10\times$ concentrated PBS added to 9 mL sterile UBM solution. UBM gelation and coating of the ABC substrates occurred at 37°C for 18 h at a ratio of $100 \mu\text{L}/\text{cm}^2$ ABC sample surface area. Excess of UBM solutions were used to assure maximum saturation of the ABC nanofibers.

2.4. Substrate characterization

Fourier transform IR spectroscopy in attenuated total reflectance mode (ATR-FTIR) was performed at RT to assess the surface chemical composition through the identification of surface functional groups. The IR spectra generated using a PerkinElmer Spotlight 300 FTIR microscope with Spectrum 100 FTIR spectrometer (USA), corresponded to the average of 100 scans measured at a resolution of $4/\text{cm}$. The vibration transition frequencies of each spectrum were baseline corrected and the absorbance was normalized between 0 and 1. Static contact angle measurements were performed using the sessile drop technique to determine the surface free energy (SFE, γ) and hydrophobicity. For SFE estimation, the van Oss-Chaudhury-Good (vOCG) method was used. Details on this methodology are described by Gonçalves et al. [20]. Substrate thickness measurements were performed using a Mitutoyo digital micrometer (N° 293-5, Japan). To assess substrate swelling, ABC substrates (coated and non-coated (control)) were first placed in PBS solution. At regular intervals for up to 12 weeks, the substrates were collected and weighed. Simultaneously to the gravimetric procedures, the handling stability of these substrates was qualitatively evaluated by manipulating the membranes with tweezers. Since the samples presented variable thicknesses (average $61.5 \pm 4.8 \mu\text{m}$), swelling data taken individually (twelve replicas) were normalized with their respective initial volume as shown in Eq. (1), where Mass_{dry} is the initial substrates' dry mass, and $\text{Mass}_{\text{wet}}^t$ is the measured mass of the hydrated substrates at each time point, t .

$$\frac{\text{Swelling}}{\text{Volume}} = \frac{\text{Mass}_{\text{wet}}^t - \text{Mass}_{\text{dry}}}{\text{Mass}_{\text{dry}} \times \text{Volume}} \quad (1)$$

To evaluate the substrates' mechanical properties, stress-strain assays were performed in tensile mode using a Shimadzu Universal Testing Machine, AG-IS with a 50 N load cell (Shimadzu Scientific Instruments, USA) at RT and with a strain rate of $0.5 \text{ mm}/\text{min}$. The samples were immersed for approximately 5 min in distilled water immediately prior to the tensile tests. Maximum stress (σ_{max}) and strain-to-failure ($\varepsilon_{\text{break}}$) were determined and the elastic modulus (E) was calculated from the stress-strain data of five replicas per substrate in the linear zone of elasticity, between 0 and 1% of strain.

BC substrates were analyzed for the presence of endotoxin using the LAL kit according to the supplier instructions. Each sterile ABC substrates of approximately 0.88 mm^3 , with and without UBM, were immersed in 40 mL pyrogen-free water. The reaction

was stopped with 25% acetic acid and the absorbance measured at 405 nm .

2.5. Cell culture

Human RPE cells (hTERT-RPE1, ATCC CRL-4000) immortalized by the transfection with human telomerase gene (hTERT) have been reported to exhibit a normal RPE phenotype [32]. Cells were grown in Dulbecco's MEM/Ham's F12 (1:1 mixture) culture media supplemented with 10% v/v fetal bovine serum (FBS), $100 \text{ U}/\text{mL}$ penicillin and $100 \mu\text{g}/\text{mL}$ streptomycin (named DMEM/F12 complete medium) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . At confluence, hTERT-RPE cells were harvested with 0.05% (w/v) trypsin-EDTA and were sub-cultured in the same medium. PBS was used for all washing steps. For the viability assays, the media used consisted of DMEM (without phenol red) supplemented with $100 \text{ U}/\text{mL}$ penicillin and $100 \mu\text{g}/\text{mL}$ streptomycin. Cells were seeded on the substrates and control surfaces at a density of $40,000 \text{ cells}/\text{cm}^2$.

2.6. Cell viability and proliferation

Cell viability was assessed after 3, 7 and 14 days, to evaluate cell proliferation, using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and the LIVE/DEAD Viability/Cytotoxicity kit for mammalian cells. The MTS assay measures the metabolic activity of viable cells via its dehydrogenase activity. Briefly, cells were washed and incubated for 2 h with $20 \mu\text{L}$ MTS solution reagent in $100 \mu\text{L}$ EM; and afterwards the absorbance was recorded at 490 nm in a microplate reader. The LIVE/DEAD kit provides a two-color 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 loss of plasma membrane integrity, respectively. Cell cultures from the different time intervals were incubated for 30 min at 37°C in 10% LIVE/DEAD working solution ($2 \mu\text{M}$ EthD-1 and $4 \mu\text{M}$ Calcein AM in PBS pH 7.4) in EM. The substrates were then observed under a Leica DMIRE2 fluorescence microscope with a DFC350FX camera (Germany). Tissue culture polystyrene (TCP) surface was used as positive control to evaluate cell response in ABC substrates with and without UBM, in three independent experiments.

2.7. Immunofluorescence

RPE cell monolayers grown on glass coverslips (positive control surface) and on ABC substrates coated with UBM were fixed in ice-cold methanol for 5 min at RT and then carefully washed 3 times in PBS, 5 min each. Cells were incubated in permeabilization and blocking (perm/block) solution (0.05% saponin and 0.5% BSA in PBS), for 45 min at RT in a humidified chamber. This perm/block solution was also used to wash the cells between the following steps. Cells were incubated with rabbit polyclonal antibody anti-ZO1 IgG (1:100, Life Technologies, 61-7300) or mouse monoclonal antibody anti-RPE65 IgG (1:200, Abcam, Ab13826) in perm/block solution for 2 h. Samples with no primary antibody's incubation were used as negative control. Afterwards, cells were incubated with the appropriate fluorophore-conjugated secondary antibodies ($1:2000$ in perm/block solution) for 30 min, namely goat anti-mouse IgG for RPE65 and goat anti-rabbit IgG for ZO-1. Finally, nuclei counterstaining using DAPI was performed and samples were embedded with Mowiol® mounting solution under a coverslip and examined with a LSM 710 Meta confocal laser-scanning microscope (Zeiss, Germany).

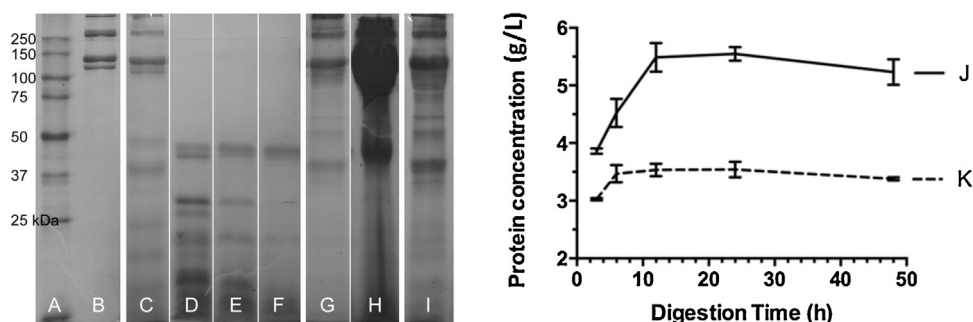


Fig. 1. SDS-PAGE protein profiles (left side) of the following samples: molecular mass marker; (A) rat-tail collagen type-1; (B) soluble fraction of urinary bladder matrix (UBM) after 6 h, (C) 12 h, (D) 24 h (E) and 48 h (F) of digestion using 10 g L⁻¹ UBM and 1 g L⁻¹ of pepsin; soluble fraction (G) and pellet (H) of UBM for 6 h of digestion using 20 g L⁻¹ UBM and 1 g L⁻¹ of pepsin; and soluble fraction of UBM after 12 h of digestion using 20 g L⁻¹ UBM and 3 g L⁻¹ of pepsin (I). Protein concentration (right side) of the UBM soluble fraction up to 48 h of digestion in two digestion conditions: (J) 20 g L⁻¹ UBM + 3 g L⁻¹ pepsin and (K) 10 g L⁻¹ UBM + 1 g L⁻¹ pepsin (results correspond to the mean $\pm$ standard error mean of three independent assays with three replicas each).

2.8. Transepithelial resistance (TER)

TER assays can be used to measure the cells epithelial barrier function through the quantification of the cell-polarized level. The TER assays were performed with cells cultured in Transwell® insert chambers and using an epithelial voltammeter (EVOM) according to the protocol described by Sonoda et al. [4]. Briefly, measurements with the electrodes of the EVOM were made at RT after 20 min of stabilization, to minimize the TER fluctuation with temperature. The values of TER were measured with cells in a confluent monolayer configuration. Near the full confluent state, the FBS was reduced to 1% to prevent cell overgrowth and to maintain a consistent cell monolayer in culture. Net TERs were calculated by subtracting from the experimental values the corresponding value of unseeded fibronectin-coated filters or filters with UBM-coated ABC substrates. Final resistance-area products (Ω cm²) were obtained by the multiplication with the effective growth area.

2.9. Scanning electron microscopy (SEM)

For the cell morphology analysis, substrates subjected to cell culture for 14 days were fixed with 2.5% glutaraldehyde in PBS pH 7.4 for 1 h, washed with distilled water, dehydrated through several ethanol changes (namely in 55, 70, 80, 90, 95 and 100%, 30 min each), sputtered with gold and transferred to the microscopic carrier. For surface observation, the substrates were only dehydrated, sputter coated with gold and transferred to the microscopic carrier for observation under a Leica Cambridge S360 scanning electron microscope (Germany) with electron beam energy of 15 KV.

2.10. Statistics

All results are presented as the mean $\pm$ standard error mean of several independent experiments. Statistical analysis of variance (ANOVA) and the Tukey multiple comparisons post-test were used to compare results between different substrate conditions. Statistical differences were assigned to groups with a *p*-value lower than 0.05.

3. Results and discussion

3.1. UBM characterization

In this work, the digestion of UBM was assayed using different protein (UBM and pepsin) concentrations, temperature and time (upto 48 h) to evaluate the effect of such parameters on the amount and molecular mass of the solubilized protein fractions. The protein's molecular mass profile of the soluble fractions was analyzed

by protein electrophoresis (Fig. 1A–I). The amount of protein in the pellets and in the UBM soluble fractions were quantified using the BCA method (Fig. 1J, K). The results showed that when using 10 g/L UBM and 1 g/L of pepsin, increasing the digestion times, increases the amount of protein with lower molecular masses (Fig. 1C–F). Well-defined bands characteristic of the collagen type-1 (Fig. 1B) can be visualized in samples C, G and I (as digested with 20 g/L UBM and 1 g/L pepsin), in accordance with the ones reported in the literature [23,28]. UBM digestion using 20 g/L UBM and 1 g/L of pepsin (Fig. 1G and K) for 6 h resulted in a soluble fraction with higher protein content. However, a significant amount of protein remained in the pellet fraction, following centrifugation (Fig. 1H). The amount of pepsin was then increased (from 1 to 3 g/L) and digestion was let to occur for up to 48 h as an attempt to further solubilize the protein. Quantitative data (Fig. 1J, K) shows that a higher initial UBM and pepsin concentration (Fig. 1J) led to higher levels of soluble protein. Under these conditions, the optimum digestion time, which corresponds to a protein release of 5.5 ± 0.2 g/L, was found to be 12 h. This higher concentration corresponds to 20–30% (w/w) of that of the initial (undigested) lyophilized UBM. Also, besides the protein bands related to collagen type-1, other bands of lower molecular masses were observed that have been reported as being related to other elements present in UBM such as glycosaminoglycans and the glycoprotein fibronectin [23]. These results allowed establishing the optimum conditions for the preparation of UBM (20 g/L UBM and 3 g/L pepsin for 12 h of digestion) to be further used in the surface modification of ABC substrates.

3.2. Substrate characterization

The substrates were characterized according to their surface chemical composition, dimensional stability, mechanical strength, surface topography, endotoxin content, surface wettability and free energy. The ABC surface composition, based on acetate related functional groups, was confirmed by ATR-FTIR spectroscopy (Fig. 2A) and is presented herein as a comparison to the data reported in Ref. [20]. The typical absorbance bands of ABC substrates correspond to the ester C=O stretch at 1750/cm, the alkane C–H bending at 1355/cm, and the two peaks relative to the ester C–O stretch at 1210 and 1025/cm [20,33,34]. After UBM coating, the substrates displayed additional absorbance bands related to the collagen type-1, namely the amide I and amide II bands at 1650/cm and 1560/cm, respectively. Three weaker bands that represent amide III vibration modes centered at 1245/cm are also characteristic of the collagen FTIR spectrum [35]. However, these were not easily detected in the spectrum, similarly to other reports of protein immobilization where the bulk phases IR adsorption is strong [36]. This is due to the several hundreds of nanometers

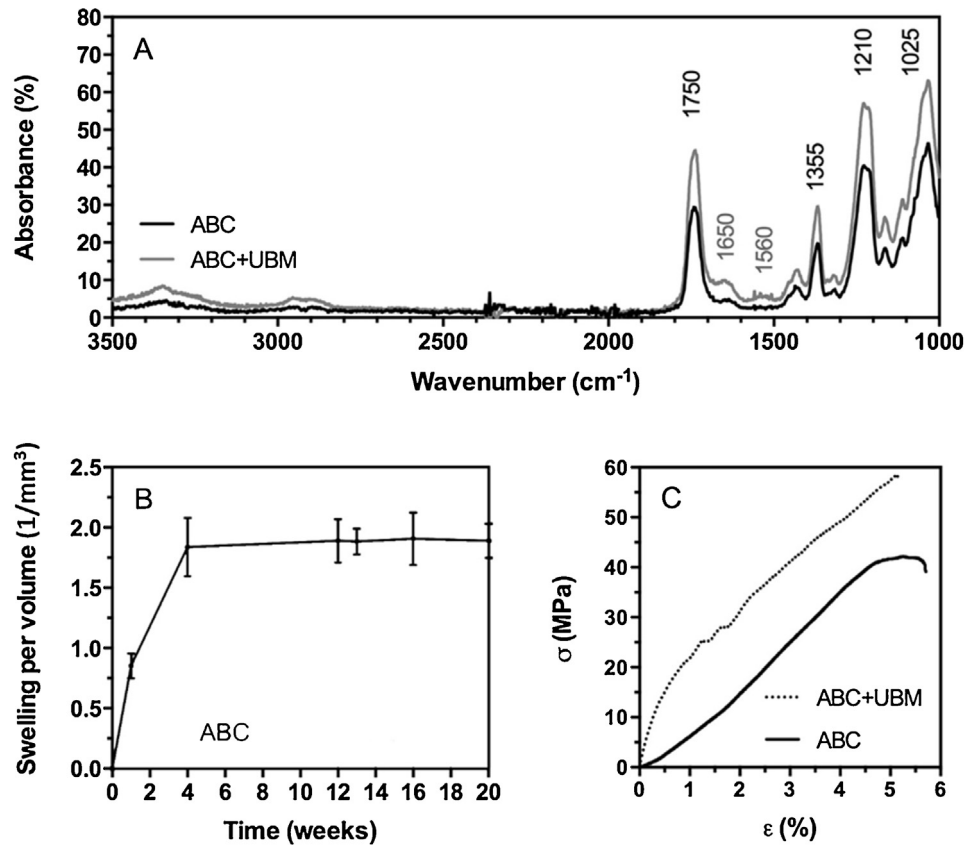


Fig. 2. (A) Infrared spectra, (B) swelling profile and (C) stress–strain measurements for bacterial cellulose substrates. The results correspond to the mean $\pm$ standard error mean of twelve replicas *per* condition for swelling assays and five replicas *per* condition for stress–strain assays.

Table 1

Contact angles, estimated surface free energy components and mechanical properties of acetylated bacterial cellulose (ABC) uncoated and coated with urinary bladder matrix (UBM).

	ABC	ABC + UBM
Contact angles ($^{\circ}$)		
WCA	64.8 ± 1.9^e	49.6 ± 1.8^f
FCA	43.7 ± 1.4^g	42.1 ± 1.8^g
BCA	16.5 ± 0.6^h	20.0 ± 0.7^i
Surface free energy components (mN/m)		
γ^+	0.3	0.1
γ^-	13.4	31.9
γ^{AB}	4.3	3.0
γ^{LW}	42.6	41.8
γ	46.9	44.8
Mechanical Properties		
σ_{max}	(MPa) 37.6 ± 4.4^a	42.5 ± 4.3^a
ϵ_{break}	(%) 4.8 ± 0.5^b	5.1 ± 1.4^b
E	(MPa) 677.5 ± 43.5^c	2047.7 ± 192.3^d

Abbreviations: σ_{max} : maximum stress; ϵ_{break} : elongation-at-break; E : elastic modulus; WCA: water contact angle; FCA: formamide contact angle; BCA: bromonaphthalene contact angle; γ^+ : acidic parameter; γ^- : basic parameter; γ^{AB} : acid-base component; γ^{LW} : Lifshitz-van der Waals component; γ : total surface free energy. Statistics five replicas per substrate in the mechanical assays and two contact angle measurements per replica with six replicas per substrate. Equal letters in superscript indicate that differences are not statistically significant.

sampling depth of ATR-FTIR, comparing to only several to tens of nanometers of protein-grafted layers' thickness. In this case, the C–O stretch at 1210/cm from the acetylation layer may be masking the amide III vibration bands at 1245/cm.

The substrates surface free energy and hydrophobicity (Table 1) were evaluated as they are known to influence protein and cellular

adhesion. The nanofibrillar nature of BC translates into a globally irregular topography, in which the measured (apparent) contact angle is obtained by the tangent between the liquid–vapor interface and the apparent (as seen macroscopically during measurements) local surface of the solid. Surface roughness and chemistry are known to affect its wettability [37]. Although following UBM coating, ABC substrates exhibited a decrease in the water contact angles from 64.8 ± 1.9 to $49.6 \pm 1.8^{\circ}$, the moderate hydrophobic character of ABC (46.9 mN/m) was maintained after UBM coating (44.8 mN/m). This decrease may be due to a lower availability of surface hydroxyl groups after UBM adsorption. The dispersive (γ^{LW}) and polar (γ^{AB}) energy components were roughly maintained after UBM coating. The acid–base forces (γ^{AB}) resulted mainly from negative/basic charges (γ^-), which showed a strong increase from 13.4 to 31.9 mN/m after UBM coating. Therefore, the UBM adsorption onto ABC was found to mainly increase the monopolar nature (γ^-) of the ABC surface.

The maintenance of the mechanical integrity (resistance to tear) and dimensional stability (neither swelling nor degradation) are relevant properties in RPE transplantation, since the transplant success depends on the correct positioning of ultrathin substrates with surgical instruments in the subretinal space [14,38]. After 4 weeks in PBS, ABC substrates (Fig. 2B) showed some swelling characterized by a two-fold increase in ABCs initial dry mass; however, this swelling stabilized afterwards. Following 20 weeks of immersion in PBS, ABC substrates showed no signs of degradation and both substrates were easily manipulated with tweezers. A nearly identical profile for ABC substrates coated with UBM was observed in the same exposure period (*data not shown*).

According to its mechanical properties (Table 1 and Fig. 2C), both samples showed similar stress–strain behavior, with stress

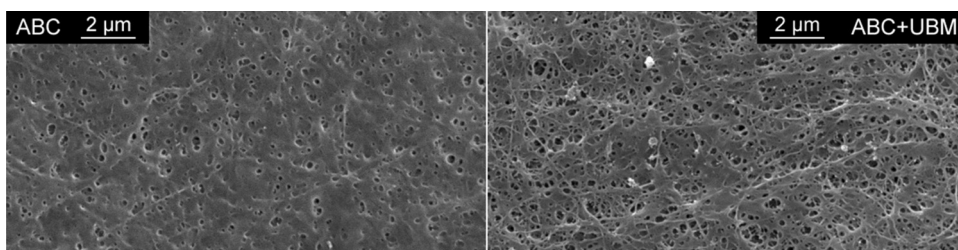


Fig. 3. Surface scanning electron micrographs of acetylated bacterial cellulose (ABC) and urinary bladder matrix coated ABC (ABC + UBM) substrates.

increasing with strain increment until the sample collapsed. The surface coating of ABC nanofibers by UBM resulted in a 3-fold increase in the elastic modulus (E). The mechanical properties of ABC substrates coated with UBM are comparable to the ones reported in a recent study, where nanofibrous membranes of poly(lactic-co-glycolic acid) (PLGA) and collagen type-I (the main component of UBM) were evaluated as potential BM substitutes [3]. The values obtained for the PLGA nanofibrous membrane were of 1.5 ± 0.4 MPa, $28.8 \pm 4.9\%$ and 131.9 ± 13.3 MPa for maximum tensile strength, tensile strain and elastic modulus, respectively. On the other hand, higher values were obtained from the collagen nanofibrous membrane, namely 10.8 ± 0.7 MPa, $70.0 \pm 4.6\%$ and 217.9 ± 15.3 MPa for maximum tensile strength, tensile strain and elastic modulus, respectively. In this work, the values obtained for the developed BC substrates are in the same range or even higher, thus suggesting its mechanical feasibility as a cell carrier substrate for the potential RPE transplantation therapy in retinal degenerative diseases. Also, surface scanning electron micrographs showed porous surfaces for both substrates (Fig. 3). The porous nature of these substrates could be advantageous since a non-biodegradable substrate has to meet the passive diffusion requirements to be considered a viable BM substitute [9].

Since BC is a microbial polysaccharide, the presence of endotoxins in BC may constitute a problem when considering biomedical applications. Endotoxins can activate RPE cells to secrete excessive amounts of inflammatory cytokines in the retina, thus promoting RPE degeneration [40]. In the USA, the regulatory endotoxin limits are 500 EU/L for general medical devices and 60 EU/L for devices that contact cerebrospinal fluid [41]. The endotoxin quantification obtained by the LAL test (determined according to the FDA guideline [41]) indicated an extremely low presence of endotoxins in ABC substrates, specifically 0.11 ± 0.01 and 2.12 ± 0.04 EU/L before and after UBM coating, respectively. UBM coating resulted in a 20-fold increase in the amount of endotoxins present in the ABC substrates. This may be due to microbial contamination of the porcine urinary bladders that led to the presence of endotoxins in the UBM solution. Similar endotoxin results have been reported for BC scaffolds produced by *G. xylinus* ATCC® 700198™ for tissue-engineered urinary conduits [39]. From the above results, the substrates could be classified as non-pyrogenic and should not induce complications in vivo. Also, purification/depyrogenation method of treating BC using aqueous 1 M NaOH solution followed by rinsing with deionized sterile water [39], was found to be effective in removing endotoxins from the BC scaffolds.

3.3. RPE cell viability and proliferation

Cell viability after 3, 7 and 14 days of cell culture on ABC substrates was assessed through metabolic activity (MTS) assays (Fig. 4) and LIVE/DEAD fluorescence microscopy (Fig. 5) of viable (green staining) and compromised (red staining of nucleus) cells. Regarding the metabolic activity after 3 days in culture, a difference in the number of adhered and metabolically active cells was observed between the ABC surfaces (coated and non-coated with

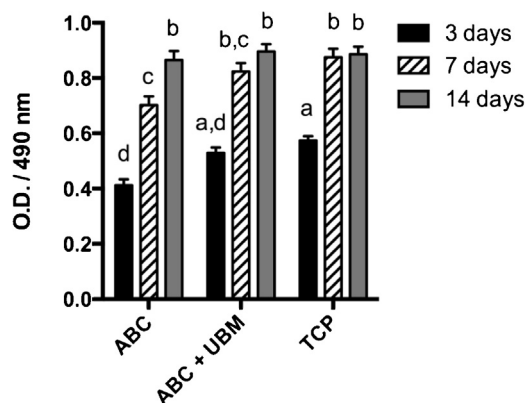


Fig. 4. Metabolic activity determined after 3, 7 and 14 days of HTERT-RPE cells cultured on acetylated bacterial cellulose (ABC), ABC with urinary bladder matrix (ABC + UBM) substrates, and tissue culture polystyrene (TCP, control surface). Three independent assays were performed, each with six replicas *per* substrate condition. Equal letters above the bars indicate results not different statistically.

UBM) and the TCP positive control. Cells cultured on the ABC substrates coated with UBM substrate and TCP surface showed the same metabolic activity after 3 days. Lower metabolic activity after 3 days was obtained for the ABC substrates without UBM coating as compared to TCP.

After 7 and 14 days, the cells were able to further proliferate and presented similar viability profiles as seen for the 3 days. Cells cultured in the TCP surface, achieved a 100% confluent monolayer configuration after 7 days. The LIVE/DEAD fluorescence micrographs were coherent with the metabolic activity results, thus confirming the positive effect of UBM in cell viability leading to a profile similar to TCP. Additionally, while a confluent monolayer was obtained in TCP after 7 days and in ABC after 14 days, in the ABC + UBM substrates it appears to be reached between 7 and 14 days (as can also be observed in Fig. 5). The moderate hydrophobic surface composed of amine groups from the UBM proteins and acetate groups from the ABC substrates further improved the cell response to a behavior similar to the one observed in the TCP positive control surface. Tezcaner et al. [42] reported that RPE cells prefer smooth rather than rough surfaces and that protein adsorption could modify the surface roughness by smoothing its irregularities. This might have contributed to the UBM positive effect on the cells response. Moreover, a similar RPE cell growth to the one obtained in the ABC substrates coated with UBM was reported with ultrathin collagen type-1 substrates and montmorillonite clay based polyurethane nanocomposite substrates [12,38].

3.4. RPE cell morphology

To evaluate the morphology of the RPE cell monolayers grown on the ABC substrates, several cell parameters may be assessed, namely the expression of RPE proteins such as ZO-1 and RPE65, the observation of a polygonal shaped cells with apical microvilli

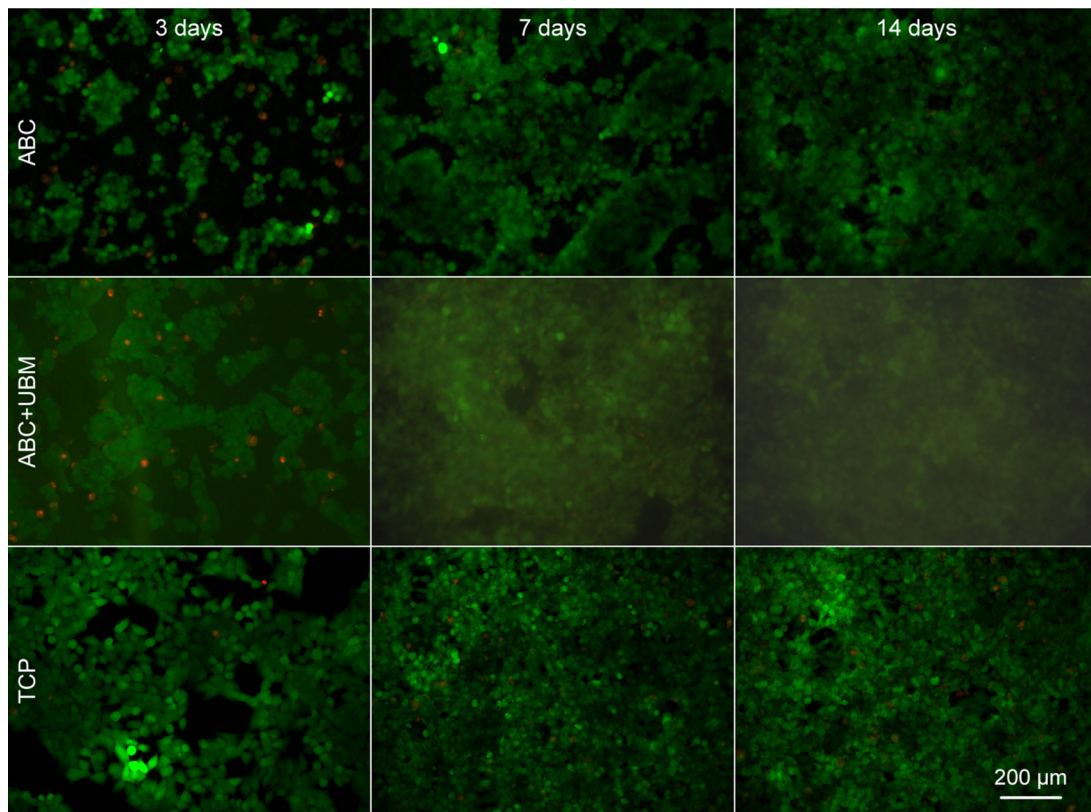


Fig. 5. Representative live/dead fluorescence micrographs of hTERT-RPE1 cells cultured for 3, 7, and 14 days on acetylated bacterial cellulose (ABC), ABC coated with urinary bladder matrix (ABC + UBM) and tissue culture polystyrene (TCP, control surface). The ABC + UBM micrographs presented higher substrate fluorescence interference. (For interpretation of the references to color in the text, the reader is referred to the web version of this article).

in a monolayer configuration and the development of TER [1,3,4]. The RPE characteristic phenotype can be ascertained by the expression of RPE specific proteins. Immunofluorescence (IF) allows the observation of specific biomolecules and its cellular distribution. Two proteins were studied: the tight junction protein ZO-1 and the chaperone RPE-specific protein 65 kDa (RPE65), which is involved in the visual cycle of retinal. These are two of the most common RPE markers used to evaluate the development of differentiated properties of RPE cells in *in vitro* culture studies [38,43–45]. In IF assays, hTERT-RPE cells grown to confluence on glass and ABC substrates coated with UBM (Fig. 6), were able to express the structural protein ZO-1 and the metabolism protein RPE65, in the intracellular side of plasma membrane and cytoplasm, respectively.

Confluent cells grown on non-coated ABC substrates were also evaluated; however, these typical RPE phenotypic features could not be observed (no fluorescence signal was detected, *data not shown*). These results suggest that although the cells were able to adhere and proliferate on the ABC substrates, they were not able to develop the morphological features typical of RPE cells. Also, this confirms the positive effect of the UBM coating, and its important role in the development of a native RPE phenotype. Moreover, although ZO-1 was clearly expressed in glass and ABC substrates coated with UBM (Fig. 6A, B), the cells cultured in the coated ABC substrates were still apparently in the process of full differentiation, as there was some ZO-1 in the cell cytoplasm. Also, the RPE65 protein was detected in the cytoplasm of both cultures (Fig. 6C, D), which was expressed in small amounts when compared to ARPE-19 cells and primary cells [3,38]. Therefore, the hTERT-RPE1 cells may need longer periods in a confluent state to further organize their structure and proteins, or simply lower amounts of this protein are expressed in this cell line. To further observe the morphology of

hTERT-RPE cells cultured in the ABC substrates coated with UBM, SEM pictures were taken (Fig. 6E–G). These results supported the observation that the cells were able to polarize in these substrates since microvilli and a monolayer configuration with polygonal epithelial cell shape (white hexagon) were clearly shown. Also, an increasing number of microvilli (white arrows) was observed with prolonged culture time (from 10 to 20 days seen between F and G, Fig. 6). The achievement of a cell monolayer between 7 and 14 days, as expected by the viability and proliferation results (Figs. 4 and 5), was confirmed in the micrographs taken after 10 days of cell culture (Fig. 6E, F). The development of microvilli confirmed the ability of RPE cells to form a cell monolayer in a polarized state when cultured in ABC substrates coated with UBM. To further assess the polarized state of RPE cell monolayers grown in these substrates, transepithelial resistance quantification assays were performed. Although only human fetal or primary RPE cells have been described to develop highly polarized RPE *in vitro*, studies on polarized RPE include the use of immortalized human RPE cell lines, such as the ARPE19 and D407 [4]. For sub-confluent or unpolarized RPE cells, no resistance to the passage of current was detected. Hence, in accordance with the previous morphological studies (IF and SEM), TER was detected in cells cultured in the ABC substrates coated with UBM. The average TER of cells in a confluent state maintained for 3 weeks in Transwell® inserts with fibronectin and in ABC substrates coated with UBM substrates was 12.0 ± 0.7 and $10.2 \pm \Omega \text{ cm}^2$, with a maximum value of 17 and $13 \Omega \text{ cm}^2$, respectively. To our knowledge, the TER values of hTERT-RPE cells have not been reported so far. The TER herein presented for hTERT-RPE cells comprises low values (below $17 \Omega \text{ cm}^2$), lower as compared to other immortalized cell lines (ARPE19 and D407) that were described to develop monolayers with TER values below $50 \Omega \text{ cm}^2$ [4,32,45,46]. Nonetheless,

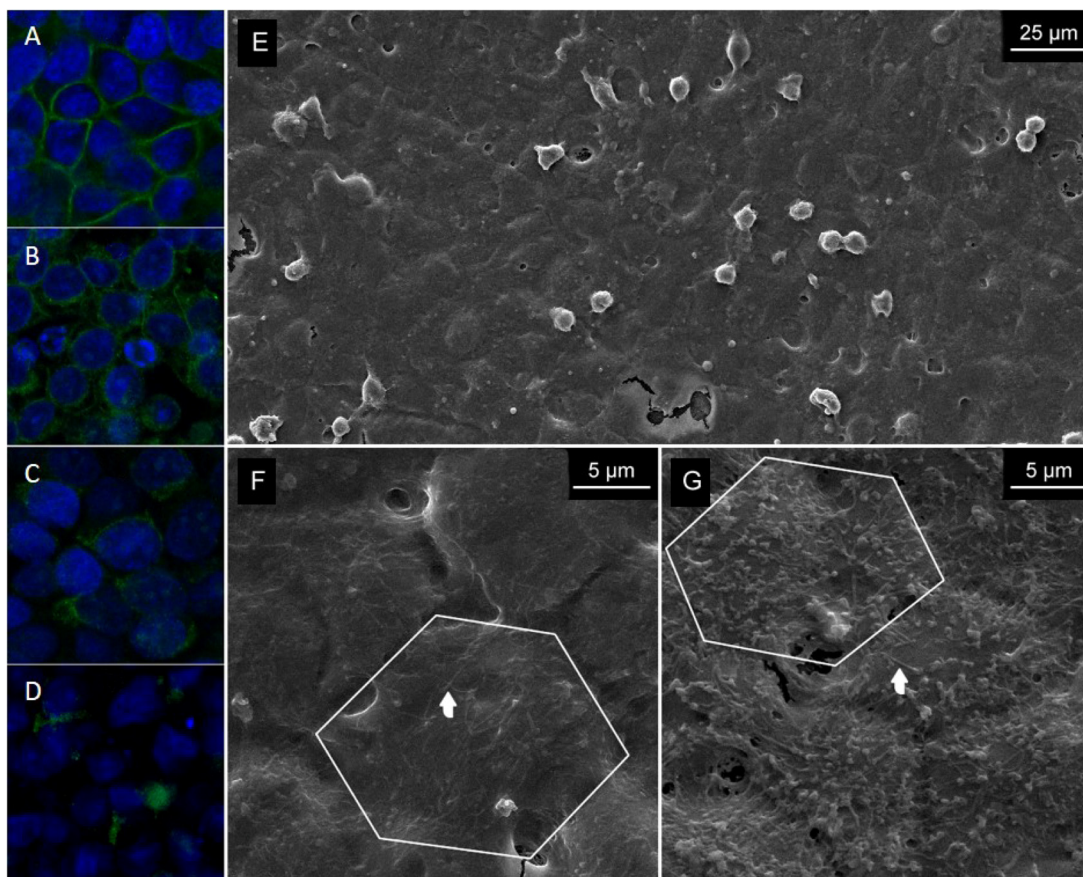


Fig. 6. Immunofluorescence micrographs of ZO-1 (A and B) and RPE65 (C and D) green staining and nucleus blue staining in hTERT-RPE cells grown in glass (A and C) and in acetylated bacterial cellulose with urinary bladder matrix (ABC+UBM) substrates (B and D). Scanning electron micrographs of hTERT-RPE cell cultured in ABC+UBM substrates for 10 days (E and F) and 20 days (G). The cell monolayer configuration is observed in E, while the presence of microvilli is pointed out with white arrows (F and G) and the white hexagons highlight the polygonal shaped cells (F and G). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

the lower values obtained in this work could tentatively be supported by the reported down-regulation of proteins attributed to cell polarization in this cell line [47].

Additionally, several *in vitro* changes have been described in immortalized RPE cells, such as decreased apical microvilli and basal infoldings, low expression or absence of RPE-specific markers, among others [2]. These changes are most likely due to the lack of direct interaction and signaling from photoreceptors to the RPE apical surface. In fact, primary cultures are known to better retain the RPE native tissue physiology and morphological characteristics [2]. Therefore, these cultures should be explored in the future studies (such as phagocytosis assays for cell function analysis) to further assess the substrates potential for RPE transplantation. Nevertheless, the polarized RPE cell monolayers formed on the ABC substrates coated with UBM, using an immortalized cell line, reinforce the high potential of this biomaterial as a cell carrier for RPE transplantation.

4. Conclusion

This study demonstrated for the first time that coating ABC substrates with UBM is advantageous for the adhesion and proliferation of RPE cells. Notably, these coated substrates led to the development of a cell monolayer exhibiting the desired RPE phenotype, *i.e.*, polygonal shaped cells with microvilli, capable of expressing cytoskeletal (ZO-1) and metabolic (RPE65) proteins. Besides, these substrates showed low swelling degree, no signs of hydrolytic degradation, easy manipulation, residual

content in endotoxins and high mechanical strength. The complex requirements of cell carrier substrates for RPE transplantation have hindered the development of viable substrates, and although many have been studied for this purpose, rarely a substrate has shown positive results in more advanced studies, either failing in mechanic robustness or in promoting an adequate cell monolayer. Hence, comparatively ABC substrates coated with UBM exhibit interesting physicochemical features and proper mechanical properties that together with their ability to support the phenotype of RPE cells, represent a very promising advance in this field of study.

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