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Desarrollo y aplicación de materiales avanzados en la producción de bio-membranas artificiales funcionales para su uso en medicina traslacional: DMRE

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ÍNDICE

Abstract
Retinal constructs comprising retinal pigment epithelium (RPE) cells supported by electrospun scaffolds have been investigated to treat Age-related Macular Degeneration (AMD). Various artificial membranes have been used as scaffolds for RPE for monolayer reconstruction. However, electrospun scaffolds studiedto-date do not mimic the structural microenvironment of human Bruch's membrane (BM), essential for native-like RPE monolayers. The aim of this study was to design, produce, and evaluate the efficacy of an artificial Bruch´s membrane (ABM) produced by bioengineering techniques for the treatment of AMD.We manufactured an ABM by electrospinning using solutions of polycaprolactone (PCL) in combination with different concentrations of collagen. These membranes were co-cultured with ARPE-19 cells for 25 days, and cell viability and expression changes in Retinal Pigment Epithelium-Specific 65 kDa and Zonula Occludens 1 were quantitatively studied. Morphology and ultrastructure of ABM and seeded cells were evaluated by Immunofluorescence (IF) and scanning electron microscopy (SEM). ABM showed no cytotoxic effect on ARPE-19 cells seeded and showed proliferative capacity, mainly at PCL/30%Gel proportion. Expression of RPE markers showed no differences in cells cultured in ABM versus controls. IF assays for RPE65-, cytokeratin-18- and actin and SEM show that ARPE-19 cells forman orientated monolayer of polygonal cells with morphological polarity on PCL/30%Gel; the apical surface of cells exhibited abundant protruding microfolds and zones of polygonal borders.
The PCL/30%Gel membrane provides a micro-architecture
mimicking a normal human BM. In conclusion, we stablished the creation of a prosthetic Bruch's membrane for RPE transplants to treat AMD, and there is not steel an ideal substrate that can harbor RPE cells to treat this pathology.

Introduction
Brunch's membrane (BM) is a unique pentalaminar structure, which is strategically located between the retinal pigment epithelium (RPE) and the fenestrated choroidal capillaries of the eye. The pentalaminar BM structure forms a single functional unit with RPE and choriocapillaris. The BM is an elastin- and collagen-rich extracellular matrix that acts as a molecular sieve. It is involved in the essential exchange of numerous biomolecules, oxygen, nutrients, and waste product between these tissues. Thisspecial extracellular matrix (ECM) undergoes significant age- related changes that are involved in age-related macular degeneration (AMD) and other chorioretinal diseases [1,2,3,4].
The changes occurring in BM with age include increased calcification of elastic fibers, increased cross-linkage of collagen fibers, and increased turnover of glycosaminoglycans. These age- related changes may not only influence the normal age-related health of photoreceptors cells, but also the onset and progressionof diseases like retinitis pigmentosa (RP) and age-related macular degeneration (AMD). AMD is a well-characterized and extensively studied disease and it is currently considered the leading cause of visual disability among patients over 60 years of age. The hallmark of early AMD is the formation of drusen, pigmentary changes at the macula, and mild to moderate vision loss [4,5,6].
Among the first damaged cells in AMD are RPE cells that are anatomically adjacent to the neurosensory retina, which is essential for vision creation and processing. The RPE is composed of a monolayer of polarized pigment cells, which lie on BM [2]. The pathophysiological changes of the BM in the AMD wet form affect the normal functions of RPE cells [7]. Thus, a potential and promising therapy for the wet form of AMD could bethe restoration of the structural characteristics of the BM. Several treatments for the wet form

of AMD have been proposed, such as antiangiogenic drugs, photodynamic therapy, and laser photocoagulation; however, these options those treatments do not restore the functions or BM structure. RPE cell replacement therapy is one of the most promising strategies to replenish or to replace RPE that has been damaged or lost. In this late setting of AMD, the presence of a basal membrane that can support the viability and nourishment of transplanted cells is essential. Several studies have shown subretinal transplantation of suspended RPE cells in the patients with AMD; however, the outcome has not been consistent. Given that BM plays an essential role to maintain andsupport the physiological function of the RPE, it has been proposed that RPE cells can be seeded on a biodegradable and biocompatible prosthetic artificial Bruch's membrane (ABM) for use in cell transplants [8].

Therefore, efforts have been conducted to develop a BM substitute that enables and supports the delivery of a functionalintact RPE monolayer patch [7,9]. Artificial BMs for RPE transplantation has been developed using a variety of thin and biodegradable and biocompatible polymers such as collagen, polyimide, polycaprolactone (PCL), poly(L-lactide-co-glycolide) (PLGA), poly(L-lactide-co-D, L- lactide) (PLDLA) and poly (L- lactide, PLLA). ABMs have been also developed using natural materials such as the modified human amniotic membrane or some biopolymers such as collagen, fibrin, silk fibroin-chitosan,and chitosan-gelatin used as RPE cells carriers [7,9,10,11].

Nevertheless, some of these previous scaffolds have failed to preserve the structural features of native BM and mainly failed to display the capability of inducing and maintaining a cells RPE-likemorphology (natural appearance) during cell culture onto the scaffolds [13]. Electrospinning is a well-known scaffold processing technology that generates thin polymeric fibrillary materials with fibers diameters ranging from below 100 nm to above several microns.

Scaffolds formed by nano- or micro-fibers can be generated by this process, and a great diversity of materials can be used to generate such scaffolds, for example, PCL [14], which is a biodegradable polyester suitable as an implantable bio-material [14,15,16]. PCL has been approved by the Food and Drug Administration (FDA) for some applications in the human body, such as drug delivery systems, sutures, or adhesion barriers [17]. Thus, particularly, fabrication by electrospinning of a novel, artificial, and biocompatible thin, micro-fibrous artificial basal membrane based on PCL could be interesting. PCL-based materials can present adequate mechanical properties for surgical management. In conjunction with the micro-fibrillar structure provided by the electrospinning fabrication, these materials can represent an artificial basal membrane with adequate topographicand mechanical properties to simulate the structural features of the native BM, and consequently, to display the capability of inducing and maintaining a cells RPE-like morphology (natural appearance) upon cell culture on them.

Nevertheless, PCL alone lacks the appropriate biological functionality to promote cell adhesion, mainly due to its hydrophobic nature and the lack of biologically favorable functional groups. In this sense, gelatin (Gel) represents a hydrophilic, greatly available, natural polymer that upon incorporation to the PCL-based membranes might provide the membranes with the required biological functionality to promote cell adhesion [18]. Gelatins obtained from collagen hydrolysis is the main component of the ECM of different tissues [18,19]. Thusthis work is aiming at developing a novel, thin, micro-fibrillar membrane through electrospinning, performed by PLC and Gel todevelop a scaffold capable of supporting the culture of viable cells that preserve their RPE cells-like features and functionality, to eventually be able to deliver a functional, intact RPE monolayer patch

(artificial BM) for the potential treatment of AMD.
The functional features of the artificial BM designed herein were analyzed using ARPE-19 cells, a human RPE cell line. Similarly, to native RPE cells, ARPE-19 cells express several specific genes such as RPE65 and Zone-Ocludens-1 (ZO-1) and phagocytose shed components from outer segments of rod and cones [7,20].
ARPE- 19 cells were cultured onto the developed PCL/Gel micro-fibrillar membranes and cell adhesion, viability, expression of characteristic markers of RPE-like cells, and morphological polarity were evaluated. Our results demonstrated that 70 wt.% PCL- 30 wt.% Gel electrospun thin micro-fibrous membranes constitute an appropriate scaffold for ARPE-19 cell culture to potentially develop an adequate artificial BM. The PCL-Gel membranes developed were no cytotoxic and allowed the proliferation of ARPE-19 cells that showed adequate eRPE-like morphology and expressed normal levels of the principal markers of native RPE cells such asRPE65 and ZO-1 after 25 days of culture on the PCL-Gel membranes.

Material and Methods

Material

Polycaprolactone (PCL) (Mn = 80,000 Da) was purchased from Sigma-Aldrich. Gelatin Type B derived from bovine skin and 99.5wt% glacial acetic acid (AcAc) was purchased from J.T. Baker, Fisher Scientific SL. MTT ([3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide]), dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich, Inc. (MO, USA). Dulbecco's modified Eagle's medium (DMEM: F12), fetal bovine serum (FBS), penicillin/streptomycin (antimycotic) 0.25%, trypsin-EDTA0.25%, phosphate-buffered saline (PBS, pH = 7.4) was acquiredfrom GIBCO. Rabbit anti-actin primary antibody (Santa Cruz Biotechnologies, CA, USA), Rabbit anti-Cytokeratin 18 primary antibody (Biorbyt Ltd. Cambridge, UK), Alexa Fluor goat anti- rabbit 488, Alexa Fluor goat anti-rabbit 594 or Alexa-Fluor goat anti-mouse 488 (1:200 dilution) was purchased from JacksonImmunoResearch Laboratories Inc.

Preparation of fibrillar scaffolds

The polymer solution of Gel-PCL for electrospinning was prepared at room temperature (RT; ≈ 18-20 °C) by dissolving PCLinto AcAc (98.5% v/v) at a concentration of 19% w/v, based on ourprevious work to obtain homogeneous defect-free electrospun PCL fibers, and simultaneously adding the adequate amount of gel to produce Gel-PCL solution with 5, 15 or 30 wt. % Gel, relative to total polymer content in solution. Gel concentration waschosen aiming to obtain homogeneous electrospun scaffolds by the single-step AcAc solution process reported herein, with Gel concentrations within the range where significant phase separation has been observed for other solvents systems (25% ≤ Gel wt.% ≤ 50%, depending on the solvent system) and where appropriate cell response has been observed (Gel wt.% ≥ 15%).
Only-PCL solution (19% w/v) in AcAc was also prepared at RT (18-20 °C) and used as a control to study the influence of Gel concentration on the scaffold's properties. The only-PCL and Gel-PCL blends solutions were stirred at 300 rpm for 48 h at RT. The viscosity of solutions was measured in a viscometer (Brookfield LVDV-E15) equipped with a small sampleadapter and their conductivity was measured in a JENWAY 3540conductivity meter. Electrospinning of scaffolds was performed using a horizontal equipment assemble in our laboratory, consisting of a syringe pump (NE-4000 2 channels, Pump System Inc.), a high voltage power supply (EH60P1.5 Glassman High Voltage Inc.) and a grounded aluminum

collector plate. Gel-PCL fibrillar scaffolds were obtained by electrospinning at 14 kV with a needle-to- collector distance of 14 cm and Gel-PCL solution pumped througha 21 Gauge metal needle at 1 mL/h. Only-PCL scaffolds were electrospun using the same solution feed rate but increasing the needle-to collector distance and the voltage to 18 cm and 18 kV, respectively. The electrospinning process was conducted at ambient conditions (temperature ≈ 20 – 21 °C and relative humidity ≈ 46 – 48%) during 40 minutes for either only-PCL or PCL-Gel blends. After electrospinning, the scaffolds were removed from the collector and sterilized under ultraviolet light (UV) for 15 min on each scaffold side. Scaffolds were named according to their composition and Gel wt.% as follows: PCL for only- PCL scaffolds and PCL /5%Gel, PCL/15%Gel and PCL/ 30%Gel for 5, 15 and 30 wt.% Gel Scaffolds, as reported in Table1.

Table 1. Chemical composition, viscosity and conductivity of electrospinning solutions and nomenclature of electrospun scaffolds.

Scaffold	Gel (wt. %)	PCL (wt. %)[a]	Viscosity[b](cps)	Conductivity[c] (µS/cm)
PCL	0	100	4750 ± 39	0.26 ± 0.01
PCL/5Gel	5	95	4547 ± 46	3.35 ± 0.23
PCL/15Gel	15	85	4445 ± 50	10.6 ± 0.15
PCL/30Gel	30	70	4139 ± 139	18.72 ± 0.28

[a]Scaffolds chemical composition according to total polymer content (100 wt.%) in electrospinning solution. [b]Viscosity of polymers solution used for electrospinning of Scaffolds. [c]Conductivity of polymers solution used for electrospinning ofscaffolds.

Physical-chemical characterization of the scaffolds

Morphology

Established membranes were subjected to an examination of their physicochemical properties, including physical morphology, porosity, and wettability. To investigate the precise scaffold micro- morphology, samples were sputtered coated with carbon and observed with a JEOL-7600 Scanning Electron Microscope (SEM)using an accelerating voltage of 10.0 kV. Fiber diameter distribution was calculated by measuring (AxioVision

Software; Carl Zeiss Microscopy GmbH) the diameter of 80 fibers in total, proportionally selected from two different SEM micrographs acquired from the air and the collector sides of the scaffolds.
Measurements from both scaffolds' sides micrographs were averaged as one group of measurements per scaffold. Averagefiber diameter ± standard deviation (SD) is reported.

IR Spectroscopy

Chemical functional groups were analyzed by Infrared Spectroscopy. FTIR spectra were acquired by means of an infrared spectrometer (Nicolet 880 FTIR) with Attenuated Total Reflection (ATR) module at 4 cm^{-1} resolution and 32 scans in a wavenumber range of 4000-400 cm^{-1}. Spectra of pristine Gel and PCL were acquired as reference for the assignment of the IR bands in the spectra of the as-electrospun PCL, PCL/5%Gel, PCL/15%Gel, and PCL/30%Gel scaffolds. FTIR spectra of the scaffold samples, as used for the in vitro cell assays (i.e. scaffoldsrinsed in water and ethanol, and left 48 h to dry at RT before UV- sterilization) were acquired before and after UV-sterilization to identify the possible UV-sterilization effects on the components ofthe scaffolds.

Wettability and water contact angle

Scaffolds hydrophilic/hydrophobic character was determined from water contact angle (WCA) measurements performed in a video- enabled goniometer (OCA 15EC; Dataphysics) via the static sessile drop method using 4 μL double distilled water drops at RT.WCA was measured on hydrated (in deionized water to their water uptake plateau) scaffolds samples to discarding the influence of the water absorption effect. WCA was automaticallycalculated (156 frames/s) using the SCA20_U software over the whole time period of video recording. WCA reported values represent the mean ± SD for the stable plateaufor hydrated samples.

ARPE-19 cells culture on the membranes

All the present study protocols adhered to the provisions of the Declaration of Helsinki for research involving human tissue. A commercially available human retinal pigmented epithelium cell line (ARPE-19) from ATCC was used to study the biological response of the EBM. Cells were first grown in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, MA,USA) with 10% fetal bovine serum (FBS) until confluence.
Thereafter, cells were harvested from plates using trypsin and seeded onto the different membrane samples at a density of 3×10^4 cells/cm^2 and cultured in DMEM with 10% FBS in 48-well plates. Cells seeded in empty wells (standard tissue culture polystyrene wells; TCPS) without scaffolds were used as controls. To ensure sterilization of membranes samples, they were initially exposed to ultraviolet (UV) radiation for 30 min in a Biostar cabinet from Telstar S.A. (Madrid, Spain) before cell seeding. Twodifferent cell seeding protocols on the EBM were probed in order to determine the best seeding protocol to increase the adhesion of ARPE-19 cells onto the membranes. In the first protocol, (total-volume seeding), 3×10^4 cells in 300 μl of culture medium were seeded onto the EBM samples. In the second protocol (drop- seeding), 3×10^4 cells in 30 μl of culture medium were seeded ontoEBM, and then, after 30 min of incubation, culture medium was added to complete 300 μl. In both protocols, DMEM complemented with 1% penicillin- streptomycin and 10% FBS wasused as culture medium. Plates containing the membranes and cells or the cells seeded on the bare TCPS wells were always kept, during culture, in an incubator at 5% CO_2 at 37°C.Theseeding protocol with the best results, that is drop seeding protocol, was used for all further in vitro experiments.

ARPE-19 cells viability onto the PCL/Gel membranes

After 24 h of the culture of ARPE-19 cells onto the EBM or ontothe TCPS wells (controls), cells were deprived of FBS to avoid any participation of FBS components in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) experiments, and further culture for 24 h. Then, viability assays were performed in reference to the MTT kit manufacturer's instructions (Cell Proliferation Kit II, MTT proliferation, ROCHE, Mannheim, Germany). *In vitro* biological performance of the different EBM was compared to TCPS wells. Three samples from each group ofcells cultured onto BM transferred to a new 24-well plate containing 200 µl of DMEM/penicillin-streptomycin per well for MTT assays, in order to measure only the viability of the ARPE-19 cells that were adhered to the membranes. Then, 50 µl of MTT solution was added to each well and MTT-added samples were cultured for 45 min at 37 °C. Finally, formazan crystals were dissolved using the dissolution medium provided by the kit and 100 µl of supernatants were individually placed into a new well of a 96-well plate by duplicate. Cell viability, regarding absorbance, was determined by spectroscopy with a microplate reader at 540 nm (Biotek St Louis Mo, USA). A standard curve of ARPE-19 cellswas used as a reference for the MTT assays to correlate the absorbance to number of viable (metabolically active) cells. All assays were individually and independently performed four times. Data were analyzed by one-way ANOVA and Friedman test usingGraphPad Prism software (GraphPad Software Inc., CA, USA) and $p< 0.05$ was regarded as statistically significant with a 95% confidence interval.

Immunodetection assays

Immunofluorescence assays were performed to corroborate the growth and distribution of ARPE-19 cells after 48 h of culture onto the different EBM. EBM cultured with cells previously seeded were washed three times with PBS /Tween 0.02% (PBS/T) for 1 hand incubated in PBS 1X/Tween 0.02% for permeabilization.

Then, a blocking solution (PBS1X/BSA 0.02%) was used for avoiding nonspecific binding. After, preparations were incubatedovernight at 4 °C with monoclonal mouse anti-RPE65 primary antibody (Novus NB-100-355), rabbit anti-actin primary antibody(1:100 dilution, Santa Cruz Biotechnologies, CA, USA) or rabbit anti-cytokeratin 18 primary antibody (1:100 dilution, Biorbyt Ltd. Cambridge, UK). After incubation, samples were washed three times using PBS1X/Tween 0.02% and incubated with corresponding secondary antibodies: Alexa fluor goat anti-rabbit488, Alexa fluor goat anti-rabbit 594 or Alexa-fluor goat anti- mouse 488 (1:200 dilution). After 2 hours of incubation with secondary antibodies, samples were washed four times using PBS/Tween for 3 min and mounted using VECTASHIELD Antifade Mounting Media with DAPI (Vector Laboratories). Finally, mounted samples were observed by fluorescence microscopy and photographs were taken at random locations with a confocal microscope Axio Imager M1 fluorescence microscope (Zeiss).

ARPE-19 cell morphology on the membranes byScanning Electron Microscopy

EBM (8-mm diameter) were placed in 48-well plates. ARPE-19 cells were drop seeded in the central part of each sample and cultured for 25 days; culture medium was changed every 3 days for SEM experiments. After the culture period, a standard preparation method for SEM observation was used for all membrane-cells samples [21,22]. Briefly, specimens were fixed with 4% paraformaldehyde/0.5%, glutaraldehyde in 0.1M sodiumcacodylate buffer (pH 7.2) at 4°C overnight. Then, washed three times with 0.2M sodium cacodylate buffer (pH 7.2) at 4°C, using Pasteur pipettes. Post-fixation treatment was carried out with

1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h. at 4°C. Then, preparations were washed and dehydrated with increasingly graded ethanol (30% v/v to absolute ethanol) until propylene oxide at room temperature. Posteriorly, were dried by the Critical Point method in a Samdri 780A desiccator (Rockville, MD, USA) using CO_2. Dried EBM samples were then mounted on aluminum stubs with silver paste and placed in a high vacuum evaporator for gold coating during 6 minutes in a metal ionizing JEOL JFC-1100 (Fine Coat® ion Sputter, JEOL Ltd, Tokyo, JP). Finally, preparations were examined with a Zeiss DSM-950 scanning electron microscope (Carl Zeiss, Jena, DE) at 25 kV anda 10 mm working distance. Electron-micrographs were recorded (55 P/N film; Polaroid Corp., Cambridge, MA) of each sample at random locations.

Real-time quantitative PCR

To confirm the expression of characteristic RPE-cells markers upon cell culture on the EBM, qRT-PCR assays were performedon the ARPE-19 cells cultured on the membranes. Cells were drop seeded in the central part of the membranes further incubated for 20 days; culture medium was changed every 3 days. RNA was independently isolated from the ARPE-19 cells cultured on the different EBM samples using the RNA extraction Kit from ZYMO Research as instructed by the kit manufacturer. The isolated RNA was treated with RQ1-RNase-free DNase (Promega, Southampton, UK) to remove any contaminating DNA.First-strand cDNA synthesis was performed in 3 µg of total RNA, using the AffinityScript QPCR cDNA Synthesis Kit at 50 °C, according to the kit manufacturer's protocol. A reaction containingno reverse transcriptase was also prepared for each RNA sampleas a control (-RT). Following cDNA synthesis, all reactions were treated with RNase H (Invitrogen) to degrade the RNA template.

PCR was performed on the first-strand cDNA synthesis reaction products, using the SuperScript III Platinum CellsDirect Two-StepqRT-PCR kit (Invitrogen) accordingly to the kit manufacturer’s protocol with gene-specific primers synthesized by Instituto Nacional de Biotecnología, UNAM, Mexico. The primers were as follow: RPE65GCC CAG GAG CAG GAC AAA AGA A FW, RPE65 GCG CAT CTG CAA GTT AAA CCA TREV,ZO-1 FW 5′-TGCCATTACACGGTCCTCTG-3′ and ZO-1 REV 5′-GGTTCTGCCTCATCATTTCCTC-3′and GAPDH specific primer pair (5′ GGAAGGTGAAGGTCGGAGTCA; 5′-CTTCCCGTTCTCAGCCTTGAC) as a reference gene for mRNA.

Duplicate PCR reactions were prepared using 5 µl cDNA with q- PCR (Brilliant II SYBR® Green QRT-PCR Master Mix with ROX, 1-Step) and 0.2 µM of gene-specific primer in a total volume of 20µl. The RT-PCR reaction was performed on a RotorGen (Qiagen,Fast Real-Time PCR System; Warrington, Cheshire, UK) according to the manufacturer’s instructions. Fluorescence signals produced by binding of SYBR Green to new double-stranded amplicons were collected after each PCR cycle. The manufacturer’s default thermal cycling conditions were followed (40 cycles of 1 s at 95 °C and 20 s at 60 °C). Data were analyzed using Rotor gen Software analysis (Applied Biosystems) and raw fluorescence data were exported into the DART–PCR spreadsheet to calculate relative gene expression normalized to the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The specificity of all primers was assessed by gel electrophoresis of amplified products and examination of the dissociation curve. The relative expression levels of RPE65 and ZO-1 of ARPE-19 cells cultured on each different membrane were assessed using SigmaStat 3.5 software (Systat Software, Inc., Chicago, IL). The results are expressed as n-fold induction or inhibition of gene expression, relative to endogenous control calculated using the $\Delta\Delta C_T$ method.Two-tailed Student *t*-tests was

performed to compare the statistical significance of the gene expression of cells cultured on each different EBM

Statistical Analysis
All results were analyzed with Prism GraphPad 8 Software. Statistical significance was determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to compare pair groups. Triplicate wells were used to calculate thefinal number of ARPE-19 cells seeded onto each different membrane. Viability assays of ARPE-19 cells cultured on the different EBM were analyzed in pairs by one-way analysis of variance (ANOVA) and Friedman tests. Data from the same experiments were pooled and are expressed as the mean ±SD. A value of $p< 0.05$ was considered statistically significant.

Results
Conductivity and viscosity of electrospinning solutions
The viscosity and conductivity of the PCL, 5Gel/PCL, 15Gel/PCLand 30Gel/PCL solutions for electrospinning were assessed andthe results are reported in Table 1. The viscosity of solutions significantly decreased with the addition of Gel to the solutions and it also significantly decreased with the increment of Gel concentration in the Gel-PCL blend solutions. Showing the opposite trend, the conductivity of the solutions significantly increased with the increment of Gel concentration in the electrospinning solutions.

Physical-chemical properties of the membranes
The overall macroscopic and microscopic aspect of the as- synthesized membranes, we can be observed that the obtainedscaffolds were flexible, resistant to the manipulation and characterized by a homogeneous fibrillar morphology. The average thickness of the synthesized scaffold as shown in the micrographs of the EBM showed that defect-free fibrillar membranes were obtained for all the different polymer compositions used; that is PCL (0.614 ± 0.574 μm), PCL/5%Gel(0.896 ± 0.491 μm), PCL/15%Gel (0.872 ± 0.205 μm) and PCL/
30%Gel (0.707 ± 0.156 μm) respectively (Figure 1). Membranes showed sub-micron average fiber diameters between 0.614 and 0.896 μm according to the membrane chemical proportion. No statistical differences were identified among the average fiber diameter for membranes at different Gel proportion in comparisonto the PCL membrane.
FT-IR spectra of pristine Gel, pristine PCL and as-electrospun PCL, PCL/5%Gel, PCL/15%Gel, and PCL/30%Gel scaffolds are shown in Figure 2. The FT-IR spectra of PCL membrane exhibitedcharacteristic IR absorption bands of polycaprolactone at 2945, 2859, 1731, 1294, 1240, 1175 and 1045 cm^{-1} that were assigned to the vasCH2, vsCH2, vC=O, vC-C, vasC-O-C, vC-O-C and vC- O vibrational modes of polycaprolactone, respectively [23].
Characteristic IR bands corresponding to the Gel structure were observed for the PCL/15% Gel and PCL/30%Gel membranes at ≈3350 cm^{-1} (vN-H amide bond), 3085 cm^{-1} (vO-H), 1650 cm^{-1} (vC=O, amide I) and 1538 cm^{-1} (δN-H, amide II) [23]. The spectrum of PCL/5%Gel membrane did not show any clear band that could be exclusively and confidently assigned to the Gel chemical structure.
Results of water contact angle (WCA) measurements as-EBM areshown in Table 2. PCL membrane displayed the highest WCA (145.2o), and it decreased as the Gel content in the membranes increased from 132.3°, 129.5° to 43.8° for PCL/5%Gel, PCL/ 15%Gel and PCL/30%Gel membranes respectively, exhibiting that Gel-PCL blend scaffolds possessed

a hydrophilic surface.

ARPE-19 cells viability assays on the differentmembranes

Cell viability studies were performed by culturing ARPE-19 cells on the EBM first, cell culture assays were performed to evaluate two different procedures for cell seeding onto the EBM, “drop seeding” and “total volume seeding”. The number of viable cells on EBM (measured by the MTT-formazan assay after 24 h of cellculture) was insignificant when cells were seeded on the membranes by a total volume seeding procedure; that is, seeding 1.5 x104cells/300 µl of medium directly on the culture wells containing the membrane samples (data not shown). In contrast, the number of viable cells on the membranes (measured by the MTT-formazan assay after 24 h of culture) significantly increased when cells were drop seeded (1.5x104cells/30µl of medium) on the center of the membrane samples to allow cellular adhesion for30 min, and then the medium was added to complete the total culture volume of 300 µl for each culture well containing the samples; Figure 3A. When cells were drop seeded onto EBM, MTT assays showed a significant increase in the optical density (490 nm) of supernatants from the dissolution of cell- metabolizedformazan crystal as Gel concentration in the membranes increased. Absorbance readings from wells corresponding to cellscultured onto PCL/30%Gel (172,800 cells) were significantly higher than readings from cells cultured onto PCL (99500 cells), PCL/5%Gel (102,000 cells) or PCL/15%Gel (124,500 cells) (Figure 3A). This indicated that a significantly higher number of viable cells adhered to PCL/30%Gel in comparison to those adhered to the other three membranes. (Figure 3B). It is importantto emphasize that from MTT assays, it is possible to obtain the degree of cell proliferation and the number of viable (metabolicallyactive) cells on the membranes from supernatants (formazan crystals dissolution) absorbance readings by obtaining a standardcurve of the number of cells vs absorbance from MTT/Formazan assays. Upon cells drop seeding, results evidenced that PCL/ 5%Gel did not exert any significant improvement on the number of viable cells adhered at the 24 h of culture in comparison to the PCL membrane (Figure 3). On the other hand, a significant increase in the number of viable cells on PCL/15%Gel (*p = 0.030) and on PCL/30%Gel membranes (*p = 0.041) (Figure 3B) was observed in comparison to PCL membrane. The number of viable cells on PCL/30%Gel was smaller than the number of cells on the positive control (TCPS); nevertheless, differences were not statistically significant (Figure3B). Thus, it can be considered that the number of viable cells was the same on PCL/30%Gel as on the positive control.

Expression of RPE cells markers for cells cultured on theEBM

To quantitatively address possible changes in the gene expression of some of the principal markers of RPE cells that might have been induced by cell culturing on the membranes, qRT-PCR was used to evaluate the gene expression of RPE65 and ZO-1 in ARPE-19 cells cultured for 25 days on the membranes. As we can observe from Figure 4A and 4B, there were non-significant changes in the gene expression of these two markers for cells cultured on the EBM in comparison to the control(cells cultured on TCPS). A slightly increasing trend for the gene expression of both markers was observed as Gel concentration in the membranes increased; however, it was not statistically significant. The largest increase for the gene expression of RPE65 and ZO-1 was observed for cells cultured on PCL/30%Gel in comparison to control (cells cultured on TCPS). Nevertheless, as we mentioned before,

this increase was not statistically significant, suggesting that gene expression patterns of ARPE-19 cells characteristic markers were not affected upon cell seeding and culturing on the membranes.

Immunodetection of characteristic proteins in ARPE-19cells onto PCL/30%Gel

The MTT assays showed better ARPE-19 cellular adhesion and viability onto PCL/30%Gel, in comparison to the control (cells on TCPS) and the other PCL/Gel membranes studied. Moreover, thestudies of gene expression (qRT-PCR) of RPE cells characteristicmarkers, RPE65 and ZO-1, exhibited that PCL/30%Gel slightly increased the expression of these markers for ARPE-19 cells in comparison to the control and the other PCL/Gel membranes. Thus, SEM and immunodetection experiments to further characterize the biological response of ARPE-19 cells to PCL/Gel electrospun membranes were onlyperformed for cells cultured on PCL/30%Gel and compared tocells cultured on PCL membrane as control. In order to observe the cell morphology and cytoskeleton of ARPE-19 cells cultured on PCL/30%Gel membrane, immunofluorescence (IF) assays were performed using antibodiesto actin cytoskeleton (Figure 5) and for RPE65 (Figure 6). A largernumber of cells and a different arrangement of the actin were observed for cells cultured on PCL/30%Gel in comparison to cellson the PCL membrane, Figure 5. To evaluate the expression of one of the most used RPE cell markers (RPE65), IF assays were performed after 25 days of ARPE-19 cells culture on PCL and PCL/30%Gel membranes. Figure 6 shows that ARPE-19 cells on PCL/30%Gel displayed an RPE- like morphology and an RPE65 expression pattern more similar to that of normal human RPE cells, in comparison to ARPE-19 cells on PCL membrane.

Structural characteristics of ARPE-19 cells on PCL/30%Gel

Figure 7 shows representative SEM images ofARPE-19 cells cultured for 25 days on PCL and PCL/30%Gelmembranes. As it can be observed from Figure 7 (A, B, D, and E), the PCL/30%Gelmembrane exhibited uniform thin fibers (red arrows) even after 25days of cell culture. These fibers exhibited differences in its nanofibrillar structure in comparison to the PCL membrane (Figure 7F), probably due to the effect of the addition of gelatin in the PCL/30%Gel. As a result of cell culture conditions, PCL membrane partially lost its fiber-based morphology after 25 days of culture; Figure 7F. Interestingly, it resulted in a more continuousstructure showing a reduction of its fibrillar characteristics. On theother hand, the PCL/30%Gel membrane seemed to better preserve its fibrillar structure upon culture (Figure 7D and E). Figures 7A, B, and C show ARPE-19 cells cultured on PCL/ 30%Gel after 25 days of culture, exhibiting cells with RPE-like cells epithelial morphology that were densely packed and presented hexagonal morphologies (green arrows) and apical microvilli (white arrows in Figure 7C). ARPE-19 cells formed a monolayer of polygonal cells on the PCL/30%Gel membrane (Figures 7A, B, and C) but not on the PCL membrane (Figure 7F). Apical surfaces of ARPE-19 cells showed a rough and furry aspect due to the presence of abundant and small protrusions of the cell membrane (Figure 7C, white arrows). These features were not observed in the very scarce ARPE-19 cells adhered to the PCL membrane (Figure 7F). It is worth to emphasize the presence of very narrow adhesion zones among the contours of the polygonal cells of the cell monolayer on PCL/ 30%Gel (Figures 7A, B and C, green arrowheads). Some areas ofdetached cell monolayer allowed observing the fibrillar structure of the PCL/30%Gel membrane (Figure 7A and B, red arrowheads), which was preserved below the cell monolayer evenafter 25 days of cell culture. The fibrillar structure of the PCL/30%Gel membrane was better observed in regions

without adhered ARPE-19 cells (Figure 7D, E). These results show that PCL/30%Gel nanofibrillar membranes provide a micro- architecture mimicking the inner collagenous structure of the human BM.

Discussion

Retinal degenerative diseases such as AMD, Stargardt´s disease, Macular hole, Diabetic retinopathy, and Retinitis pigmentosa are the leading causes of blindness worldwide. There are some treatments like anti-angiogenic drugs, laser photocoagulation or RPE cell replacement therapy [4,19,20,16,24,21,22]. However, there is not a completely effective therapy to stop the progressionand more importantly, to restore the lost vision in these ocular pathologies. The RPE cell replacement treatment is an excellent option since it is directed to replace and restore the RPE, and thus, to stop and even reverse the progression of AMD. This treatment has been performed in animal models and human patients of AMD [4,25,26,27]. But these therapies are far from being perfect, and there is still ample room for improvement.

Recent scientific reports showed advances in the design, construction, and functionality of artificial BMs for RPE reconstruction in different ocular pathologies, such as AMD [21,22,28,29,30]. Many artificial carrier substrates, both natural and synthetic, have served as BM prosthesis to support the attachment and growth of RPE cells. However, despite these advances is limited biodegradability, and reduced ability to support RPE cells after long-term co-culture. It is expected that apotential, artificial, and functional BM presents a similar ultrastructure to thatof the natural BM, resistance to biodegradability, and adequate properties to support RPE cell culture.

In this study, we used EBM of PCL at different Gelatin concentrations of (5, 15, 30 wt.%) as potential artificial BM-like membranes, showing that PCL/30%Gel membrane was capable of supporting the culture of viable and functional ARPE-19 cells, maintaining the morphological characteristics and gene and protein expression of RPE-like cells markers upon culture for up to 25 days. Gel-PCL fibrillar scaffolds with 30 wt.% gel were successfully electrospun using an environmentally friendly, single-step solution procedure, where AcAc was used as a “green” sole solvents to straightforwardly produce Gel-PCL solution with Gel concentration ≥ 30 wt.% and suitable for electrospinning.

First, when ARPE-19 cells were drop seeded in a small volume ofmedium (30µl) and cultured for 30 minutes on the membranes, and before adding the rest of the culture medium, it was shown that the number of viable cells on the membranes increased in comparison to total volume cell seeding (300µl) (Figure 1B). We suggest that this result was a consequence of having allowed the cells to stay on (not floating away) and adhere to the membranes before completing the total volume of medium in the wells. From these observations, the drop seeding procedure was performed for all the other sets of experiments. When a standard curve of cells was used as a reference to obtain the number of viable cellson the different membranes after 24 h of culture, results showed asignificant increase of the number of ARPE- 19 cells on PCL/ 15%Gel and PCL/30%Gel membranes in comparison to those on PCL membrane. The remarkably different properties of Gel/PCL scaffolds in dry and hydrated conditions emphasize the importance of assessing the mechanical properties of Gel/PLC scaffolds in their hydrated state, which better intended for tissue engineering applications. Thus, most probably, these two membranes promoted a higher cellular adhesion on their surfacesin comparison to PCL (Figure 1B) due to the biologically favorableeffect of Gel. There is a previous report by [14] using PCL/Gel-based electrospun for tissue engineering; though, Ketoprofen, a non-steroidal anti-inflammatory

drug, and a more diluted concentration of Gel were used for that preparation, in comparison with the present study. Basar et al. 2017 fabricated PCL/Gel/Ketoprofen membranes as a drug delivery system intended for wound covering, showing that L929 mouse fibroblasts seeded onto these membranes highly proliferated andthat certain cells overgrowth showing agglomeration [14,31] usedBombyx mori silk fibroin (BMSF) as a mat for human RPE cells culture, exhibiting that BMSF in combination with ECM proteins (laminin, fibronectin, and vitronectin) increased cell attachment inlong-term culture of ARPE-19 cells and in primary cultures of human RPE cells [31]. Nevertheless, their results showed a slower growth for human RPE cells seeded onto vitronectin- coated-BMSF mats [31].

In our results, there was no agglomeration of the ARPE-19 cells on the membranes and MTT assays did not show any reduction of the number of viable cells on PCL/30%Gel when compared to the number of cells on TCPS wells (control). The control, TCPS, presented a slightly higher number of viable cells in comparison toPCL/30%Gel; however, this difference was not statistically significant. This effect might be ascribed to the capabilities of the PCL/30%Gelsamples to retain the contact between the liquid phase and a solid surface (wettability). PCL/30%Gel was the only hydrophilic membrane (WCA ~ 43.8°) among the different membranes studied in the present work (Table 2). It is well known,that highly hydrophobic polymers such as PCL tend to exhibit relatively poor cell attachment performance, while hydrophilic materials tend to improve cell attachment (Azimi et al. 2014; Hotaling et al. 2016). Interestingly, as we said before, the PCL/ 30%Gel membrane showed a similar attachment performance in comparison to TCPS and better performance compared to the PCL membrane. This can be then an effect of the hydrophilic nature of Gel that decreased hydrophobicity (water contact angle; Table 2) of the here-developed membranes as Gel concentration in the membranes increased. It is also important to mention, that Gel is a hydrophilic compound that resembles the structure of collagen (a major component of the ECM of different tissues which have been shown to improve cell attachment and viability [32]. According to Davidenko et al. 2016 and Basar et al. 2017 [14,32], another favorable effect improving cell attachment and proliferation on the electrospun membranes could be addressed to the high porosity of their fibrillary structure (Figure 1). Thus, PCL/30%Gel membranes, without any other factor or chemical compound added, supported ARPE-19 cell culture and might represent an excellent option as an RPE cell carrier. Regarding the nanofibrillarcharacteristic of this membrane, it is clear that it provides a micro-architecture mimicking the inner collagenous structure of the human BM.

In addition to supporting ARPE-19 cells attachment, our membrane, or any material to be used as a scaffold to develop RPE-like artificial membranes must support and allow the development of a fully differentiated RPE cells monolayer. Among the principal characteristics of a well-differentiated and functional RPE cells, the monolayer is the tightly hexagonal morphology of the cells, which can be observed using some RPE markers such as ZO-1 and RPE65 or the peripheral distribution of F-actin fibers and cytokeratin [19,31,33,34]. We performed IF assays to observe actin and cytokeratin 18 distributions, as well as RPE65 in ARPE-19 cells cultured on PCL and PCL/30%Gel membranes.The results showed differences in the actin and cytokeratin distributions in cells cultured on PCL or PCL/30%Gel. ARPE-19 cells on PCL showed a more centralized distribution and more stress fibers for both actin and cytokeratin 18. In contrast, ARPE-19 cells on PCL/30%Gel exhibited a reduction of stress fibers and a more peripheral arrangement of actin as well as of cytokeratin 18.

These results are similar to previous reports, where the analysis of F-actin showed a similar distribution pattern to the one observed in the present studies in long-term RPE cell cultures

grown on BMSF membranes [31] (Shadforth et al. 2012). It has been demonstrated that low-phagocytic RPE cells possess an abnormal F-actin distribution characterized by the lack of peripheral and circumferential F-actin, less apical F-actin, and thepresence of stress fibers [7,35]. Our results showed a tendency for re-arrangement of actin and cytokeratin 18 to the cell periphery, suggesting that ARPE-19 cells on PCL/30%Gel were recovering their normal phagocytic activity; however, further studies are needed to prove this.

Regarding the arrangement of cytokeratin18, we observed differences between the expression pattern forARPE-19 cells onPCL and on PCL/30%Gel. It has been demonstrated that a continued cytokeratin18 expression helps to preserve RPE epithelial phenotype in long term cultures [11,28,29,30,31,36]. Using both cytoskeleton markers, cytokeratine18, and actin, we could observe the ARPE-19 cells on the PCL and the PCL/30%Gel membranes. However, cells on PCL/30%Gel membrane exhibited a tighter RPE monolayer, according to their cytoskeleton distribution and the characteristic polygonal structure of RPE-like cells, suggesting that ARPE-19 cells were more functional on PCL/30%Gel, in comparison to cellson PCL. Finally, no significant differences were evident between PCL and PCL/30%Gelatin membranes concerning to the intracellular localization of RPE65 protein by IF; nevertheless, cells on PCL/%30Gel exhibited different morphological characteristics in comparison to those on PCL. Cells on PCL/ 30%Gel presented a marked polygonal morphology, suggesting that PCL/30%Gel membrane promoted the formation of a healthy, mature, and more differentiated RPE monolayer in comparison toPCL.

Related to this, we performed qRT-PCR assays to evaluate if these slight differences in ARPE-19 morphology were related to changes in gene expression of RPE markers, RPE65, and ZO-1. Although no significant increased expression of RPE65 or ZO-1 was observed for cells cultured on any of the studied membranes (PCL, PCL/5%Gel, PCL/15%Gel and PCL/30%Gel) in comparisonto control, there was a trend evidencing a slight increment of the expression of both markers with Gel concentration in the EBM (Figure 4). This suggested that herin electrospun membranes did not disturb normal RPE-cell gene expression but increased it as Gel concentration in the membranes increased. Some authors have suggested that the increased expression and maintenance of RPE genes, such as RPE65 and ZO- 1,over long-term culture, is an indication of properly attached RPE cells proliferating and forming functional monolayers on the scaffolds [11,36,37,38,39].

Although no significant changes in the expression of RPE65 and ZO-1 were observed, the qRT- PCR analysis showed a ~0.25-foldincrease in the expression of both markers for cell culture on PCL/30%Gel in comparison to control. This is a remarkable finding because it has been demonstrated that RPE65 deficiency leads toalterations in mammalian vision and failures in the retinoid cycle producing human blindness, in contrast, the increase of RPE expression leads to the differentiation of Human Stem Cells to RPE phenotype [40].

ARPE-19 cells on PCL/Gel membranes expressed the tight junction complex protein ZO-1 without significant changes in respect to the control (Figure 4). ZO-1 is a junctional adaptor protein that interacts with multiple other junctional components, including the transmembrane proteins of the claudin and JAM families. It has been proved that ZO-1 depletion leads to selectiveloss of tight junction proteins, barrier formation, and mechano-transducers such as vinculin and PAK2, inducing vinculin dissociation in ARPE-19 cells [41-43]. On the other hand, an increment of ZO-1 expression has been correlated with the acquired epithelial morphology characteristics of the native RPE cells [34,44]. Thus, it is possible to suggest that the PCL/30%Gelmembrane supported the culture of healthy ARPE-19 cells sinceno reduction of the RPE65 and ZO-1 normal expression was observed upon cell culture on this membrane.

Finally, ARPE-19 cells cultured on PCL/30%Gel showed a more "natural appearance" compared to cells on PCL and on other membrane-like scaffolds previously reported [45-48]. From micrographs of cells cultured on PCL/30%Gel (Figure 8), it was possible to observe a fibrillary network supporting and maintainingthe culture of differentiated ARPE-19 cells. The membrane made of only PCL did not exhibit the characteristics fibrillar network of as-electrospun PCL membrane after culture with ARPE-19 cells.

Contrary to what was expected, due to the fastest degradation of the Gel component in comparison to pristine PCL, PCL/30%Gel membrane retained its fibrillar characteristics and exhibited a porous membrane, probably making it more permeable to nutrientexchange regulated by BM. Besides, the surface topography and nanofiber arrangement preserved in this membrane upon culture might have increased cellular adhesion and maturation positively influencing RPE cell attachment and monolayer formation.

ARPE-19 cells line has been broadly used as an alternative to human RPE cells, since this cell line presents epithelial cell morphology and specific markers of native RPE cells such as RPE65 (abundantly expressed in the RPE), as well as expression of the cellular retinaldehyde-binding protein (CRALBP), a retinoid- binding protein involved in the regeneration of visual pigment.

This cell line also performs the assimilation of outer photoreceptorsegments (POS) by phagocytosis [7,49,50]. Thus, the use of ARPE-19 cells to study the biological behavior of the electrospun membranes allowed us to hypothesize that the here-developed PCL/30%Gel membrane represents a potential option as a scaffold to grow, support and differentiate primary human RPE cells, and consequently a potential option to develop an artificialBM.

According to Xiang et al. 2014; Fereshteh et al. 2016 [19,22], the ideal artificial BM would exhibit properties such as a thickness of less than 5 mm, porous ultrastructure, biocompatibility for cell adhesion and growth, no changes in gene expression of RPE cells, preservation of the physiological RPE cells features like their polygonal shape and formation of tight junctions, phagocytosis of ROS, the formation of apical microvilli, polarized secretion of neurotrophic factors, and would not induce inflammation after implantation. Our results showed that the here-developed PCL/30%Gel membrane exhibited some of these idealBM characteristics. However, longer-term investigation and in vivostudies are still needed.

Conclusion

In conclusion, our results showed that PCL/30%Gel nanofibrillar membranes provide a micro-architecture mimicking the inner collagenous structure of the human BM and this provides an artificial niche for ARPE-19 cells that allowed such cells to maintain their bio functionality and morphological characteristics of RPE-like cells. Finally, these are appropriate for potential surgical management and could be used in translational medicine for AMD patients. However, pre-clinical studies are needed in order to demonstrate it.

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