Electrospun ultrathin scaffold for Bruch's membrane regeneration in retinal tissue engineering

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***Abstract*— Currently there are no retinal tissue engineered grafts for clinical applications. Our project aims at designing a functional retinal tissue that could be implanted in patients suffering from age-related macular degeneration. To this end we will apply 3D electrospun ultrathin scaffolds and characterize their mechanical and biological properties using suitable cells.**

***Keywords*—Retina, Tissue engineering, Electrospinning, Ophthalmology.**

I. Introduction

RETINA is a complex multi-layered tissue that absorbs, modulates, and transmits visual stimuli from the external world to the brain.

The outer retina is composed of retinal pigment epithelium (RPE). The RPE consists in a monolayer of polarized pigment cells which collaborate closely with the adjacent photoreceptors in maintaining visual function. RPE cells perform important roles including absorption of stray light, phagocytosis of outer segment of photoreceptors, maintenance of blood-retinal barrier, secretion of growth factors, and nourishment of the retinal visual cells [1]. The RPE lies on Bruch's membrane (BM) that is a thin (2-4 μm), acellular, extracellular matrix located between the retina and the choroid. Thanks to its permeable nature, the BM regulates the diffusion of biomolecules, nutrients, oxygen, fluids and metabolic waste between the outer retina and the choroidal blood supply [2]. As a vessel wall of the choroid, another crucial function of the BM is structural. Indeed, it serves as physical support for intact and functional RPE formation [2]. A healthy RPE together with a normal BM are essential for maintaining survival, integrity, homoeostasis, and function of the adjacent photoreceptors, and thus vision.

The dysfunction or degeneration of RPE cells results in different types of retinal diseases such as age-related macular degeneration (AMD). AMD is the principal cause of blindness in the elderly worldwide, affecting globally about 30-50 million individuals [3]. AMD consists in a progressive degeneration of the central retina due to age-related changes in BM and in the RPE. Aberrant neovascularization characterizes the wet form of AMD, which is responsible for 90% of AMD-associated severe visual impairment [4]. Currently, periodic intravitreal injections of anti-vascular endothelial growth factor (anti-VEGF) drugs are the gold standard therapy in the management of wet-AMD [5]. However, these treatment protocols are unable to restore tissue functionality, are expensive, and may lead to adverse effects, such as chorioretinal atrophy [5]. Hence, new strategies are needed to alleviate the progression of, or to recover the lost vision associated with this disease.

Subretinal surgery with resection of changes in the BM and subsequent RPE cell replacement therapy is one of the most promising approaches to restore retinal function. However, the subretinal injection of suspended RPE cells can lead to lack of structural organization into monolayer, significant cell death, and retinal fibrosis [6]. In addition, the RPE malfunction is not the only challenge for treatment, as the BM is also compromised in AMD. In fact, the BM should provide a proper microenvironment to support cell attachment and survival while maintaining cell functionality over time. For this reason, it has been proposed that RPE cells can be transplanted on a proper scaffold that acts as a BM substitute supporting mechanically and physically cell attachment, cell proliferation, and formation of a functional intact monolayer [6].

Many research efforts have focused on developing a biocompatible prosthetic BM to reconstruct retinal tissue *in vitro* [6]. In most studies, 2-D membranes with a closed dense structure have been utilized as scaffold for RPE monolayers [7]-[9]. Nevertheless, such a structure is in contrast to the open fibrillar structure of a native BM and can prevent nutrient diffusion. Furthermore, cells accomplish their function in a three-dimensional (3D) environment that resembles their natural habitat [10]. Therefore, the ideal BM-like scaffold probably needs to be designed as a 3D fibrillar mesh.

Recently electrospinning has been suggested as a promising technique to fabricate RPE scaffold as it permits generating 3D nanofibrous network topographies that are highly permeable for solutes thus facilitating cell adhesion and proliferation [11]. Polycaprolactone (PCL), a biodegradable aliphatic polyester with high tensile and elongation properties, has been widely used for tissue engineering applications as it was approved by U.S. Food and Drug Administration (FDA) [1]. Electrospun PCL nanofibers have been demonstrated to support the growth and proliferation of retinal cells [1], [12]. However, PCL hydrophobicity and hence the lack of recognition sites for cell adhesion limit its use as prosthetic BM material. Previous studies showed that the blending of silk fibroin (SF) with PCL and other synthetic polymers, significantly improved cell adhesion due to SF good biocompatibility [12]-[13]. Compared to synthetic materials, silk degradation products (peptides) do not considerably affect the pH or osmolarity at the implantation site.

Consequently, the aim of this study was to design and develop an ultrathin nanofibrous electrospun membrane composed of *Bombyx mori* silk fibroin (BMSF) blended with polycaprolactone (PCL) that would closely mimic the natural fibrous architecture of the human BM.

II. Materials And Methods

A. Electrospinning

The polymer solutions with a concentration of 15% (wt/v) were prepared by dissolving *Bombyx mori* silk fibroin (BMSF, Crea) and Polycaprolactone (PCL, Sigma) with a weight ratio of 5:95 (BMSF/PCL) in 98% formic acid (Sigma). EF300 electrospinning system (SKE Research Equipment, Leonardino s.r.l.) was used to fabricate the BMSF and PCL nanofibrous membranes. A voltage of +18 kV was applied to the needle 15 cm distant from the surface of the grounded collector. The polymer solution was delivered at a flow rate of 1.3 ml/h. Electrospinning was performed at a constant temperature (33.5 °C) and a relative humidity of 22%.

B. Characterization of electrospun membranes

Membranes were subjected to an examination of their physico-chemical and mechanical properties including physical morphology, stress-strain relation, elasticity, and permeability. To investigate the morphology, scaffolds were sputter coated with gold and observed with a scanning electron microscopy (SEM, Stereoscan 360, Cambridge Instruments) at 10 kV. ImageJ software (National Institute of Health, USA) was used to determine the membrane thickness, the fibre diameter, and packing density. 20 random fibres were measured to calculate the average fibre diameter. The packing density, presented as a percentage, was computed by counting the number of the fibres across each image, multiplying this by the average fibre diameter and then dividing by the width of the image.

For the mechanical measurements, scaffolds were cut into 5 x 32 mm samples. A material testing machine (Synergie 200, MTS Systems) equipped with a 100 N loading cell was used for the tensile test. Displacement was applied at 0.1 mm/s. Samples were first preconditioned four times and then pulled to failure. Measurements were repeated five times. The same protocol was applied to hydrated samples, i.e. samples submerged in saline solution for 1 hour. Young's modulus was calculated by measuring the slope of the initial linear region of the stress-strain curve.

To estimate permeability, a custom-made apparatus was employed containing two stainless-steel cylinders, a polyethylene filter, an O-Ring, and a capillary. Briefly, after inserting the sample properly between the two cylinders, we applied a constant hydrostatic pressure to the sample and measured the fluid volume through the sample over time. We then calculated permeability using Darcy's law, as in Eq. (1).

$$k = \frac{\Delta V * \mu * h}{\Delta t * A * \Delta P} \quad (1)$$

Where k is the permeability, $\Delta V/\Delta t$ is the fluid volume over time, h is the sample thickness, A is the flow area, and ΔP is the applied hydrostatic pressure. Four different values of pressure were applied to each sample. The measurements were repeated three times.

C. The culture of ARPE-19 cells

ARPE-19 cells, a human retinal pigment epithelium (RPE) cell line obtained from American Type Culture Collection (ATCC), were cultured with DMEM/F12 (ATCC) supplemented with 10% fetal bovine serum (FBS, ATCC), 1% 100 U/ml penicillin (Gibco™), and 100 µg/ml streptomycin (Gibco™). Medium was changed regularly every 3 days until confluence. Cells were cultured at 37 °C and 5% CO_2.

D. Biocompatibility

To investigate the *in vitro* biocompatibility of the BMSF/PCL scaffolds, a direct contact cytotoxicity test was carried out. Scaffolds were soaked in 75% ethanol for 6 h followed by washing three times with sterilized phosphate saline buffer (PBS, Gibco™). The ARPE-19 were seeded on 6-well plates at a density of 20000 cells/cm^2 and cultured in direct contact with the electrospun membranes for 3 days. Changes in cell morphology and cell viability were assessed using a fluorescent microscope (Olympus IX70). For cell viability, cells were stained with Live/Dead assay (Invitrogen™).

E. Statistical analysis

Results are presented as mean ± standard deviation (SD). Statistical analyses were performed using Graphpad Prism Version 8 (Graphpad Software). Differences were considered statistically significant if the *p*-value was less than 0.05.

III. Results

A. Scaffold characterization

In this study, the BMSF/PCL membranes were successfully produced by electrospinning and appeared to be uniform as shown in Figure 1 (Fig. 1).

Fig. 1: Appearance of electrospun BMSF/PCL scaffolds.

The resulted scaffolds were constructed of randomly oriented fibres and thoroughly interconnected pore structures (Fig. 2). The electrospun membranes displayed a thickness of 44 µm, an average fibre diameter of 1217 ± 101 nm, and a fibre packing density of 63.76 ± 1.2%.

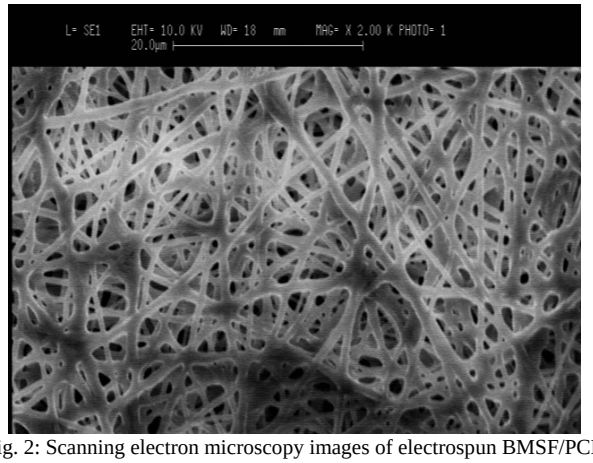

Fig. 2: Scanning electron microscopy images of electrospun BMSF/PCL scaffolds. Scale bar: 20 µm.

The mechanical properties of both hydrated and dry membranes were reflected by typical tensile stress-strain curves (Fig 3).

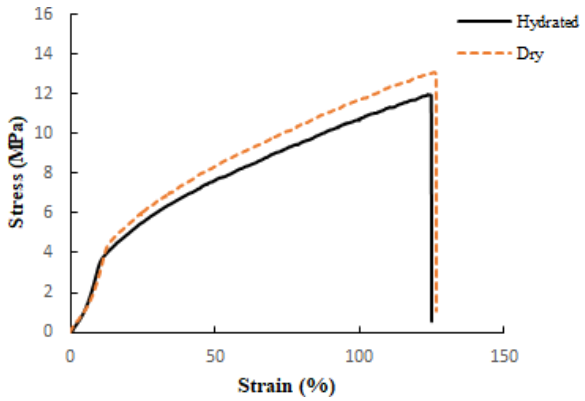

Fig. 3: Tensile stress versus strain curve of BMSF/PCL hydrated (black continuous line) and dry (dotted orange line) scaffolds.

The ultimate tensile strength of the dry membrane was 9.72 ± 2.47 MPa, with an ultimate strain of 100.4 ± 31.6% and a Young's modulus of 17.37 ± 1.99 MPa. The hydrated membrane had slightly higher values of ultimate tensile strength, ultimate strain and Young's modulus, of 10.234 ± 2.21 MPa, 118.6 ± 25.9% and 18.57 ± 4.47 MPa, respectively. No significant difference was found between mechanical properties of hydrated and dry scaffolds (Fig. 4).

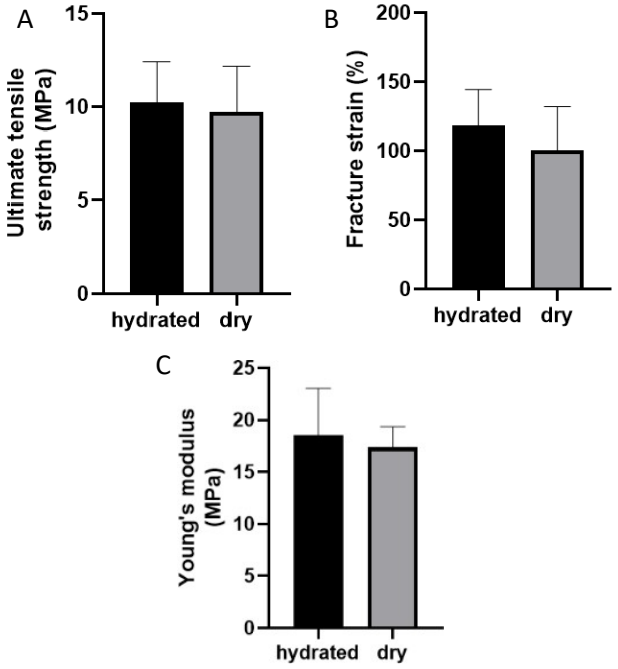

Fig. 4: **(A)** Ultimate tensile strength of hydrated (black column) and dry (grey column) scaffolds. **(B)** Fracture strain of hydrated (black column) and dry (grey column) scaffolds. **(C)** Young's modulus of hydrated (black column) and dry (grey column) scaffolds.

Membrane permeability was performed to assess how the fluid flows through the material. For instance, at a pressure of 3090 Pa the average permeability was $5.07\times10^{-18} \pm 1.99\times10^{-18}$ m^2.

Generally, the average permeability resulted to decrease with the increase of hydrostatic pressure ΔP (Fig. 5).

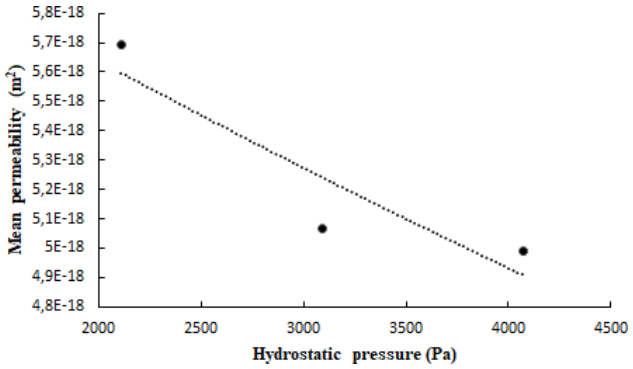

Fig. 5: Average permeability of BMSF/PCL scaffold versus hydrostatic pressure curve

B. Biocompatibility

We carried out preliminary tests to assess the scaffold biocompatibility *in vitro*. According to initial results (not shown) of these preliminary tests, ARPE-19 cells seemed to be viable after 3 days of direct contact with the retinal membranous scaffold. However, we will need to further investigate this hypothesis to confirm the scaffold suitability for retinal regeneration.

IV. Discussion and Conclusion

Impairment of the native RPE monolayer is a major pathologic feature of AMD. Transplantation of RPE with a carrier, instead of injections of cell suspension, seems to improve RPE survival, engraftment, and integration [13]. However, an ideal substrate for constructing a prosthetic BM with attached RPE cells has yet to be found. The perfect retinal scaffold should be thin enough to accommodate the subretinal space (5-90 µm), be permeable to allow biomolecules transfer, exhibit a non-linear stress/strain behavior with a Young's modulus of 6-14 MPa consistently with the native tissue, present an appropriate degradation time matched to synthesis of new extracellular matrix by cells, should favor cell adhesion, and maintain cell viability and normal function including the formation of a pigment epithelium [14].

In our study, the obtained scaffold showed structural and mechanical similarity to human BM, which has a random fibrillar network, a packing density of 48%, and a Young's modulus ranging from 6 to 14 MPa [11], [15]. In addition, we found out that scaffolds are suitable for cell culture and we are carrying out additional investigation with ARPE-19 cells in both static and dynamic conditions, as bioreactors support cell viability of 3D constructs by perfusion or diffusion system [16, 17]. However, to more closely imitate the BM, fibre diameters, membrane thickness, and the packing density need to be further reduced. The successful outcome of this study will inform the treatment of an optimal substrate as a basal support for RPE. Moreover, this work may lead to new studies where a second layer of photoreceptors can be bioprinted on the RPE.

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REFERENCES

[1] P. Xiang, *et al.*, "A novel Bruch's membrane-mimetic electrospun substrate scaffold for human retinal pigment epithelium cells," *Biomaterials*, vol. 35, pp. 9777-9788, September 2014.

[2] C. A. Curcio and M. Johnson, *Structure, function, and pathology of Bruch's membrane*, London: Elsevier, Inc., 2013, ch. 20.

[3] Colijn, Johanna M., *et al.*, "Prevalence of age-related macular degeneration in Europe," *Ophthalmology*, vol. 124, no. 12, 2017, pp. 1753–1763.

[4] N. C. Hunt, D. Hallam, V. Chichagova, D. H. Steel, and M. Lako, "The application of biomaterials to tissue engineering neural retina and retinal pigment epithelium," *Advanced Healthcare Materials*, vol. 7, 1800226, 2018.

[5] Fernández-Robredo, P., et al. "Current treatment limitations in age-related macular degeneration and future approaches based on cell therapy and tissue engineering." *Journal of Ophthalmology*, vol. 2014, pp. 1–13., January 2014.

[6] S. Royce Hynes and H. B. Lavik, "A tissue-engineered approach towards retinal repair: Scaffolds for cell transplantation to the subretinal space," Graefes Arch. Clin. Exp. Ophthalmol., vol. 248, pp. 763–778, February 2010.

[7] G. K. Srivastava, *et al.*, "Elastin-like recombinamers as substrates for retinal pigment epithelial cell growth," J Biomed Mater Res A, vol. 97, pp. 243–50, 2011.

[8] J. T. Lu, C. J. Lee, S. F. Bent, H. A. Fishman, and E. E. Sabelman, "Thin collagen film scaffolds for retinal epithelial cell culture," *Biomaterials*, vol. 28, pp.1486–94, 2007.

[9] E. Santos, R.M. Hernández, J. L. Pedraz, G. Orive, "Novel advances in the design of three-dimensional bio-scaffolds to control cell fate: translation from 2D to 3D," *Trends Biotechnol*, vol. 30, pp. 331–41, 2012.

[10] M. A. Asnaghi, *et al*., "Trends in biomedical engineering: focus on regenerative medicine," J Appl Biomater Biomech, vol. 9 no. 2, pp. 73-86, 2011.

[11] P. H. Warnke, *et al.* "Primordium of an artificial Bruch's membrane made of nanofibers for engineering of retinal pigment epithelium cell monolayers," *Acta Biomaterialia*, vol. 9, pp. 9414-9422, 2013.

[12] D. Zhang, et al., "Electrospun SF/PLCL nanofibrous membrane: a potential scaffold for retinal progenitor cell proliferation and differentiation", Scientific Reports, vol. 5, Article 14326, 2015.

[13] S. Popelka, *et al*. "A frame-supported ultrathin electrospun polymer membrane for transplantation of retinal pigment epithelial cells," *Biomedical Materials*, vol. 10, Article 045022, 2015.

[14] Y. S. E. Tan, P. J. Shi, C. Choo, A. Laude, and W. Y. Yeong, "Tissue engineering of retina and Bruch's membrane: a review of cells, materials, and processes," *Br J Ophthalmol*, vol. 102, pp. 1182–1187, 2018.

[15] W. H. Chan, *et al.* "Young's modulus of Bruch's membrane: Implications for AMD." *Investigative Ophthalmology & Visual Science*, Vol. 48, no. 13, pp. 2187, 2007.

[16] S. Mantero, N. Sadr, S. A. Riboldi, S. Lorenzoni, and F. M. Montevecchi, "A new electro-mechanical bioreactor for soft tissue engineering," *Journal of Applied Biomaterials & Biomechanics*, vol. 5 no. 2, pp. 107-116, 2007.

[17] L. Urbani, *et al.* "Multi-stage bioengineering of a layered oesophagus with in vitro expanded muscle and epithelial adult progenitors," *Nature Communications*, vol. 9, pp. 1-16, 2018.