Using Humidity to Control the Morphology and Properties of Electrospun BioPEGylated Polyhydroxybutyrate Scaffolds

Leslie J. R. Foster,* Rodman T. H. Chan, Robert A. Russell, and Peter J. Holden

ABSTRACT: Electrospinning produces nanofibrous scaffolds with potential for tissue engineering and wound repair. Spinning parameters control scaffold morphology and properties. BioPEGylation of polyhydroxybutyrate (PHB) introduces terminal hydrophilic groups into the hydrophobic chain, making this natural–synthetic hybrid copolymer more susceptible to humidity. Varying the humidity from 10 to 50% RH during electrospinning had a relatively little effect on polyhydroxybutyrate (PHB) average fiber and pore diameters, which remained around 3.0 and 8.7 μm, respectively. In contrast, fiber and pore diameters for electrospun bioPEGylated PHB scaffolds varied significantly with humidity, peaking at 30% RH (5.5 and 14.1 μm, respectively). While scaffolds showed little change, hydrophobicity decreased linearly with humidity during electrospinning. Compared to solvent-cast films, electrospun scaffolds showed significantly greater average cell spread. A 108% increase for olfactory ensheathing cells (OECs) cultivated on bioPEGylated PHB scaffolds was proportionally greater than their counterparts on electrospun PHB scaffolds, (70%). OECs grown on BioPEGylated PHB scaffolds were over twice the size, 260 ± 20 μm diameter, than those on PHB electrospun scaffolds, 110 ± 18 μm diameter. Electrospun scaffolds also promoted cell health compared to their solvent-cast counterparts, with increases in the mitochondrial activity of 165 ± 13 and 196 ± 13% for PHB and bioPEGylated PHB, respectively. OECs cultivated on electrospun scaffolds of bioPEGylated PHB had significantly better membrane integrities compared to their counterparts on solvent-cast films, 47 ± 5% reducing to 17 ± 6%. The combination of bioPEGylation and humidity during electrospinning permitted significant controllable changes to scaffold morphology and properties. These changes resulted in the significantly greater promotion of cell growth on electrospun bioPEGylated PHB scaffolds compared to their solvent-cast counterparts and electrospun PHB.

1. INTRODUCTION

Biomaterials used in tissue engineering applications serve as support scaffolds and substrates for cells during in vitro culture prior to implantation. The design and structure of scaffolding biomaterials can significantly influence the development of engineered tissues, and a variety of biopolymers have emerged as potential candidates supporting cell adhesion, proliferation, and differentiated function. Biopolymers from the family of polyhydroxyalkanoates (PHAs) have been investigated for such roles, with poly(hydroxybutyrate) (PHB) the most studied. Biocompatible and biodegradable PHB is approved by the U.S. Food and Drug Administration (FDA) and regulatory bodies in various other countries; its application in biomedical devices including sutures, bone plates, and tissue scaffolds has been readily explored. However, PHB’s highly crystalline structure produces brittleness that makes it difficult to process and reduces the scope of its potential application.

Poly(ethylene glycol) (PEG), a synthetic FDA-approved polymer, is readily utilized in protein purification processes, as a drug carrier and various other pharmaceutical applications. The PEG family displays a wide range of molecular weights from 106 (diethylene glycol (DEG)) to 20 000. The chemical structure of PEG provides strong hydrophilic properties as well as mild hydrophobicity. While reports suggest that DEG has an estimated single-dose oral toxicity for humans of 1.0 mL/kg and between 1.0 and 1.63 g/kg of body weight, it is also reported to enhance cellular biocompatibility when blended or grafted to biocompatible polymers. PEG is considered an excellent conjugate for polymer graft materials. PEG is used to improve the pharmacokinetic properties of biologicals; data show that there is a large therapeutic window of about 600-fold between maximum PEG burden from a biological agent and doses associated with human toxicity. PEG-modulated bioprocessing of PHB produces a natural–synthetic hybrid of PHB-b-PEG with a reduced crystallinity, making this biomaterial relatively less brittle and more flexible than PHB.
Chemosynthetic and chemical modifications of polyhydroxyalkanoates with the aim of introducing various functional groups including hydroxyl moieties and improving their properties have been reported. However, BioPEGylation often results in significantly greater molecular weights and more appropriate mechanical properties. Additionally, we have previously demonstrated that bioPEGylation of PHB introduced a degree of amphiphilicity to the polymer, which influenced the physiochemical and mechanical properties. Furthermore, the responses of nerve-derived olfactory ensheathing cells (OECs) to bioPEGylated PHB were significantly improved. OECs are a type of glial cell vital in promoting the regeneration of nascent neurons in the olfactory system. They can be easily collected from adults through a minimally invasive procedure and, in contrast to Schwann cells, show no interaction with astrocytes. Furthermore, OECs are the only type of cell that continuously ensheath axons from the peripheral to central nervous system (PNS and CNS), respectively. The potential of PHB as a biomaterial for nerve repair is suggested by Kalbermatten et al., who reported its use as an implant carrying Schwann cells for regeneration of nerves in the CNS.

The introduction of hydrophilic groups to the hydrophobic PHB through bioPEGylation suggests that humidity can be used to control morphology, and by extension cellular attachment and response. Indeed, we have previously demonstrated that bioPEGylated PHA chains can self-assemble into disordered microporous films when fabricated through solvent casting under high humidity. Similarly, the presence of microdroplets of water in PHB-PEG solvated in chloroform leads to a stable microcrystalline solution.

Electrospinning has been used to fabricate matrices composed of fibers with diameters ranging from microns to nanometers. Electrospun nanofibrous matrices are thought to support cell and tissue growth by imitating the architecture of the extracellular matrix (ECM). Consequently, electrospun biomaterials have found applications in various fields including tissue engineering and regenerative medicine. A variety of biomaterial scaffolds fabricated by electrospinning PHB with various blends have been reported, and their potential for applications in engineering of bone, cartilage, nerve, retinal, and muscle tissues among others have been explored. While PHB scaffold morphology and function are influenced by copolymerizing and blending, Wang et al. have reported that fiber diameter and pore size in electrospun PHB matrices can also be significantly altered by changes to fabrication parameters, including temperature, voltage, polymer concentration, and distance from the collector plate. Ricotti et al. spun PHB nanofibers under climate conditions of 23 °C and 40% humidity and showed that the scaffold morphology influenced cell proliferation. However, the influence of humidity control during electrospinning of PHB has yet to be reported. In this study, we report on the manipulation of electrospun PHB scaffolds using humidity control, as well as its combination with DEG when introduced through bioPEGylation.

2. RESULTS AND DISCUSSION

2.1. Biopolymer Production. Consistent with previous reports, the biopolymer produced by bioprocessing of Cupriavidus necator in this study was confirmed as PHB using 1H NMR. Similarly, DEG-modulated cultivation resulted in end-capping of the PHB chains to form a natural–synthetic hybrid of PHB-b-DEG (Figure 1). BioPEGylation resulted in a significant reduction (87%) in molecular weight ($M_w$) to 145 from 1143 kg/mol for PHB. The bioPEGylated PHB also possessed a narrower polydispersity, 3.02, compared to 6.71 for PHB (Table 1 and Figure 2). Zanzig et al. also demonstrated

![Figure 1. 400 MHz 1H NMR spectrum of PHB-b-DEG produced from C. necator cultivated with 20 g/L glucose and 2% (v/v) DEG. The enlarged 3–4.5 ppm region of the spectrum displays proton peaks of DEG and a covalent bond between PHB and DEG.](https://dx.doi.org/10.1021/acsomega.0c02993)

![Figure 2. GPC/SEC traces obtained for (a) biopolymer PHB and (b) natural–synthetic hybrid PHB-b-DEG produced through bioprocessing of C. necator.](https://dx.doi.org/10.1021/acsomega.0c02993)
$M_r$ reduction for bioPEGylated PHB synthesized in Acaligenes latus, 74 from 403 kg/mol for PHB (81%). While the melting point of both polymers remained about 177 °C, the combination of bioPEGylation and molecular weight reduction significantly reduced the glass-transition temperature from 0.1 °C for PHB to −1.7 °C for its bioPEGylated counterpart. Fusion enthalpy was also significantly reduced from 64.5 to 55.9 J/g, suggesting a crystallinity reduction of 14.6% (Table 1, Figure 3).

Figure 3. DSC traces obtained for (a) biopolymer PHB and (b) natural–synthetic hybrid PHB-b-DEG produced through bioprocessing of C. necator.

The presence of the hydrophilic DEG group in the hybrid can significantly affect chain conformation, with implications for morphology. For example, when solvent cast under humid conditions, bioPEGylated PHA chains exhibited a degree of self-assembly, leading to disordered microporous films.23 In the study here, control of morphology in electrospun matrices was explored through bioPEGylation and varying the relative humidity during the spinning process.

2.2. Morphology of Electrospun BioPEGylated PHB Scaffolds. Consistent with previous studies, electrospun PHB scaffolds consisted of thin fibers and thicker “beads.”35,36 Scaffolds of PHB fabricated by electrospinning at a relative humidity (RH) of 10% showed a variety of irregular fibers with obvious variations in diameter both between and within individual fibers as well as beading, defined as comparatively larger bulbous regions of short duration (Figure 4a). Figure 4b shows that the density of these PHB beads increased almost linearly from 95 ± 5 cm$^{-2}$ in scaffolds spun at 10% RH to 276 ± 34 cm$^{-2}$ for those fabricated at 40% RH, before decreasing to 86 ± 34 cm$^{-2}$ at 50% RH. In contrast, bioPEGylated PHB scaffolds displayed no obvious beads until a spinning humidity of 50% RH was used (Figure 4c). Bead formation may be a consequence of surface tension, with poor interaction between the polymers and the solvent under the spinning conditions.39

Figure 4a–e shows the distribution of fiber diameters for PHB scaffolds electrospun at humidity varying from 10 to 50%.

Scaffolds fabricated at 10% RH exhibited two notable peaks of fiber diameters with the majority (75%) around 1–3 μm and 20% at approximately 4–5 μm (Figure 4a). Increasing the electrospinning humidity to 20% (RH) changed the diameter profile of PHB fibers, the main peaks shifted from 1–3 to 3–5 μm, and the emergence of fibers with diameters ranging from 5–6 μm (15%) was observed (Figure 4b). PHB scaffolds fabricated by electrospinning at 30% RH showed a similar diversity of fiber diameters with four notable diameter peaks, the majority (75%) with diameters below 2 μm (Figure 4c). Fiber diameters remained diverse for scaffolds spun at 40% RH (Figure 4d), before returning to three notable peaks in diameters at 1–2 (25%), 2–3 (40%), and 4–5 μm (25%) when spun at 50% RH (Figure 4e).

Electrospun PHB-b-DEG scaffolds showed a different trend in their fiber diameter distributions with increasing humidity compared to their PHB counterparts (Figure 5f–j). PHB-b-DEG fibers spun at 10% (RH) exhibited two peaks of fibers possessing diameters in the ranges of 2–3 and 4–5 μm of 45 and 30%, respectively (Figure 5f). When spun at 20–30% RH, fiber diameters increased, before decreasing for fibers spun at 40% RH (Figure 5g–i). However, the diversity of diameters for bioPEGylated PHB fibers spun at 40% RH was less than that for PHB fibers spun under the same conditions (Figure 5i). At 50% RH, electrospun PHB fibers showed three notable peaks in their diameter distributions, while scaffolds of
bioPEGylated PHB showed less variation in fiber diameters, with 70% within 2–3 μm (Figure 5j).

It is common practice to summarize gel- and electrospun scaffolds in terms of average fiber and pore diameters.24,28–30,33–41 At 3.50 μm, the average fiber diameter for scaffolds of bioPEGylated PHB produced by electrospinning at 10% RH was approximately 0.5 μm greater than their PHB counterparts (Figure 6a,b). As the spinning humidity increased, the average fiber diameter for PHB scaffolds was relatively unaffected, remaining around 3.0 μm, which is similar to previous reports (Figure 6a).24 In contrast, bioPEGylation introduced hydrophilic groups, which responded to the increase in humidity and the average fiber diameter increased to a peak value of 5.0 μm (Figure 6a), 2.5 μm greater than their PHB counterparts (Figure 6b) at 30% RH. However, as humidity increased further, the differences in average diameter decreased such that at 50% RH, the electrospun PHB-b-DEG nanofibers were approximately 50 μm less than their PHB counterparts (Figure 6b).

Changes in electrospinning humidity influenced not only the fiber diameters but also pore sizes (Figure 6c). Similar to the diameter analyses, the average pore diameter for PHB scaffolds did not vary significantly with spinning humidity; however, humidity exerted a notable effect on the bioPEGylated PHB scaffolds, which possessed greater average pore diameters compared to their PHB counterparts and followed a similar trend with changing humidity (Figure 6d). Thus, the average pore diameter for bioPEGylated PHB matrices was 3.5 μm greater than their PHB counterparts when electrospun at 10% RH; this difference increased to 5.5 μm for scaffolds spun at 30% RH before decreasing (Figure 6d).

Increases in fiber diameter for PHB-b-DEG scaffolds were most likely a consequence of changes to viscosity and the DEG hydrophilic properties, which more readily absorbed moisture compared to the PHB; humidity changes also extended the solvent evaporation rate and lowered its boiling point during the electrospinning process.39,41 Similarly, pore formation may have been caused by the water droplets leaving an imprint on the fibers, the motion of the jet, and air-casting phase separation. Casper et al.41 have demonstrated that the frequency of pore formation was increased with polystyrene fibers when the humidity was set above 30%.

2.3. Characterization of Electrospun BioPEGylated PHB Scaffolds. The X-ray diffraction pattern for PHB scaffolds was consistent with that reported by Hurrell et al.42 and was composed of three signature peaks, 14, 17, and 21°; there were no apparent differences for PHB-b-DEG (Figure 7a). However, the intensity of the PHB-b-DEG peaks was comparatively lower, consistent with a reduction in PHB crystallinity due to bioPEGylation, from 50.5 to 38.3% at 10% spinning humidity. Increasing the humidity during electrospinning of PHB resulted in scaffolds signifi- cantly in crystallinity from 50.5 (±2.0) to 54.7 (±1.1)% (Figure 7b). In contrast, increasing the spinning humidity during PHB-b-PEG scaffold fabrication had no significant effect on their crystallinity.

Changes in fiber distribution and pore structure of PHB scaffolds significantly influenced their apparent surface hydrophobicity (Figure 8a,b). Water contact angles decreased linearly from 115.3 ± 2.1° for scaffolds spun at 10% RH to 104.0 ± 2.8° for those fabricated at 50% RH (Figure 8c). These are considerably greater than those previously reported for solvent-cast PHB films (69.8 ± 1.3°).15 this may be due to comparatively greater surface roughness in the scaffolds, which created multiple contact points on the surface of the water droplet.14 Electrospun scaffolds of bioPEGylated PHB also
showed a linear decrease in hydrophobicity, as measured through water contact angles. However, the presence of PEG groups significantly reduced the initial surface hydrophobicity, with scaffolds spun at RH 10% exhibiting a contact angle of 97.4 ± 1.2°, compared to 89.4 ± 1.2° for those fabricated at 50% RH (Figure 8c).

Our study shows that control of humidity during electrospinning had a significant influence on the morphology of nanofibrous scaffolds as well as their crystallinity and surface hydrophobicity. BioPEGylation of PHB introduced hydrophilic groups, which, coupled with these humidity changes, showed proportionally greater changes to the scaffold morphologies compared to their non-bioPEGylated counterparts. Changes in surface morphology and porosity have been shown to affect cell proliferation and metabolic activity. Similarly, hydrophobicity reflects the surface energy of a biomaterial, influencing protein adsorption as well as cell attachment and proliferation.

2.4. Cell Proliferation and Health. Consistent with previous reports, OECs cultivated on solvent-cast films of PHB displayed characteristics consistent with healthy growth, including significant filopodia (Figure 9). Electrospun scaffolds are reported to promote cell growth and have potential applications in wound healing and tissue engineering. In the study, electrospun scaffolds promoted OEC growth, increasing the average cell spread from 110 ± 18 to 187 ± 16 μm; a 70% increase (Figures 9 and 10). In contrast, the average spread of OECs on electrospun bioPEGylated PHB scaffolds was 260 ± 20 μm, approximately twice the size of those determined on solvent-cast films of PHB-l-DEG (+108%, Figure 10). Thus, while electrospinning improved OEC spread for both PHB and PHB-l-DEG, the improvement was proportionally greater for the bioPEGylated polymer. Cell attachment to the electrospun scaffolds was stronger than that of OECs on solvent-cast films; we were unable to release all cells from the scaffold, rendering any cell density measurements inaccurate.

The relatively greater degree of OEC proliferation on electrospun scaffolds compared to their solvent-cast counterparts was also evident in their respective mitochondrial activities, which can be used to quantitate cell health (Figure 11a). OECs cultivated on PHB and PHB-l-DEG showed activities, as determined by MTS release, of 89 ± 9 and 95 ± 11%, respectively. In contrast, OECs grown on electrospun scaffolds of the same polymers showed MTS levels that were 165 ± 13% of the control for PHB scaffolds, but significantly higher, 196 ± 13%, for OECs grown on scaffolds of bioPEGylated PHB (Figure 9a). Membrane integrity, measured as lactate dehydrogenase (LDH) release from OECs grown on the solvent-cast films, was similar to those in the control of cells in asynchronous growth (58 ± 7%, Figure 11b). OECs cultivated on electrospun PHB scaffolds showed a slight but significant increase in LDH release compared to their counterparts grown on solvent-cast films, 68 ± 5 and 52 ± 5%, respectively. In contrast, electrospun PHB-l-DEG scaffolds significantly reduced membrane leakage to 17 ± 6 from 47 ± 5% for cells grown on solvent-cast films (Figure 11b). Thus, while bioPEGylation of PHB promotes the growth and health of OECs on solvent-cast films, these benefits are significantly magnified when films are electrospun, where humidity can be used to control morphology.

3. CONCLUSIONS
Although a predominantly hydrophobic biopolymer, humidity during electrospinning of PHB could be used to significantly alter the resulting scaffold morphology and properties. The nanofibrous structure of the scaffolds supported a reduction in surface hydrophobicity and promoted the attachment and spreading of OECs compared to PHB solvent-cast films. BioPEGylation of PHB introduced hydrophilic groups, which, coupled with humidity changes during electrospinning, showed...
proportionally greater changes to the scaffold morphologies compared to PHB. Scaffold morphology and its significantly lower hydrophobicity promoted cell attachment and spreading such that OECs on the bioPEGylated scaffold showed a 108% increase in spread compared to those cultivated on its solvent-cast equivalent. Similarly, improvements in cell health were also significantly greater for the bioPEGylated PHB samples.

Thus, humidity control during electrospinning of PHB can be used to manipulate fiber morphology and properties to enhance cellular attachment. The introduction of hydrophilic groups into the biomaterial significantly magnifies the impact of humidity, permitting greater control.

4. MATERIALS AND METHODS

4.1. Materials and Reagents. Polyhydroxybutyrate (PHB) of natural origin, diethylene glycol (DEG), and dimethylformamide (DMF) were purchased from Sigma-Aldrich (Sydney, Australia). Chloroform and dimethyl sulfoxide (DMSO) were purchased from Ajax (Sydney, Australia). C. necator (ATCC 17699) was used to produce PHB and PHB-b-DEG. All chemicals were of analytical grade with a minimum 98% purity.

4.2. Biopolymer Production. PHB and its bioPEGylated hybrid were produced using C. necator (ATCC 17699) in a 15 L bioreactor (Applikon Biotechnology, the Netherlands) with a working volume of 10 L using a modification of the method by Shi et al. Briefly, cells were grown in minimal salt medium with glucose (total of 10 g/L) at 30 °C, pH 6, and 30% dissolved oxygen tension (DOT) for 48 h. Biomass was subsequently harvested by centrifugation (6000 g, 30 °C, 30 min), the supernatant discarded, and the cell pellet resuspended in nitrogen-free minimal salt medium with 20 g/L glucose and 2% (v/v) DEG before incubating as before for a further 24 h. Biomass was then harvested by centrifugation (8000g, 4 °C, 30 min), washed with reverse osmosis (RO) water, and lyophilized for 24 h. Polymer extraction into
chloroform and purification through cycles (×3) of precipitation in cold methanol were carried out as per Shi et al. The pure polymer was dried under vacuum at 25 °C.

Chemical structures of the polymer samples were determined using a 1H NMR spectrometer (400 MHz, CDCl₃, Ultrashield 400, Bruker, Switzerland). The purified samples were dissolved in deuterated chloroform in an NMR tube (10−20 mg/mL). Chemical shifts were recorded in ppm (D₁ = 5 s, scans = 16).

4.3. Characterization: Molecular Weights. Molecular weights of PHB and PHB-b-DEG samples were determined using GPC with a PL gel column (5 μm 2x mixed-C, 30 cm × 7 mm) at constant temperature (30 °C) inside a refractive index detector (Varian, PL-GPC 50 Plus). Chloroform was used as the eluent at a flow rate of 1 mL/min. The sample concentration was 10 mg/mL. The GPC was calibrated with a series of polystyrene standards (American Polymer, Standard Corporation) processing low polydispersity (<1.1).

4.4. Characterization: Thermal Properties. Thermal properties were determined using a DSC-1 Star System (Mettler Toledo). Samples (5 mg) were sealed in pans and heated at 10 °C min⁻¹ from 25 to 220 °C to obtain the melting temperature (T_m) and enthalpy of fusion (ΔH_f). The samples were then cooled at the same rate from 220 to −50 °C and reheated from −50 to 220 °C to obtain glass-transition temperatures (T_g). The polymer (PHB-Xc) phase crystallinity was calculated using eq 1.

\[ \text{PHB - } X_c = \frac{\Delta H_f}{\Delta H_f^o} \times 100 \]  

where \( \Delta H_f^o \) is the enthalpy of fusion for PHB (146 J/g). Means of at least three samples were determined (n ≥ 3).

4.5. Electrospinning. Solutions of PHB and PHB-b-DEG were prepared (6% w/v in a solvent mix of chloroform/dimethylformamide (DMF) in a ratio of 7:3) in sterile sealed vessels (160 rpm, 50 °C, for 12 h). Samples of each solution were poured into 3 mL Luer-lock syringes equipped with blunt-ended 0.8 mm needles. A multiphaser programmable pump (NE-1000, New Era Pump System, Inc.) was connected to a syringe, and the pump rate was set at 2 mL/h. Polymer solutions were electrospun (EC-CLI, IME Technology, Netherlands) at voltage differences of 12, 15, 20, and 25 kV; a collection distance of 20 cm; and a temperature of 40 °C for 30 min. The influence of humidity was investigated by spinning at 25 kV and in the range of 10−50% RH. Electrospun scaffolds were dried under vacuum (40 °C, 48 h) before atmospheric equilibration (25 °C, 48 h). Electrospun samples were produced in triplicate (n = 3).

4.6. Scaffold Morphology. Electrospun samples were rinsed twice with 1% phosphate-buffered saline (PBS) and fixed for 4 h at 22 °C in glutaraldehyde (2.5%, 0.1 M PBS, pH 7.2). Samples were then washed with PBS, four times each of 5 min duration to remove unbound cells, then dehydrated for 10 min in a series of ethanol washes, 30, 50, 70, 80, 90, 95, and 100% prior to critical point drying using liquid CO₂ to prevent loss of cells. All specimens were mounted on aluminum stubs and surface-coated with a layer of gold particles using a sputter coater (Emitech K550x, England). Samples were subsequently examined using scanning electron microscopy (Hitachi S3400-N, Japan, UNSW analytical center) at 15 kV and 30 mA. Fiber thickness, pore sizes, bead densities, and appearances were measured by ImageJ analysis software (National Institutes of Health) from SEM images at 10k magnification. Fiber
diameters and pore sizes were measured using the linear dimension and freehand function of ImageJ; an average of three individual measurements were taken at any one point, for five different fibers or pores per 20 different SEM images (n = 20 × 5). Fiber thickness and pore sizes were expressed as diameters for simplicity and consistency with previous reports.

4.7. Scaffold Crystallinity. X-ray diffraction patterns of the biopolymer scaffolds were acquired using a Philips X’Pert Material Research Diffraction (MRD) System (Holland, UNSW analytical center). Samples (20 mm × 20 mm) were secured on glass slides and aligned with 2θ, z-axis, and omega scans (scattering angle range of 2θ = 10−30° and scan step size of 0.02° continuous scan type).

Crystallinity (X_c) were calculated as per eq 2 using X’Pert HighScore Plus software

\[
\text{crystallinity} (\%) = \left[ \frac{F_c}{(F_c + F_a)} \right] \times 100\%
\]

(2)

where Fc and Fa are the areas of crystal (peak) and noncrystal regions (under the curve), respectively.

4.8. Scaffold Hydrophobicity. Surface hydrophobicity was determined through water contact angles using a sessile drop method (25 °C, KSV cam 200, Finland). Electrospun samples (20 mm × 20 mm) were placed on the stage, and water droplets from a microsyringe were placed onto their surfaces. The contact angles of each sample were calculated using KSV instrument software. Means of 10 readings were recorded for each sample (n = 10).

4.9. Cell Proliferation and Morphology. Adherent OECs were cultivated in sterile T75 tissue flasks containing DMEM supplemented with 10% FBS (37 °C, 5% CO2) and harvested at 70% confluence. Populations of approximately 4 × 10^4 cells/mL were used to inoculate samples (13 mm × 13 mm) and incubated (37 °C, 5% CO2). Controls inoculated in the absence of the polymers were simultaneously conducted. At periodic intervals, the samples were sacrificed by rinsing twice with PBS (10 mL) containing trypsin (2 mL, 2.5% v/v) before further incubating for 2 min (37 °C, 5% CO2). Cell morphology and proliferation were visualized after 10 days using SEM. Cell sizes were determined by averaging 4 dimensions per cell for a minimum of 15 different cells taken from five randomly selected scaffold regions at a magnification of 1.00k (n ≥ 3 × 5).

4.10. Mitochondrial Activity and Membrane Integrity. Mitochondrial function in the OEC populations was assessed using a Cell Titer 96 Aqueous one solution cell proliferation assay. OECs were cultured in DMEM with 10% FBS, harvested by trypsinisation, counted, and plated into 96-well plates containing the electroposn samples. The cells cultivated in the absence of the biomaterials were used as controls. The cells (3 × 10^3) were cultivated in each well (48 h, 37 °C, 5% CO2). MTS solution (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, 30 μL) was then added to each well, and the plate was incubated again (4 h, 37 °C, 5% CO2). MTS concentrations were determined through spectrophotometry at 490/690 nm (Spectra Max 3400, Molecule Device). A mean of five samples was determined (n = 5).

Leakage of lactate dehydrogenase (LDH) was used to measure membrane integrity in the OEC populations as a consequence of their incubation with the polymer films. At 45 min prior to the incubation end point, samples of lysis solution (10 μL) were added to five of the wells which served as positive controls. After incubation, plates were centrifuged (300g, 5 min, 22 °C, RH 30%) and the samples of the supernatants (50 μL) were transferred into each well. LDH mixture (100 μL) was then added to each well before incubating in the dark (30 min, 37 °C, 5% CO2). Analysis was performed at 490/650 nm using a microtiter plate spectrophotometer. Means of five replicates per sample were determined (n = 5).

4.11. Statistical Analysis. Mean values with standard deviation of each group were calculated. Statistical analyses were performed using Student’s t-test and Bonferroni analyses to a significance with 95% confidence (P = 0.05) where appropriate.

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