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Research Article

Nerve Tissue Engineering Using Blends of Poly(hydroxyalkanoates) for Peripheral Nerve Regeneration.

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Abbreviations: COLI1, collagen type I 1; ECM2, extracellular matrix; LPS3, lipopolysaccharides 3; mcl-PHAs, medium chain length poly(hydroxyalkanoates); NCIMB,

National Collection of Industrial and Marine Bacteria; NGCs, nerve guidance conduits; NTE, nerve tissue engineering, PGA, polyglycolic acid; PHAs, poly(hydroxyalkanoates); PLCL, poly(DL-lactide-ε-caprolactone); PNI, peripheral nerve injury, P(3HB), poly(3-hydroxybutyrate); P(3HB-co-3HHx), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate); PVA, polyvinyl alcohol; RGD, L-arginine-glycine-L-aspartic acid; Rq, root mean square roughness; scl-PHAs, short chain length poly(hydroxyalkanoates).

Practical application

The standard treatment for peripheral nerve repair is the nerve autograft, which has several limitations including donor site morbidity, scar tissue invasion, scarcity of donor nerves, inadequate return of function and aberrant regeneration. Although artificial nerve guidance conduits (NGCs) made from various biomaterials have been clinically approved, they have not been able to overcome these limitations and can induce scar tissue and release compounds that are detrimental to the nerve regeneration process. Therefore, in this study, blends of poly(hydroxyalkanoates) (PHAs), that have not been used before in nerve tissue engineering were analysed for their potential use in the manufacture of multi-channel and electrospun NGCs. PHAs displayed properties that could overcome some of the limitations of the available NGCs such as controllable surface erosion, lower acidity of their degradation products after biodegradation and longer-lasting stability compared to their synthetic counterparts.

Abstract

The only types of poly(hydroxyalkanoates) (PHAs) that have been explored for their use in nerve regeneration are poly-3-hydroxybutyrate P(3HB) and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(3HB-co-3HHx)). However, the regeneration displayed by these PHAs is still inferior to that displayed in using autologous nerve grafting. The aim of this work was to study PHA blends as resorbable biomaterials for their use in the manufacture of nerve guidance conduits (NGCs). PHA blend films with varying ratios of poly(3-hydroxyoctanoate)/poly(3-hydroxybutyrate) (P(3HO)/P(3HB)) were produced using the solvent-casting method. Neat films of P(3HO) and P(3HB) along with 25:75, 50:50 and 75:25 blend films of (P(3HO)/P(3HB) were characterised with respect to their chemical, material and biological properties in order to evaluate them as potential base materials for nerve tissue engineering. In the surface analysis the blends exhibited the highest values of roughness compared with the neat films. The DSC values of the blends confirmed that P(3HO) and P(3HB) formed immiscible blends. FTIR and XRD analysis of the blends showed a decrease in the chrystallinity with the increase of the proportion of P(3HO). However, an increase in the stiffness of the blends was observed when the proportion of P(3HB) increased. Although all of the blends were biocompatible with NG108-15 neuronal cells, the 25:75 P(3HO)/P(3HB) blend showed not only significantly better support for the growth and differentiation of these cells, but also displayed suitable mechanical properties for its use as a base material for the manufacture of NGCs.

1 Introduction

Peripheral nerve injuries (PNI) affect about 2.8% of trauma patients, many of who suffer life-long disability [1]. Peripheral nerves are able to repair when the injuries present a gap of less than 5mm to bridge [2, 3]. For injuries resulting in nerve damage with gaps of more than 5mm, treatment is most commonly attempted using autologous nerve graft repair [4, 5]. When nerve damage is even more extreme and gaps exceed 3cm, allografts, vascularized nerve grafts and nerve grafts without vessels are used [5]. Peripheral nerve repair using nerve autografts has several limitations including donor site morbidity, scar tissue invasion, scarcity of donor nerves, inadequate return of function and aberrant regeneration [5, 6]. Currently there are several clinically approved artificial nerve guidance conduits (NGCs) made from various biomaterials that have overcome some of the limitations of these nerve autografts. Conversely, NGCs made from synthetic materials can trigger immune responses, induce scar tissue and can release compounds that are detrimental to the nerve regeneration process [5].

During the past two decades a large variety of materials including nano-structured materials and biochemical factors have been explored in attempts to improve the quality of nerve conduits, and currently there are several commercial nerve conduits approved by U.S Food and Drug Administration (FDA) and Conformit Europe (CE) [2]. All of the models currently available take the form of a simple hollow tube with a single lumen. They possess no internal substructure, are made from either synthetic or natural materials and are available in different designs and sizes [7]. The materials that have been used for their manufacture include poly(DL-lactide-ε-caprolactone) (PLCL), polyglycolic acid (PGA), polyvinyl alcohol (PVA), collagen type I (COLI) and extracellular matrix (ECM). Furthermore, a large diversity of materials have been used experimentally to produce nerve guidance conduits such as aliphatic polyesters, polylactic acids, polycaprolactones, polyurethanes, silicones, collagens, glycoproteins, polypeptides, poly(hydroxyalkanoates) (PHAs), polysaccharides, proteins and acellular or extracellular matrices.

PHAs possess great potential as materials for use in the manufacturing of NGCs to assist axonal regeneration. Their prominent properties such as: controllable surface erosion; variability in material properties; lower acidity of degradation products and longer stability compared to their synthetic counterparts are all of special interest in this field.

Currently, P(3HB) and P(3HB-co-3HHx) are the only type of PHA that have been explored for their use in nerve regeneration. P(3HB) conduits have been shown to repair nerve gaps of 10mm [8, 9, 10, 11, 12] and 40mm [13, 14] in rat sciatic nerves and rabbit peroneal nerves respectively. Hollow P(3HB-co-3HHx) conduits have also been used to bridge 10mm defects in rat sciatic nerves [15]. Although these studies showed low level of inflammatory infiltration and suitable reabsorption time for nerve repair, the regeneration obtained was not statistically comparable with the regeneration obtained by using autologous nerve grafting.

The aim of this work was to investigate PHA blends as resorbable biomaterials for their use in the manufacture of NGCs. Mechanical, physical and chemical properties of P(3HO), P(3HB) and their blends were characterized. The biocompatibility of these materials with NG108-15 neuronal cells was also studied. As P(3HO) displays mechanical properties similar to those of the peripheral nerve and P(3HB) have shown to be biocompatible to neuronal cells these PHAs were chosen for evaluation as improved materials for nerve tissue engineering.

2 Materials and Methods

2.1 Production and extraction of P(3HO) and P(3HB)

Production, extraction and purification of P(3HO) were performed as described previously [16]. The extraction method used to extract P(3HO) was dispersion of chloroform and sodium hypochlorite [16]. Production, extraction and purification of P(3HB) were carried out as described previously [17].

2.2 Film preparation

Films of P(3HO), P(3HB) and three different blends of P(3HO)/P(3HB) were prepared using the solvent casting method. The PHAs were dissolved in chloroform in order to obtain a total polymer concentration of 5 w/v%. The P(3HO)/P(3HB) blends were prepared in ratios of 75:25, 50:50 and 25:75 by dissolving the required amounts of polymers in 10 mL of chloroform. After polymer dissolution, the solutions were homogenised by sonication and then cast in 6-cm glass petri dishes. The films were air dried for two weeks and produced in triplicate to obtain a total of fifteen films with varying thicknesses of 0.09 – 0.15mm.

2.3 Scanning electron microscopy (SEM) of the films

Surface topography of the films was analysed using a FEI XL30 Field Emission Gun Scanning Electron Microscope (Eindhoven, the Netherlands). All the samples were previously sputter-coated with a 20 nm film of palladium using a Polaron E5000 sputter coater. The operating pressure of the sputter coating was $5x10^{-5}$ bar with a deposition current of 20 mA for a duration of 1m 30s. The images were then recorded at different magnifications at 5kV using the FEI software.

2.4 Surface wettability of the films

The static contact angle of the films was carried out as described previously [27].

2.5 Profilometric surface analysis

The surface roughness of the films was analysed using a Sony Proscan 1000 Laser Profilometer (Tokyo, Japan). The laser used was model 131A, which has a measuring range of 400 μm, a resolution of 0.02 μm and a maximum output of 10 mW. Scans of 0.5 mm^2 were obtained from each sample. Nine random coordinates were selected from each specimen in order to measure the root mean square roughness (Rq).

2.6 X-Ray diffraction analysis

Crystallization analysis of the films was performed using a Brüker D8 Advance diffractometer in flat plate geometry, using Ni filtered Cu Ka, radiation. Data was collected from 10 to 40° with a primary beam slit size of 0.6 mm. A Brüker Lynx Eye silicon strip detector was used and a step size of 0.02° and a count time of 0.1 s per step.

2.7 Fourier transform infrared spectroscopy (FTIR) of the films

The FTIR of the P(3HO), P(3HB) and (P3HO)/P(3HB) blend films was performed as described previously [16].

2.8 Static tensile test of the films

Mechanical analysis of the films was conducted using a Perkin Elmer Dynamic Mechanical Analyser 7 (Norwalk, USA). The sample dimensions were 1.66 mm - 2.05 mm in width; 5mm - 6mm in length and had a thickness of 0.05 mm - 0.18 mm. The load was set within a range of 1 nN – 6000 mN with a rate of 200 mN/min^{-1} at 24°C. The mechanical properties were determined using the software. This included analysis of Young's modulus, ultimate tensile strength and elongation at break.

2.9 DSC

Thermal analysis of neat polymers and their blends was conducted as described previously [27].

2.10 NG108-15 neuronal cell culture

NG108-15 neuronal cells were obtained from The European Collection of Cell Cultures (ECACC) and grown in Dulbecco's Modified Eagle Medium (DMEM) under a humidified atmosphere of 5% CO_2 at 37°C. The DMEM was supplemented with 10% (v/v) foetal calf serum, 1% (w/v) glutamine, 1%(w/v) penicillin/streptomycin, and 0.5% (w/v) amphotericin B. Cells were only used in the experiments once they were 80% - 90% confluent. Cells were

trypsinized and $3x10^4$ cells were seeded directly onto the PHA film samples within 12-well plates in 3 mL of DMEM. The cultures were maintained for 4 days, with half of the medium being removed and replaced with fresh serum-free DMEM on day 2 to trigger experimental differentiation. NG-108-15 neuronal cells were used between passages 10 and 20.

2.11 Live / dead measurement of NG-108-15 neuronal cells

After growing cells for 4 days, the culture medium was removed and replaced with fresh serum-free DMEM containing 0.0015% (w/v) propidium iodide (Invitrogen) and 0.001% (w/v) Syto-9 (Invitrogen) at 37°C/5% CO_2 for 15 min. After washing with PBS (x3), the cells were imaged with an upright Zeiss LSM 510 confocal microscope. A helium-neon laser was used for the detection of propidium iodide (λex= 536 nm/λem=617 nm) while an argon-ion laser was used for Syto 9 (λex= 494 nm/λem=515 nm). Three fields-of-view were imaged containing 20-500 cells per sample, so as to express the data as a percentage of live versus dead cells ± SEM. Quantification of live and dead cells was performed using the software Image J.

2.12 Immunolabelling of NG108-15 neuronal cells

To assess the differentiation of neuronal cells, samples were inmunolabelled using β III-tubulin as the primary antibody and with Alexa Fluor® 488 goat anti-mouse IgG as the secondary antibody. Sample films containing cultures of NG108-15 neuronal cells previously washed with PBS (x3), were fixed with 4% (v/v) paraformaldehyde for 20 mins, then permeabilized with 0.1% (v/v) Triton X-100 for 20 mins, before being washed with PBS (x3). Unreactive binding sites were blocked with 3% (w/v) bovine serum albumin (BSA) with the cells being incubated overnight with mouse anti β III-tubulin antibody (1:1000) (Promega, USA) diluted in 1% BSA at 4°C. Cells were then washed three times with PBS before being incubated with Alexa Fluor® 488 goat anti-mouse IgG antibodies (1:200 in 1 % BSA) (Sigma Aldrich) for 90 min. After washing the cells once with PBS, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Aldrich) (1:500 dilution in PBS) was added to label nuclei. Cells were then immersed

for 15 mins at room temperature before being washed again with PBS (x3). Cells were then imaged using an upright Zeiss LSM 510 confocal microscope. Nuclei ware visualised by two photon excitation using a Ti:sapphire laser (716 nm) for DAPI (λex = 358 nm/λem = 461 nm). For imaging the neuronal cell body and neurites of NG108-15 cells, a helium-neon laser (543nm) was used to detect the Alexa Fluor® 488 goat anti-mouse IgG (λex= 589 nm/λem=615 nm). The differentiated cells were then counted using ImageJ software, identified as neuronal cells expressing neurites.

2.13 Statistical analysis

Statistical analysis was conducted using Graph Pad Prism 6 software. A Shapiro - Wilk and Bartlett's test was previously performed to verify the normality and homogeneity of the data respectively. To analyse the difference between data, a one-way ANOVA test ($p < 0.05$) was conducted followed by Turkey's post-test ($p < 0.05$). Data was reported as mean ± SEM.

3 Results

3.1 Scanning electron microscopy of PHA films

Scanning electron microscopy images of the films were obtained in order to compare their surface morphology. The P(3HO) film (Fig. 1A) displayed the presence of pores with sizes ranging from 0.1µm to 5µm. The 75:25 (Fig. 1B) and 25:75 (Fig. 1D) blends presented smaller and less abundant pores (0.1µm to 3µm) compared to the P(3HO) film. Conversely, pores were not detected in the 50:50 blend (Fig. 1C), which showed protrusions uniformly distributed on the surface. The P(3HB) film (Fig. 1E) displayed the smoothest and most homogenous surface without the presence of pores. It was observed that the presence of P(3HO) in the blends increased the porosity compared with the neat P(3HB).

3.2 Profilometric surface analysis

The root mean square roughness (Rq) of the films was determined using a laser profilometer. The roughness of P(3HO) and P(3HB) films were significantly different (3.69 ± 0.20nm versus 2.60 ± 0.09nm, $p < 0.05$). Although the P(3HO) film was the most porous, its roughness (3.69 ± 0.20 nm) was not significantly different to the roughness of the 75:25, 50:50 and 25:75 blends (4.00 ± 0.15nm, 4.23 ± 0.37nm, 4.16 ± 0.25nm, $p > 0.05$). On the other hand, statistical analysis showed that the roughness of P(3HB) (2.60 ± 0.09 nm) was significantly different to that of the blends 25:75, 50:50 and 75:25 (4.00 ± 0.15nm, 4.23 ± 0.37nm, 4.16 ± 0.25nm, $p < 0.05$). As expected, the neat P(3HB) film presented the lowest value of roughness, which correlates with its surface having the smoothest appearance of all the films, as can be observed in the SEM analysis (Fig. 1F).

3.3 X - Ray diffraction analysis

The solvent-casted films were characterised by wide-angle X-ray diffraction spectroscopy. The neat P(3HB) film exhibited two intense peaks at 2θ values of 13.5° and 16.4° (Fig. 2A). The peak positions correspond to the reported values in previous studies of P(3HB) [18, 19] crystalline structure where they were assigned to the (020) and (110) planes of the orthorhombic unit cell. Moreover, a series of peaks were observed in 2θ range between 18° and 34°. These peaks are most likely attributed to the crystalline lattice planes of (021), (120), (111) and (101). In contrast to neat P(3HB), the diffraction pattern of P(3HO) was characterised by the presence of a broad amorphous halo located around 2θ=20°. However, in the P(3HO) diffractogram the amorphous halo was superposed with a series of diffraction peaks around 17°, 19° and 21°. Thus a significant fraction of semi-crystalline P(3HO) was in amorphous phase. X-ray diffractograms of all the blends showed intense peaks of P(3HB) (020) and (110) planes indicating that P(3HB) crystallised in the presence of P(3HO) even when the concentration of

P(3HO) was 75%. However, peaks corresponding to P(3HO) crystallites were not detected in the diffractograms of the blends. Also the weak amorphous halo was observed only in the 75:25 blend while other blend compositions did not show P(3HO) amorphous phase. Comparing the spectrum of the pure P(3HB) with those of the blends, it was seen that peak positions corresponding to P(3HB) were constant. This indicated that the P(3HB) unit cell did not change in the blends.

3.4 DSC

A representative thermogram of aged P(3HO)/P(3HB) blends (Fig. 2B) shows the presence of two endothermic events with peak temperatures around 50 °C and above 150 °C corresponding to the melting temperatures (T_m) of P(3HO) and P(3HB) respectively. After erasing the thermal history and material cooling at 20°C/min, neither material including neat P(3HO) showed the lower temperature melting event. Thus such cooling conditions do not allow crystallisation of P(3HO). The blending of these two polymers did not have any effect on the Tm of P(3HB). These PHAs have distinctive glass transitions (T_g) namely -39 and 2 °C for P(3HO) and P(3HB) respectively. Blends of these polymers were characterised by the presence of two glass transition events. Similar to melting, positions of both glass transitions were not affected by the presence of the second polymer. Interestingly, cold crystallisation was observed for the blends with high P(3HO) content (50:50 and 75:25) as shown by the relatively narrow exothermal peaks between 40 to 70 °C. In contrast, the cold crystallisation of neat P(3HB) and the 25:75 blend were barely detectable, they manifested as slight depreciation of the baseline at temperatures above 60 °C lasting until the endothermic melting of the P(3HB) crystals. It appears that P(3HB) crystallises to the same degree as P(3HB) in blends with P(3HO) content up to 50%. Specific enthalpy of fusion (ΔH_f) had slight fluctuations for P(3HB), 25:75, 50:50 blends, namely 73.4, 74.4, 71.6 J per gram of P(3HB) respectively. However, it significantly decreased to 28.8 J per gram of P(3HB) for the 75:25 blend.

3.5 FTIR of the films

The makers or signatures commonly used to rapidly detect and identify PHAs are the bands of the C=O group stretch and ester C-O-C group stretch. Furthermore, C-O-C stretch vibrations are also useful not only for the identification of PHAs but also for the determination of the crystallinity index (CI) [20]. In the infrared spectra of PHAs, C=O stretch bands are detected in the region 1719-1744 cm^{-1}, whereas ester C-O-C stretch bands are observed in the range of 1160-1300 cm^{-1}. Although in the FTIR spectra, the above bands are the ones most commonly used to analyse PHAs, the peaks of other detectable groups confirming the presence of PHA molecules such as CH_2 and CH_3 were also identified and are presented in the table 1 [21, 22, 23].

In figure 2C, it was observed that the intensity of the peaks was higher when the concentration of P(3HB) in the blends was increased. As absorbance (A) is proportional to the concentration of the molecules in the sample, the higher A values observed in the films containing P(3HB) could indicate a higher number of molecules of P(3HB) compared to P(3HO), regardless of blend composition. The P(3HO) film showed the carbonyl band at 1725.45 cm^{-1}, whereas the P(3HB) film showed it at 1718.99 cm^{-1} (Fig. 2C, Table I). The 75:25, 50:50, 25:75 P(3HO)/P(3HB) blends showed their carbonyl bands at 1726.53 cm^{-1}, 1719.37 cm^{-1} and 1719.29 cm^{-1} respectively. The carbonyl bands presented by both P(3HO) and the P(3HO)/P(3HB) 75:25 blend at around 1725 cm^{-1} are characteristic of the crystalline phase of medium chain length PHAs (mcl-PHAs). Similarly, the C=O stretch band at around 1719 cm^{-1} presented by the P(3HB) film and both the 50:50 and 25:75 P(3HO)/P(3HB) blends are attributed to the crystalline phase of short chain length PHAs (scl-PHAs).

In the region 1126-1317 cm^{-1} multiple C-O-C corresponding to the amorphous and crystalline state of the polymers are observed (Fig. 2C, Table I). The C-O-C stretch bands around 1160cm^{-1} and 1180 cm^{-1} are most common C-O-C fingerprints studied in the FTIR analyses of PHAs. These bands were similar for the P(3HO), 75:25 and 50:50 P(3HO)/P(3HB) films (1161.12cm^{-1}; 1161.33cm^{-1}; 1167.95cm^{-1}). Similarly, the C-O stretch bands were also comparable for the

P(3HO) and 25:75 P(3HO)/P(3HB) films (1179.25cm^{-1}; 1177.57cm^{-1}). The bands around 2900cm^{-1} correspond to the stretching vibration of the C-H aliphatic groups of the polymer backbones including the side chains. As expected, these bands were stronger for P(3HO) compared with P(3HB) due to the longer aliphatic chains present in P(3HO) molecules (Fig. 2C, Table I).

3.6 Surface wettability

Surface wettability of the PHA films was analyzed by measuring the water contact angle. Wettability describes how easy a fluid spreads or adheres across a solid surface. A high contact angle signifies low wettability whereas a low contact angle means high wettability. When the contact angle between distilled water and the surface of a solid substrate is less than 90°, the material is said to be hydrophilic or wet. When the angle is greater than 90°, the material is called hydrophobic or water-repellent [24]. The water contact angle of the P(3HO)/P(3HB) films decreased as the content of P(3HO) decreased ((P(3HO), 103.56 ± 0.95; 75:25, 94.41 ± 1.16; 50:50, 84.40 ± 0.70; 25:75, 77.36 ± 0.81; P(3HB), 69.69 ± 1.63) (Fig. 2). This is due to the long aliphatic chains present in P(3HO) that constitute its hydrophobic character. The statistical analyses showed that the differences in water contact angle between all the films P(3HO)/P(3HB) were significant (p-value < 0.05). As the water contact angle of both the P(3HO) and the 75:25 P(3HO)/P(3HB) films were greater than 90°, they are considered to be hydrophobic in nature. By contrast, the water contact angles of the 50:50, 25:75 blend films and the P(3HB) films were less than 90° and are therefore hydrophilic.

3.7 Static tensile test of the films

Mechanical properties of the films were measured through the static tensile test. Young's Modulus (E values), tensile strength and elongation at break of the different films are shown in Table IV. Young's modulus, a measure of the stiffness of materials, was determined by calculating the slope of the linear region of the stress-strain curve. The tensile strength is the

maximum load that a material can sustain during the test, whereas the elongation at break is the ratio between the final length before breakage and the initial length of the specimen. The *E* values of the blends and P(3HO) were significantly different to P(3HB) *E* value (p-value < 0.05). The tensile strength values were all significantly different when compared against each other excepting P(3HO) and the blend 75:25 of which difference was not significant. All the values of percentage strain were significantly different to P(3HO) percentage strain (p-value < 0.05). When the percentage strain values of the blends were compared against each other the only significant difference was found between the blends 50:50 and 25:75. When the % strain of P(3HB) was compared with the blends the only significant difference found was when compared with the blend 50:50. Stiffness of the blends increased when increasing the content of P(3HB) excepting for the 75:25 P(3HO)/P(3HB) blend. In the other hand, the pliability of the blends decreased when increasing the P(3HB) content.

3.8 Live/dead measurement of NG-108-15 neuronal cells

A live/dead cell measurement was conducted in order to compare the attachment and survival of NG-108-15 neuronal cells on the P(3HO)/P(3HB) films using PCL and glass as controls. In Figure 4, representative confocal images of the cells grown in the different substrates can be seen. Images (C) and (D) correspond to the 50:50 and 25:75 blends respectively and displayed the highest density of cells compared with the other images.

The percentage of live cells grown on P(3HO), 75:25, 50:50, 25:75 and P(3HB) films was 80.54 ± 6.26%, 89.73 ± 3.68%, 95.12 ± 1.02%, 95.59 ± 1.23%, 88.40 ± 2.99% and on PCL was 89.29 ± 3.06%. This was higher when compared with glass substrate (62.68 ± 3.15%) (Fig 4G). Statistical analysis showed that the difference in the percentage of live cells was significant for all the substrates including PCL when compared with that of glass ($p < 0.05$). The difference in the percentage of live cells between the 75:25, 50:50, 25:75 P(3HO)/P(3HB) and PCL substrates was not significant ($p > 0.05$). The only significant difference in the percentage of live cells found between the substrates was when the P(3HB) (80.54 ± 6.26%) and the 25:75

blend (95.59 ± 1.23%) were compared ($p < 0.05$). Hence, the percentage of live cells obtained using the P(3HO)/P(3HB) 25:75 film was significantly higher than that obtained with P(3HB).

In Figure 4H, the average number of cells in the different substrates is shown. The 50:50 and 25:75 blends were associated with the highest number of adhered neuronal cells per view (385.11 ± 77.69 cells and 456.00 ± 75.67 cells respectively). No significant differences were found in the number of adhered neuronal cells on 50:50 and 25:75 polymer blends. Statistical analysis demonstrated that the number of cells on the 50:50 and 25:75 blends were significantly higher than the other substrates, including PCL and glass. The difference in the number of live cells found between the P(3HO), 75:25, P(3HB), PCL and glass substrates was not significant (23.78 ± 5.96, 84.56 ± 19.92, 54.22 ± 14.04, 27.78 ± 4.07, 19.00 ± 2.52).

3.9 Neurite outgrowth assessment

NG108-15 neuronal cells were immunolabelled with the anti-β III-tubulin antibody to assess neurite outgrowth on the substrates. The protein β-III-tubulin is considered a neuron-specific marker as this molecule is expressed in neuronal cell bodies, dendrites, axons and axonal terminations. Therefore, this protein is widely used as an indicator of neuronal cell differentiation. Figure 5 (A-F) shows the confocal images of neuronal cells grown on each of the substrates, where neurite outgrowth can clearly be observed. However, two important characteristics of neuronal cells grown on the 25:75 blend, P(3HB) and PCL films were both the presence of several neurite-bearing neurons and the appearance of longer neurites compared with those cells grown on the P(3HO), 25:75 and 50:50 P(3HO)/P(3HB) blends.

Figure 5G shows the percentage of cells containing neurites on each substrate. Statistical analysis showed no significant difference in the percentage of cells with neurites for the P(3HO), 25:75, 50:50, 25:75, P(3HB) (91.42 ± 2.99; 80.90 ± 11.39; 86.15 ± 5.76; 99.30 ± 0.40; 97.98 ± 0.73), PCL (97.00 ± 1.30) and glass (81.85 ± 9.64), ($p < 0.05$). Fig. 6H, shows the number of cells with neurites in the different substrates. There was no significant difference in the number of cells with neurites on PCL (303.00 ± 77.94), glass (273.00 ± 77.94), 75:25

(280.00 ± 147.31), 25:75 (854.00 ± 243.02) and P(3HB) (591.00 ± 159.34) ($p < 0.05$). However, the number of cells with neurites on 25:75 blend was significantly higher compared to the P(3HO) and 50:50 blends ($p < 0.05$).

Discussion

The presence of two glass transitions and melting events in P(3HO)/P(3HB) blends, which occurs at the same temperatures as neat polymers (Fig. 2B) indicates that the PHAs used in this study were immiscible in both amorphous and crystalline phase. The independence of specific enthalpy of fusion of P(3HB) for blends with P(3HO) content up to 50% indicated that P(3HB) crystallises as separate phase in the presence of P(3HO). Although the aged blends show melting of P(3HO) crystals, the crystallisation was slower than P(3HB) and occurred when crystallisation of main part of P(3HB) was complete. Apparently the presence of mcl-PHA, a polymer with lower glass transition temperature, accelerates the kinetics of P(3HB) crystallisation. This was observed in the P(3HB) non-isothermal crystallisation, which was characterised by cold crystallisation for blends containing up to 50% of P(3HO). Such influence of mcl-PHA on the crystallisation of P(3HB) could be due to the increase of polymer chain mobility in the system containing polymer in rubbery state. However, in the 75:25 blend crystallisation of P(3HB) was significantly suppressed.

Crystallisation of P(3HB) was also confirmed by XRD. Although intensities of the peaks in the diffractograms of P(3HO)/P(3HB) blends decreased with the increase of P(3HO) content their positions were essentially the same as the peaks on the diffractogram of P(3HB). The peaks characteristic for P(3HO) were not detectable even in the blend with the highest P(3HO) content. That might imply that P(3HO) co-crystallised with P(3HB). However, considering that DSC experiment showed two separate melting events exactly matching the melting temperatures of the neat polymers, it is unlikely that these two PHAs could co-crystallise. One might suggest that crystallites of P(3HO) did not develop sufficiently in the blends in order to be detectable in XRD.

The surface of the P(3HB) film was found to be smooth whereas the surface of the P(3HO) film was porous. The smooth surface observed in P(3HB) film was similar to that characterized by Wang et al. (2012), where the degradation of P(3HB) by polyhydroxybutyrate depolymerase was investigated. Conversely, this smooth appearance contrasts with the porous morphology of a P(3HB) film characterised by Kai et al. (2003) in their study of P(3HB-co-3HHx)/P(3HB) blends. Although the surfaces of P(3HO) films have shown to be smooth and non-porous in previous studies [16, 27] it is worth noting that there is a lack of information available relating to the characterisation of P(3HO) for comparison.

The smooth appearance shown in the SEM images of P(3HB) film was in accordance with the profilometric analysis in which this film presented the lowest root square mean roughness. The higher Rq values of P(3HO) compared to that of P(3HB) might be attributed to its porous surface. The higher Rq values of the 75:25, 50:50 and 25:75 P(3HO)/P(3HB) blend films compared to the P(3HO) and P(3HB) film corroborated the phase separation process detected in the blend films by DSC analysis. Furthermore, it has been shown that when smooth blend films are produced using a wide compositional range, the polymers have a high level of compatibility [28]. Most of the Rq values of P(3HO) and P(3HB) films found in the available literature have been obtained using atomic force microscopy (AFM) and not by profilometric analysis. As it has been found that there is a discrepancy in the resultant Rq values between these two techniques, comparisons with these values were not feasible [29].

The crystalline phases of P(3HO) and P(3HB) were also detected in all the films by FTIR. The bands around 1725 cm^{-1} and 1719 cm^{-1} correspond to the C=O stretching vibrations of the crystalline phase of P(3HO) and P(3HB) respectively. When mcl-PHAs are in the crystalline phase, the band of the carbonyl group is detected at 1728 cm^{-1}, whereas in the amorphous phase this band is detected at 1743 cm^{-1}. When the scl-PHAs are in the amorphous phase the C=O group band is detected at 1740 cm^{-1} [30]. The peaks detected in the FTIR spectra of all the films showed the amorphous and crystalline portions of both polymers.

It has been shown that the forces affecting the wettability in a solid substrate are the surface tension of the substrate, the surface tension of the liquid and the interfacial tension. The hydrophobicity of blend materials can change due to compositional variations and the arrangement of polymer molecules in surface layers. When polymer films are formed from polymer solutions the solution interface is exposed to the hydrophobic air environment. Thus, polymeric molecules in the surface may re-orientate their hydrophobic groups towards the surface of the material, resulting in a less wettable surface. The neat P(3HO) film was the most hydrophobic one. This polymer has a longer aliphatic chain per monomer unit with four more methyl groups compared with P(3HB). These methyl groups may have rotated towards the hydrophobic interface through chain rotation. This orientation is more favourable energetically and decreases the surface free energy [31]. Therefore, the observed decrease in the wettability of the films as the P(3HO) content increased demonstrates the higher number of hydrophobic chains present in the films.

The Young's modulus, tensile strength and elongation at break values obtained for the P(3HO) and 50:50 blend differed slightly from the mechanical properties determined for similar films previously examined [27]. The higher Young's modulus values obtained in this study for the P(3HO) and 50:50 blend could be the result of variations in the molecular weight of the polymers. It has been shown that lower molecular weights of the same polymer increase Young's modulus and tensile strength values [32]. Therefore, these results suggest that the molecular weight of the P(3HO) used in this study could be higher than that used by Basnett et al. (2013). It is well known that cultivation conditions can affect the molecular weight of PHAs. Tomizawa et al. (2011) and Agus et al. (2012) showed that the molecular weight of P(3HB) decreased with an increase in culture time and temperature. The mechanical properties of P(3HB) obtained in this study agreed with values obtained in similar studies [35]. The Young's modulus of the 75:25 P(3HO)/P(3HB) blend (1.25 ± 0.18MPa) was the most similar to that of peripheral nerves in rats (0.58MPa) [36], suggesting that it could be a good base material for the manufacture of NGCs. However, the tensile strength and % strain of the 25:75 P(3HO)/P(3HB)

blend (17.80 ± 0.80MPa and 41.30 ± 1.73% respectively) were the most similar to that of a rabbit peripheral nerve, which has *in situ* tensile strength and *in situ* % strain of 11.7MPa and 38.5% respectively [37]. Therefore, the 25:75 P(3HO)/P(HB) blend would provide the appropriate resistance and elasticity that NGCs require to provide adequate flexibility at the site of implantation.

The inferior cell attachment observed in the P(3HO) and 75:25 P(3HO)/P(3HB) films could be related to higher levels of endotoxins in these films when compared with the 50:50 and 25:75 P(3HO)/P(3HB) films. P(3HO) was produced using the gram-negative bacteria *P. mendocina*, which contain endotoxins (lipopolysaccharides) within their cell wall. A higher level of lipopolysaccharides is expected with the increased concentration of the P(3HO) in the blend. In previous work, the endotoxicity of P(3HO), extracted using the dispersion method, was 4.3 EU/mL [16]. Considering that the United States Pharmacopeia permits a maximum of 20EU per medical device, further efforts of purification are needed to remove more endotoxins from P(3HO), such as the use of activated charchoal or soxhlet extraction [38]. The superior biocompatibility displayed in the 50:50 and 25:75 blends could be attributed to the higher roughness values featured in these films and their lower hydrophobicity. It is well known that cell attachment is enhanced by the roughness, porosity and hydrophilicity. However, in the neurite outgrowth test, the 50:50 blend presented a very low number of cells with neurites compared with the 25:75 blend, which supported the highest number of cells containing neurites. Therefore, these findings indicate that the 25:75 blend support significantly better the growth and differentiation of NG108-15 neuronal cells. Biocompatibility studies of NG108-15 neuronal cells with poly(hydroxyalkanoates) have only been performed on P(3HB) substrates. P(3HB) has demonstrated high biocompatibility not only with NG108-15 neuronal cells [39] but also with neuronal cells in animal models [8, 9, 10, 11, 12, 13, 14]. Armstrong et al. (2007) have used NGC made from P(3HB) as a substrate to investigate the effect of Schwann cells (SC) on neurite outgrowth NG108-15 neuronal cells.

The growth of NG108-15 neuronal cells was characterized by their irregular distribution in various layers on all substrates showing a random migration of cells. Neuronal migration is highly dependant on the expression of cell adhesion proteins, which can also be involved in neuronal differentiation. In neuronal cells, including NG108-15, different families of proteins regulate cell-cell and cell-substrate interactions: the Efh family, α-β-hydrolase fold family, and three families of CAMs; the inmunoglubolin (Ig) superfamily CAMs, cadherins and integrins [40, 41, 42, 43, 44, 45]. It has been shown that cell adhesion to polymeric surfaces such as PHA films is mediated mainly by integrins through the interactions between proteins and the polymers [46, 47]. Proteins coming from the serum, the surrounding medium or those produced by the cells could be adsorbed into the surface of the films and recognized by integrins through the Arg-Gly-Asp (RGD) sequence which is present in a considerable number of proteins.

In summary, although all of the P(3HO)/P(3HB) blends and P(3HO) were able to support neuronal growth, only the 25:75 P(3HO)/P(3HB) and P(3HB) films displayed suitable properties for supporting better neurite extension. Although P(3HO) and the 75:25 P(3HO)/P(3HB) blend presented suitable stiffness for the manufacture of NGCs, their biocompatibility was found to be inferior to that of the other blends. As the 25:75 P(3HO)/P(3HB) blend displayed not only tensile strength and % strain similar to that of the peripheral nerve, but also presented superior biocompatibility compared with the other substrates tested. Thus, the 25:75 P(3HO)/P(3HB) blend proved to be the most appropriate base material for the manufacture of NGCs.

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Table 1. Assignments of the peaks in the FTIR spectra of the blends P(3HO)/P(3HB)

Assignment	Frequency (cm-1) of P(3HO)/P(3HB) films				
	P(3HO)	**75:25**	**50:50**	**25:75**	**P(3HB)**
C-O stretching mode, ether	1043.41	1044.83	1044.31	1043.89	1043.18
	1096.72	1096.62	1098.65	1099.14	1099.11
C-O-C stretching mode, crystalline state	1126.13	1128.02	1130.47	1128.33	1127.35
C-O-C stretching mode	1161.12	1161.33	1167.95	1177.57	1179.25
C-O-C stretching mode, crystalline state	1224.61	1223.98	1226.77	1227.18	1225.86
C-O-C stretching mode, amorphous state	1250.24	1258.96	1258.84	1260.77	1260.10
C-O-C stretching mode, crystalline state	1272.12	1273.13	1275.63	1276.46	1274.32
C-O-C stretching mode, crystalline state	1296.77	1286.06	a	a	a
C-O-C stretching mode, amorphous state	1317.80	1314.52	a	a	a
CH_3 symmetric deformation	1379.03	1378.82	1379.00	1378.87	1378.49
CH_3 asymmetric deformation	1436.38	1436.09	a	1453.50	1451.56
CH_2 deformation	1456.66	1454.20	1457.25	a	a
C=O stretching mode, crystalline	1725.45	1726.53	1719.37	1719.29	1718.99
CH_2 stretching mode	2857.84	2858.29	2855.64	2850.79	2843.94
CH_3 symmetric stretching mode	a	a	a	2871.67	2864.78
CH_2 asymmetric stretching mode	2927.46	2928.68	2928.46	2930.67	2929.89
CH_3 asymmetric stretching mode	2954.67	2955.19	2955.39	2965.62	2968.97

[a)], band not detected in the FTIR spectrum

Table 2. Mechanical analysis of the P(3HO)/P(3HB) films

P(3HO)/P(3HB) Films	*E* (MPa)	Tensile strength (MPa)	% Strain
100:0	1.88 ± 0.12	4.91 ± 0.33	286.42 ± 18.48
75:25	1.25 ± 0.18	0.71 ± 0.08	73.82 ± 18.08
50:50	21.83 ± 0.95	2.17 ± 0.92	94.18 ± 3.52
25:75	143.40 ± 2.16	17.80 ± 0.80	41.30 ± 1.73
0:100	1160 ± 185.87	28.60 ± 1.76	9.6 ± 2.62

Figure legends

Figure 1. Characterization of the surface topography of P(3HO)/P(3HB) films by scanning electron microscopy analysis. (A) P(3HO); (B) 75:25; (C) 50:50; (D) 25:75, (E) P(3HB) and (F) 25:75 blend at 500 x. The films P(3HO) (A), 75:25 (B) and 25:75 (D) showed porous topography whereas the P(3HB) presented smooth surface. The surface observed on the blend 50:50 (C) presented protrusions uniformly without pores. The figure F taken with a higher magnification (500 x) of the 25:75 blend shows the porous structure of this blend.

Figure 2. ■P(3HO); ■75:25 P(3HO)/P(3HB), ■50:50 P(3HO)/P(3HB), ■25:75 P(3HO)/P(3HB), ■P(3HB). **A)** XRD spectra of P(3HO)/P(3HB) films showing crystalline (sharp peaks) and amorphous (broad peaks) phases of the polymers. As the content of P(3HB) increased in the blend the number the peaks increased. **B)** DSC thermograms of neat polymers and their blends. Dotted line is a representative thermogram of the first heating scan of aged 75:25 blend. Solid lines are thermograms of the second heating run. **C)** FTIR spectra of P(3HO)/P(3HB) films showing the characteristic peaks of P(3HO) and P(3HB). The FTIR spectra of the P(3HO) and 75:25 blend showed peaks in similar shift positions. Similarly the films 25:75 and P(3HB) presented peaks at similar frequencies.

Figure 3. Static water contact angle of P(3HO)/P(3HB) films**.** The water contact increased as the content of P(3HO) increased in the blend. The water contact angle was significantly different for all the blends (mean ± SEM, n = 9 independent experiments $P < 0.05$).

Figure 4. Confocal micrographs of NG108-15 neuronal cells labelled with propidium iodide (red) and Syto-9 (green) after four days in culture on P(3HO)/P(3HB) films and PCL. (A) P(3HO) ; (B) 75:25; (C) 50:50; (D) 25:75; (E) P(3HB) and F) PCL. Cell growth was randomly oriented on each of the flat substrates. (G) Live/dead analysis of neuronal cells on the P(3HO)/P(3HB) blends, PCL and glass (control). Percentage of live neuronal cells on all the

blends and PLC was higher in comparison to glass (control) (mean ± SEM, n = 9 independent experiments $^{*}P < 0.05$. Percentage of live neuronal cells on P(3HO) was lower compared to the 25:75 blend (mean ± SEM, n = 9 independent experiments #$P < 0.05$. (H) Number of live cells on the P(3HO)/P(3HB) blends, PCL and glass (control). The number of live cells on P(3HO), 75:25, P(3HB), PCL and glass decreased in comparison to the 50:50 and 25:75 blends (mean ± SEM, n = 9 independent experiments $^{**}P < 0.05$ compared to 50:50, ##$P < 0.05$ compared to 25:75.

Figure 5. Confocal micrographs of NG108-15 neuronal cells inmunolabelled for beta-III tubulin after four days in culture on P(3HO)/P(3HB) films and PCL. (A) P(3HO); (B) 75:25; (C) 50:50; (D) 25:75; and (E) P(3HB) and F) PCL. Neurite outgrowth was randomly oriented on each of the flat substrates. (G) Percentage of cell with neurites on the P(3HO)/P(3HB) blends, PCL and glass (control). Percentage of neurites on all the blends, PLC and glass was similar (mean ± SEM, n = 6 independent experiments, $P < 0.05$. (H) Number of cells with neurites on the P(3HO)/P(3HB) blends, PCL and glass (control). The number of cell with neurites on P(3HO) and 50:50 was lower in comparison to 25:75 blend (mean ± SEM, n = 6 independent experiments, $^{+}P < 0.05$.