Acta Biomaterialia 10 (2014) 2086–2095

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Hierarchically structured nerve guidance channels based on poly-3-hydroxybutyrate enhance oriented axonal outgrowth

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ARTICLE INFO

Article history:
Received 19 August 2013
Received in revised form 7 December 2013
Accepted 26 December 2013
Available online 7 January 2014

Keywords:
Polyhydroxybutyric acid
Porosity
Nerve guide conduit
Nerve regeneration
Neural cell

ABSTRACT

Traumatic peripheral nerve lesions can cause local anesthesia, paralysis and loss of autonomic control. Reconstruction using engineered nerve guidance conduits (NGCs) is rarely successful due to the sub-optimal characteristics of the conduits. To address the demands of clinical practice, we developed a hierarchically structured NGC from slowly resorbing poly(3-hydroxybutyric acid) (P3HB). The NGC consists of a permeable single-lumen tube and melt-spun fibrillar lumen fillers. Permeable tubes were constructed from P3HB/poly(ε-caprolactone) (PCL) blends or poly(3-hydroxybutyric acid-co-4-hydroxybutyric acid) (P(3HB-co-4HB)). Polyvinylpyrrolidone was used as a porogen in solvent-free thermoplastic processing, followed by selective polymer leaching. All tested material compositions showed hydrolytic degradation after 16 weeks in phosphate buffered saline, whereas P3HB/PCL tubes maintained mechanical strength compared to (P(3HB-co-4HB)). The porous scaffolds allowed diffusion of large molecules (~70 kDa). *In vitro* studies demonstrated that mouse fibroblasts survived and proliferated inside closed porous tubes. An *in vitro* model of axonal regeneration using dorsal root ganglia and sympathetic cervical ganglia demonstrated that the NGCs successfully supported neuron survival and neurite outgrowth. The introduction of fibrillar lumen fillers promoted oriented neurite growth and coating with extracellular matrix proteins further increased ganglia attachment and cell migration. In this study we show that P3HB-based NGCs scaffolds have potential in long gap peripheral nerve repair strategies.

1. Introduction

Loss of motor function or sensation of an injured extremity reduces the quality of life for a patient affected by nerve trauma. For short nerve gaps (up to 3 cm), therapies such as autologous nerve grafts or engineered nerve guidance conduits (NGCs) are reported to be successful [1]. However, complete functional recovery of larger defects is rare with the currently applied tubes and autografts. The main focus of research in this area is to improve the current scaffold design of single lumen tubes and choice of material (silicone, polyglycolic acid or collagen are in use now) to improve the outcome of larger defects.

The characteristics of an ideal NGC have been discussed in various publications [1–5]. In order to improve the axonal regeneration process and achieve functional recovery over larger distances, the NGC should be designed as a protective lumenized structure. The material should be mechanically stable and resorbable with interconnected porosity. The microarchitecture in the lumen should contain surface patterning, multichannels and lumen fillers [5–11], and incorporate extracellular matrix (ECM) proteins and/or neurothrophic factors [5,12–16]. Seeding the conduit with supportive cells, such as Schwann cells, results in a higher degree of regeneration [7,10,13,17,18]. Furthermore, electrical stimulation has been shown to be beneficial for cells and growth factor expression [2,19,20].

The first generation of NGCs was made from silicone and often resulted in serious complications, such as inflammation and the appearance of a compression syndrome, due to the non-resorbable and non-permeable material [1,21,22]. These side-effects motivated further research and development of degradable NGCs based on biomaterials such as collagen, polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL) or polyvinyl alcohol (PVA) [1,2,12,21–26]. Despite all the efforts, new materials did not significantly improve the performance of the NGCs. Issues concerning

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http://dx.doi.org/10.1016/j.actbio.2013.12.053
1742-7061/

mechanical stability, biocompatibility, permeation, swelling, acidic degradation, leeching of residues (during animal and clinical studies) and the lack of fundamental structures on a cellular and biomolecular level all became prominent. So far, these complications have prevented the introduction of new artificial NGCs as a routine therapeutic option in clinical practice [24,27–30].

Poly(3-hydroxybutyric acid) (P3HB) is a promising material for neuro-tissue engineering due to its natural origin, slow resorption rate without toxic degradation products and mechanical stability, and because it can be thermoplastically processed [31–35]. Despite the inexplicit P3HB degradation mechanism, the relatively slow resorption rate of P3HB in the organism, when compared to commonly used biopolymers such as PGA or PLA, seems to be highly advantageous for biomedical applications where slow tissue regeneration is expected [36–39]. However, due to laborious and expensive production procedures P3HB has rarely been used in tissue engineering. Moreover, its thermoplastic processing parameters are very limited due to the inherent brittleness and thermo-oxidative degradation. Thus, the material would benefit from intensive research.

Hazari et al. made the first attempt to utilize P3HB in neural engineering by implanting "wrap around" P3HB fibre mats, with a longitudinally oriented fibre structure, in peripheral nerves of cats and rats [40,41]. It resulted in improved nerve regeneration but a similar inflammatory reaction to primary epineural repair. Due to the fibrous material structure, the implant degraded significantly 6 months after implantation, while the neural repair was still insufficient. The constructs were used for the repair of larger defects, up to 40 mm, suggesting that additional guiding cues could have improved cell migration and nerve regeneration [15,42]. In fact, cues in the form of aligned PCL nanofibres [18], microstructured PCL filaments [43], PGA microfibres [5] and linear ordered collagen [12] have been shown to support alignment and migration of the glial cells which guide regrowing axons. Additionally, Novikova et al. demonstrated that using P3HB fibres as carrier scaffolds for matrix components and cell lines supported neuronal survival and regeneration after spinal cord injury [44].

In this study we show a P3HB-based NGC of a hierarchical structure that mimics the fundamental architectural characteristics of the native nerve tissue while fulfilling the requirements of mechanical stability, cell protection and permeability. The presented NGC is composed of a flexible, but mechanically stable, open-porous tube containing laminin and collagen coated fibrous lumen fillers. The open-porous conduit resembles the perineurium, which surrounds the nerve fibres and allows for transport of nutrients coming from the vasa nervorum in vivo. The laminin and collagen coated fibres, on the other hand, resemble the endoneurium, which is mainly composed of different extracellular proteins and gives support to the Schwann and neuronal cells. The presented scaffold is a promising alternative to already existing single lumen guidance channels due to the highly advantageous properties of the P3HB material, i.e. mechanical stability and slow degradation, as well as the qualities of the NGC design, i.e. permeability and cellular guidance structures.

2. Materials and methods

2.1. Materials

High-molecular-weight P3HB Biocycle® 1000 was obtained from Biocycle, Brazil. Poly(3-hydroxybutyric acid-co-4-hydroxybutyric acid) (P(3HB-co-4HB)) with 7.5 wt.% 4HB was synthesized in cooperation with the Saxon Institute for Applied Biotechnology (Leipzig, Germany). Poly(ε-caprolactone) (PCL) Capa®6800 was purchased from Perstorp Chemicals GmbH, Germany and polyvinylpyrrolidone (PVP) PVP K-30 from Alfa Aesar GmbH & Co. KG, Germany. Chloroform and methanol, both analytical grade, were purchased from Merck Chemicals, Germany.

2.2. Preparation techniques

2.2.1. Preparation of blends

Blends were obtained by dissolving both polymers in chloroform, followed by precipitation of the mixture in methanol. After precipitation, the material (flakes) was dried at 40 °C for 24 h. Compositions of P3HB/PCL 70/30 and P3HB/PCL 50/50 were chosen based on earlier studies which describe physical and mechanical characteristics of these blend systems [45].

2.2.2. Fabrication of porous single-lumen tubes by microextrusion and leaching

For the fabrication of tubular structures by microextrusion (DSM Xplore, The Netherlands) special nozzles were generated to create single lumen tubes of various diameters and wall thicknesses. The inner diameter (d) varied between 1.0 and 2.0 mm and the wall thickness (wt) between 250 and 500 μm. The microcompounder was heated to 160 °C for P(3HB-co-4HB) or 185 °C for P3HB/PCL blends and the rotation speed of the conical screws was set to 50 rpm for filling and 100 rpm for mixing and output. The polymer mixture was molten and mixed for 2 min prior to output. For each run, 3 g of material was used. To evaluate the porous system, various amounts of the porogen agent PVP were tested: 10, 20, 30, 40, 50 and 60 wt.%. After tube fabrication, PVP was extracted in deionized water for 24 h at room temperature using an ultrasonic bath. For cell culture, porous tubes were kept in phosphate buffered saline (PBS) containing 1% penicillin/streptomycin prior to in vitro studies.

2.2.3. Fabrication of lumen fillers

Multifilament fibres were fabricated from P3HB by high-speed melt spinning as previously described [46]. In brief, the polymer was molten and pre-mixed using a twin-screw extruder adjusted to various temperature zones heated from 150 to 192 °C. The melt was pumped at a constant mass flow rate through an appropriate spinneret (24-hole) and multifilament fibres were taken up with special drawing equipment at high speed (~1500–1800 $m \cdot min^{-1}$). For in vitro studies, P3HB fibres were fixed at each end with medical grade adhesive silicone (Nusil Silicone Technologies, Med-1511) on P3HB foils or glass coverslips.

2.2.4. Fabrication of P3HB foil

P3HB foil was made by compression moulding (TP400/Fontune Holland). Briefly, 2 g of P3HB granules was placed between heated stainless steel plates (185 °C) and pressed with a force of 45 kN for 5 min. Plates were taken off the press and cooled to room temperature before removing the foil. Foils had a thickness of ~80 μm and were translucent. Foils strips were cut (9×1.8 mm^2) and served as substrates to glue P3HB fibres onto it. Fixed P3HB fibres where then placed inside the tube for in vitro characterization and removed out of the tube for observation by laser scanning microscopy. Table 1 shows an overview of the used preparation techniques, the resulting structure and the method applied.

2.3. Surface modification

To hydrophilize the tube surface, porous tubes were treated with 20% aqueous ethylenediamine (ED, Sigma, E 26266) solution for 2 h at 50 °C. Subsequently, tubes were rinsed in double distilled water until a pH of 7 was reached. With this treatment amino groups can be introduced, as amino groups of the ED react with ester groups of P3HB. The effect of ED treatment, i.e. introduction of