A new nerve guide conduit material composed of a biodegradable poly(phosphoester)

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Abstract

There is a resurgence of interest in the development of degradable and biocompatible polymers for fabrication of nerve guide conduits (NGCs) in recent years. Poly(phosphoester) (PPE) polymers are among the attractive candidates in this context, in view of their high biocompatibility, adjustable biodegradability, flexibility in coupling fragile biomolecules under physiological conditions and a wide variety of physicochemical properties. The feasibility of using a biodegradable PPE, P(BHET-EOP/TC), as a novel NGC material was investigated. Two types of conduits were fabricated by using two batches of P(BHET-EOP/TC) with different weight-average molecular weights (Mw) and polydispersity indexes (PI). The polymers as well as conduits were non-toxic to all six types of cell tested, including primary neurones and neuronally differentiated PC12 cells. After in situ implantation in the sciatic nerve of the rat, two types of conduits triggered a similar tissue response, inducing the formation of a thin tissue capsule composed of approximately eight layers of fibroblasts surrounding the conduits at 3 months. Biological performances of the conduits were examined in the rat sciatic nerve model with a 10 mm gap. Although tube fragmentation, even tube breakage, was observed within less than 5 days post-implantation, successful regeneration through the gap occurred in both types of conduits, with four out of 10 in the Type I conduits (Mw 14,900 and PI 2.57) and 11 out of 12 in the Type II conduits (Mw 18,900 and PI 1.72). The degradation of conduits was further evidenced by increased roughness on the tube surface in vivo under scanning electron microscope and a mass decrease in a time-dependent manner in vitro. The Mw of the polymers dropped 33 and 24% in the Type I and II conduits, respectively, in vitro within 3 months. Among their advantages over other biodegradable NGCs, the PPE conduits showed negligible swelling and no crystallisation after implantation. Thus, these PPE conduits can be effective aids for nerve regeneration with potential to be further developed into more sophisticated NGCs that have better control of the conduit micro-environment for improved nerve regeneration. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Poly(phosphoester); Biodegradable polymer; Nerve guide conduits; Biocompatibility; Axonal regeneration

1. Introduction

Current treatments for injury-induced nerve defect typically rely on donor tissues obtained from the patient. This has raised the issue of function loss at the donor sites, formation of potential painful neuromas, structural differences between donor and recipient nerves and shortage of graft material for extensive repair. To circumvent these problems, synthetic nerve guide conduits (NGCs) have been developed to bridge the nerve gaps by inserting the severed nerve stumps into the two ends of the conduit. There has been a long interest in the use of biodegradable polymers to fabricate NGCs [1]. This approach has an obvious advantage over using biodurable NGCs in dispensing with a second surgery to remove implanted tubes. This second operation is often necessary to eliminate the potential problems such as chronic tissue response and nerve compression, caused by the long-term presence of a rigid tube in the body [2,3].

Several types of synthetic biodegradable polymers, including polyester urethane [4] and \( \varepsilon \)-caprolactone-lactide copolymer [5–11], have been examined for
promoting peripheral nerve regeneration (for a review see [12]). There is still room for developing new biodegradable materials with distinct characteristics in nerve tissue engineering. Important parameters such as surface properties, porosity and biodegradation that affect cell–substrate interaction and/or communication with the external environment of the conduits have yet to be optimized. In our development of tissue engineering scaffolds, we have been studying poly(phosphoester)s (PPE), a class of biodegradable polymers with a variety of attractive properties [13–15]. The phosphoester bond in the PPE backbone can be cleaved by water under physiological conditions. The ultimate hydrolytic breakdown products of the polymers are phosphate, alcohol and diol. By rational design of the building blocks, PPE has great potential to be biocompatible. PPE polymers are adjustable; manipulation of either the backbone or the sidechain structure would readily alter their physicochemical properties. In addition, the pentavalency of phosphorus atoms of a PPE polymer allows for the chemical linkage of drug molecules or proteins to the polymer. PPE polymers have been successfully used for controlled drug delivery [16].

Here we describe studies that were undertaken to evaluate the feasibility of PPE conduits in promoting peripheral nerve regeneration. Poly(bis(hydroxyethyl)terephthalate-ethyl ortho-phosphorylate/terephthaloyl chloride) or P(BHET-EOP/TC) has been selected among other PPE polymers as a NGC material based on its good processability (Fig. 1). Two batches of the polymer with different weight-average molecular weights and polydispersity indexes were synthesized and used to prepare NGCs. The data obtained from the present study suggests that P(BHET-EOP/TC) can be an effective aid for nerve reconstruction.

2. Materials and methods

2.1. Polymer synthesis and fabrication of NGCs

The polyphosphoester, P(BHET-EOP/TC) was synthesized by a two-step solution polycondensation according to the reported method [15]. Two batches of the polymers were synthesized with a weight average molecular weight (Mw) of 14,900 and 18,900, respectively (Table 1). A 34% (w/w) solution of the polymer in chloroform was prepared by magnetic stirring. A Teflon mandrel of diameter 1.27 mm was vertically dipped into the polymer solution at a speed of 8.3 mm/s where it remained for 30 s. The mandrel was withdrawn at 24 mm/s and immediately immersed into water where it was allowed to stand for 5 min. The mandrel was subsequently rotated horizontally for 5 min to reduce variations in the wall thickness along the axis of the tube and at the same time, to facilitate the process of air drying. Three coating steps were used to obtain a polymer tube with wall thickness of about 170 μm. Finally, the coated mandrels were equilibrated in water overnight, frozen at −20 °C and subsequently freeze-dried using a Modulyo Freeze-drying Unit at a pressure of 0.1 Torr for at least 1 week. The tubes were slipped off the mandrel after the freeze-drying and cut to 14 mm length for implantation.

2.2. Biocompatibility assessment

In vitro cytotoxicity and tissue histological analyses were used for biocompatibility assessment of the P(BHET-EOP/TC) and the NGCs made from this polymer. Cell types used in this study included rat primary neurons from the dorsal root ganglion (DRG), the hippocampus and the cerebral cortex, undifferentiated rat pheochromocytoma PC12 cells and neuronally differentiated PC12 cells, as well as rat primary fibroblast cells. The cells were cultured in DMEM supplement with 10% fetal calf serum. For PC12 cells, 5% of horse serum was also added. Extracts for in vitro assessment of neuronal cytotoxicity were prepared by placing 5, 10 or 20 mg of

![Chemical structure of the P(BHET-EOP/TC) repeating unit; For EOP/TC = 80/20, the ratio x:y = 4:1.](image-url)
P(BHET-EOP/TC) or conduits in 200 μl of the cell culture medium for 3 days with an extraction temperature of 37°C. The materials were disinfected with 70% ethanol before the extraction. Control solution was prepared in the same way without the test materials. To challenge cells with the extracts, the cells were grown to 50–80% confluence in a 24-well plate. The 200 μl of extracts plus 200 μl of fresh culture medium were added into each well of the plate. The cells were allowed to grow for another 3 days before morphological examination. At the end of the experiment, some of the dishes with neuronal cells were rinsed with PBS, fixed with 4% paraformaldehyde and prepared for immunocytochemical analysis with a mouse monoclonal antibody against the 68 kDa neurofilament protein. In vivo tissue response was evaluated after toluidine blue staining of 1 μm semi-thin sections from conduit explants (see below).

2.3. NGC implantation

Twenty-two male Wistar rats (200–250 g) were used for implantation, 10 for Type I and 12 for Type II conduits. After anesthetizing with pentobarbital, the right sciatic nerve of the rat was exposed through a 3 cm long skin incision on the thigh and retraction of the gluteus maximus muscle. The nerve was freed from the surrounding tissue and transected at the mid-thigh level, proximal to the tibial and peroneal bifurcation. A 5–7 mm piece of the nerve was removed and then the proximal and distal nerve stumps were pulled 2 mm into each opening of the PPE tubes, leaving a 10-mm interstump gap. The two stumps were fixed within the tubes with a single 10-0 perineurial suture (Ethilon). Before the proximal suture was pulled into the tube opening, the tube was filled with saline. Eight rats received an autograft repair, in which the transected nerve was reversed and sutured back between the nerve stumps. The surgery was performed under an Olympus operating microscope. The muscle layers were closed with 4/0 silk sutures and the skin closed with Michel clips.

2.4. Tissue processing, immunostaining and morphometric analyses

A total number of 29 nerve samples were used for evaluation of nerve regeneration (Table 2). Rats were anesthetized again 3 months after implantation. The sciatic nerves together with tubes were re-exposed and carefully isolated from the surrounding tissue. The nerve segment distal to the tube was pinched with a pair of forceps. Contraction of muscle on the back or retraction of the leg indicates the presence of regenerating sensory fibers in the pinched segment [17]. The rats were then sacrificed with an overdose of pentobarbital. The PPE conduits were excised together with nerves. After removing the conduits, the regenerated nerves were fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in PBS overnight before immunostaining. Twenty-micrometer thick longitudinal sections were cut on a cryostat and collected onto gelatin-coated glass slides. The sections were stained with mouse monoclonal antibodies against the 68 kDa neurofilament protein. For morphometric analyses, the nerves were fixed for at least 3 days in the above solution and then post-fixed with 1% osmium tetroxide. After dehydration and embedding in Epon, the nerves were cut into 1 μm thickness of transverse

Table 2
Morphometric analysis of the regenerated nerves at the midpoint of PPE conduits
A: Mean ± SEM

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Fiber diameter (μm)</th>
<th>G-ratio</th>
<th>Fiber population</th>
<th>Fiber density (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I NGC (15kDa PPE)</td>
<td>4</td>
<td>3.48 ± 0.10</td>
<td>0.64 ± 0.05</td>
<td>8080 ± 240</td>
<td>41,042 ± 14,129</td>
</tr>
<tr>
<td>Type II NGC (19 kDa PPE)</td>
<td>11</td>
<td>3.71 ± 0.38</td>
<td>0.67 ± 0.04</td>
<td>6684 ± 2155</td>
<td>34,388 ± 6562</td>
</tr>
<tr>
<td>Autograft</td>
<td>8</td>
<td>3.75 ± 0.26</td>
<td>0.68 ± 0.02</td>
<td>16,975 ± 949</td>
<td>38,104 ± 6706</td>
</tr>
<tr>
<td>Normal control</td>
<td>6</td>
<td>7.41 ± 0.41</td>
<td>0.44 ± 0.02</td>
<td>7991 ± 2257</td>
<td>14,225 ± 2768</td>
</tr>
</tbody>
</table>

B: Tukey test for multiple comparisons in groups with significant ANOVA

<table>
<thead>
<tr>
<th>Group comparisons</th>
<th>Fiber diameter (μm)</th>
<th>G-ratio</th>
<th>Fiber population</th>
<th>Fiber density (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I vs. Type II</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Type I vs. graft</td>
<td>NS</td>
<td>p &lt; 0.001</td>
<td>NS</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Type I vs. control</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>NS</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Type II vs. graft</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Type II vs. control</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>NS</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Graft vs. control</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>
sections, and stained with toluidine blue. Quantitative evaluation was carried out at the middle of the regenerated nerve cables using UTHSCSA Image Tool (The University of Texas Health Science Center in San Antonio) 3 months after implantation. For each sample, six areas of about 500 μm² from two cross sections and about 200–300 fibers were evaluated. The samples were analyzed for fiber diameter, fiber density, total number of myelinated fiber and G-ratio (axon diameter/myelinated fiber diameter). For examination of the orientation of fibrin matrices, the longitudinal sections were cut from the middle of tissue cables collected from P(BHET-EOP/TC) conduits 3 days after surgery and stained with toluidine blue.

2.5. Characterization of NGCs

2.5.1. Swelling of NGCs

Conduits were placed in phosphate buffer pH 7.4 at 37°C. At selected time points, the conduits were removed from the solution, blotted with an absorbent tissue and weighed for weight increase. Seven samples were used for Type I and eight for Type II conduits. Due to the breakage of Type I conduits, weight increase for swelling was not measured after 4 weeks. The percentage of water absorption (WA%) was calculated according to the following equation:

\[ WA(\%) = 100 \times \frac{(W_0 - W_t)}{W_0}, \]

where \( W_0 \) is the initial weight and \( W_t \) the weight of the swollen conduit. Swelling was also analyzed by measuring changes of wall thickness and tube diameter of conduits using UTHSCSA Image Tool.

2.5.2. Degradation of NGCs

To measure weight loss, conduits were placed in phosphate buffer pH 7.4 at 37°C. At selected time points, the solution was removed, the conduits were dried to constant weight under vacuum and weighed. The percentage of weight loss (WL%) was calculated according to the following equation:

\[ WL(\%) = 100 \times \frac{(W_0 - W_t)}{W_0}, \]

where \( W_0 \) is the initial weight and \( W_t \) the residual weight of the dry conduit.

To measure changes in molecular weight, the conduits were rinsed in water, dried under vacuum and dissolved in chloroform. NGC explants from the rat body were separated from surrounding tissue and treated with 0.5 mg/ml of collagenase Type II for 24 h to remove fibrous tissue and other protein impurities. These were subsequently treated in the same way as the in vitro samples for measurement of molecular weight. Gel permeation chromatography (GPC) was performed in chloroform with respect to polystyrene standards to determine the weight-average molecular weight (Mw), number-average molecular weight (Mn) and polydispersity index (PI).

To examine the morphology of the NGC explants with scanning electron microscopy (SEM), the samples were gold-coated using Fine Coat Ion Sputter JFC-1100 and micrographs were obtained using a JEOL JSM-T220A scanning electron microscope at an accelerating voltage of 15 kV.

2.5.3. Differential scanning calorimetry (DSC)

Samples for DSC were also enzymatically treated to remove protein impurities, as described above. DSC was performed on a DSC 2010 differential scanning calorimeter with a refrigerated cooling system (TA Instruments, New Castle, DE, USA). Thermograms were obtained for the temperature range from −20 to 200°C, at a heating rate of 10°C/min.

2.6. Statistical analysis

The data were subjected to an analysis of variance (ANOVA) using the SPSS statistical software. Multiple comparison tests were performed with Tukey tests where ANOVA showed overall statistical significance.

3. Results

3.1. Biocompatibility

Compared to controls, extracts made from various amounts of P(BHET-EOP/TC) polymer and conduits induced neither morphological alteration nor cell death after treatment for 3 days in any of the cell types tested. These included rat primary neurons from the dorsal root ganglion (DRG), the cerebral cortex and the hippocampus, undifferentiated rat pheochromocytoma PC12 cells and neuronally differentiated PC12 cells, as well as rat primary fibroblast cells. Immunostaining of DRG neurons or neuronal PC12 cells with antibodies against the 68 kDa neurofilament protein, one of the major structural components within neurons, showed no difference between extract-treated and negative control cells in the intensity and pattern of the staining (Fig. 2A and B). In one experiment, 30 mg of the polymer (which is about 4 times the weight of a conduit) was extracted for 2 weeks in PBS at 37°C and primary DRG neurons were treated with the extract for 7 days. No deleterious effects were observed after the immunostaining of the 68 kDa neurofilament proteins.

The assessment of the tissue response to PPE-made conduits was conducted after histological staining of the explants collected at various time points post-implantation. The two types of tubes displayed a similar effect. The formation of a thin fibrous tissue capsule around the tube could be seen at 3 days. New capillaries had
penetrated and dispersed within the tube structure within the first 4 weeks. An initial inflammatory response during this period was characterized by a weak accumulation of lymphocytes and macrophages. This was more pronounced in Type I conduits. At 3 months, the inflammatory reaction was not notable. A well-organized fibrous tissue capsule was present on the outer surface of the conduit, with about eight layers of fibroblasts and a thickness of about 20 μm (Fig. 2C). Some macrophages could be seen between the fibrous layers and the conduits. None of the implanted conduits triggered edema or necrosis.

3.2. Nerve regeneration

One day after implantation, some of the NGC chambers (three out of four Type II conduits) had become filled with a solid structure that bridged the two nerve stumps. This structure appeared like blood clot and was loosely attached to the stumps. At day 3, the solid structure was present in all tubes examined (Fig. 3B). By microscopic examination, small threads distributed with a predominantly longitudinal orientation were observed (Fig. 3C), suggesting an accumulation of fibrin matrices [18,19]. The structure had become firmly connected with the stumps 5 days post-implantation. Regenerated axons could be observed 2 weeks later in the proximal part of regenerated cables and 1 month later in the distal sciatic stump, 5 mm distal to the suture line. Most of them were unmyelinated, present together with numerous Schwann cells (data not shown).

After 3 months, positive reflex responses were observed in 40% of the rats that were implanted with Type I and 92% of those with Type II conduits when the nerve
trunks distal to the conduits were pinched in the anesthetized animals (Table 1). All these rats had a regenerated cable inside the conduits, which had bridged a 10 mm gap between the nerve stumps (Fig. 3D). The regenerated cables were centrally located within the conduits, surrounded by a fine epineurium. The cables contained numerous fascicles of myelinated as well as unmyelinated axons. Most of the axons in the distal nerve trunks were already myelinated. Immunostaining with an antibody against the NF68 protein confirmed axon distribution through the whole regenerated cables 3 months after implantation. The staining was stronger than that in the normal undamaged control nerve at the proximal nerve trunks but weaker at the distal part (Fig. 4).

Transverse sections through the mid-point of the 10 mm gaps were analyzed to determine the number of myelinated axons, fiber density, and fiber diameters (Table 2, Fig. 5). There were no significant value differences in all these parameters between regenerated cables from Type I and II conduits. The values were comparable between the PPE conduits and nerve autographs, except the total number of myelinated axons, which was significantly higher in the nerve autographs. Compared with normal control nerves on the other side of the rats, both the PPE conduits and nerve autographs had smaller diameter axons and fibers, thinner myelin sheaths, but much higher fiber density. The fiber population was also higher in the nerve autograft than in normal controls, but did not differ significantly between the PPE conduits and the controls.

3.3. NGC degradation

3.3.1. In vitro study

Since the phosphoester bond in a PPE backbone is cleaved by water, the more readily water penetrates, the greater the bond cleavage and the faster its degradation rate [13]. Yet deformation of a conduit caused by swelling could impede outgrowth of regenerating nerves [9]. In vitro experiments were therefore conducted to characterize swelling of P(BHET-EOP/TC) conduits. In one experiment, weight increases were measured after placing conduits in PBS at 37°C for various periods of time. After 4 weeks, Type I conduits had a 30% weight increase while Type II swelled slightly more than 15% (Fig. 6). In vitro swelling of those conduits was also characterized by
measuring wall thickness (Fig. 6). A 45% increase of wall thickness was observed in Type I conduits while no significant increase was seen for Type II. Tube diameter was not affected in both groups of conduits (data not shown).

Degradation of the PPE conduits in PBS at 37°C corresponded well to their swelling extent, i.e. when more water penetrated into a conduit, greater degradation occurred. Weight loss and decrease in weight-average molecular weight in a time-dependent manner documented this (Fig. 7). About 20% of weight loss was seen in Type I conduits after 12 weeks in PBS. Corresponding loss in Type II was 10%. GPC analysis showed that the higher molecular weight fraction of P(BHET-EOP/TC) had shifted to the lower end, confirming cleavage of polymer chains. For Type I NGC, the weight-average molecular weight had decreased from 15,000 to 10,000 after 12 weeks, a drop of 33%. For Type II NGC, the molecular weight dropped 24%, from 17,000 to 13,000 after 12 weeks.

3.3.2. In vivo characterization

In a pilot study, in vivo tube integrity was examined during the first week post-implantation. Both types of tubes were rigid enough to be easily manipulated during microsurgery. They became slightly softer after saline injection within the tubes. After 1 day, small cracks were visible in many of the Type I tubes. After 3 days, some cracks started to appear in Type II tubes. By day 5, the cracks became larger and were seen in almost all tubes. Four out of 6 Type I and one out of 5 Type II conduits broke down in the middle and fell into two parts connected with the proximal and distal nerve stumps, respectively, leaving a gap between them without tube.

Fig. 4. NF68 immunoreactive axons in Type II PPE conduits 3 months after implantation. (A) At the proximal stump; (B) at the distal stump; (C) comparison of the NF68 immunoreactivity expressed in arbitrary units.
Fig. 5. Micrographs of semi-thin sections showing myelinated axons. (A) control; (B) autograft; (C) Type I and (D) Type II conduits. Note the smaller diameter of the axons and higher fiber density in grafts and PPE conduits. Toluidine blue staining. The original magnification ×1000.

After 3 months post-implantation, all tubes had fractured into pieces. More than half of Type I tubes fell apart. Some of Type II tubes also fell into two parts, which were, however, connected by a tissue cable inside and a tissue capsule outside. In most of Type II tubes, the fragments were held together by connective tissue (Fig. 8C).

Under scanning electron microscope, some areas on the surface of Type I NGCs had changed from relatively smooth to very rough after 6 weeks (Fig. 8B). This became more widely distributed after 3 months. The main microstructure change observed in Type II conduits at 3 months was numerous small cracks spreading all over the tube (Fig. 8E). The tube surface had become rough with an increased porosity (Fig. 8F).

GPC analysis of NGC explants collected after 3 months of implantation showed a drop of 16 and 10% in the weight-average molecular weight in Type I \((n = 2)\) and II \((n = 3)\) conduits, respectively. The polydispersity index decreased from 2.22 to 1.76 for Type I and from 1.70 to 1.62 for Type II conduits.

DSC revealed the absence of any crystalline melting peaks for the explant materials, which would have been expected in the range \((-20–200°C)\) analyzed if crystallinity had been present in the samples [15]. This implies that crystallinity did not develop in the P(BHET-EOP/TC) NGC in vivo, at least up to a time period of 3 months.

4. Discussion

Current treatments for injury-induced nerve gaps, although fairly successful, do not provide the optimum
therapy. NGCs provide opportunities to control the microenvironment for enhanced nerve regeneration. This report has presented the results of our systematic analysis of the feasibility of NGCs made from a novel type of synthetic PPE polymer, P(BHET-EOP/TC), in repairing peripheral nerve injury in a rat model. Understanding the features associated with these conduits may help us develop more effective artificial grafts that produce favorable circumstances for nerve regeneration.

4.1. Biocompatibility of P(BHET-EOP/TC) conduits

Low inflammatory responses have been reported for PPE polymers, characterized by minor encapsulation and slight or no lymphocyte, giant cell, or macrophage activity [13,15]. The cytotoxicity test of P(BHET-EOP/TC) showed no effects on morphology and proliferation of human embryonic kidney cells and no mutagenicity in the Ames test [15]. Our studies concentrated on the nerve system with several in vitro assessments of cytotoxicity and showed no toxic effects of P(BHET-EOP/TC) polymers and conduits on primary neurons and neuronal cell lines tested. Our studies also included a sensitive immunostaining method using antibodies against the 68kDa neurofilament protein for detecting neuronal degeneration. Neurofilaments, composed of three polypeptides with molecular masses of 68, 150 and 200kDa, respectively, exist in the axoplasm of neurons to give form and tensile strength to the cell processes. The 68kDa protein is a primary target in the process of neurofilament breakdown after a toxic insult [20]; its degradation could be detected before morphological alterations in neurons [21]. No differences between experimental and control cultures in the NF68 staining as shown in this study indicate no adverse effects of P(BHET-EOP/TC) on neurons.

The potential toxicity of a biodegradable NGC is a factor that should be considered together with in situ degradation rates and local tissue clearances. The examination of in situ tissue response to implanted P(BHET-EOP/TC) conduits has shown a low inflammatory reaction, comparable to previous reports. This low reaction is clinically desirable in minimizing adhesions of an implanted conduit to surrounding tissues, especially to tendons of an injured hand.

4.2. Biological performance of P(BHET-EOP/TC) conduits

Biodegradable polymers with lower molecular weights can facilitate a fast degradation. One concern with using this kind of material to fabricate NGCs is the possibility of impeding nerve regeneration and/or impairing
maturation of the regenerated nerves due to a lower mechanical stability and an early disintegration of the tube. PPE polymers used for our NGCs possessed molecular weights of 14,900–18,900, probably the lowest molecular weights for polymers used in NGC fabrication. We observed an early fragmentation with these tubes. This was associated with a tube breakage in Type I conduits made from P(BHET-EOP/TC) with a lower Mw and a higher PI value, which had certainly stopped the formation of tissue cable between two nerve stumps and caused a lower successful rate of regeneration. Even though Type II tubes degraded quickly and displayed fragmentation as well after 5 days, these tubes had already aided nerve regeneration across a 10 mm gap in 92% of animals. A development of connective tissue capsules may have helped to prevent the disintegration of these fragmented tubes. Meanwhile, a fast accumulation and solidification of acellular material may have helped to maintain adequate space within the tubes for later migration of cellular components and nerve regeneration. This suggests that tube integrity for a biodegradable NGC is probably required only at the very beginning of
regeneration, during the formation of an extracellular matrix scaffold. This process starts with fluid exudation from the nerve stumps into the tube chamber and may be complete within 1 week [18,19]. When a fresh-cut nerve is repaired with our PPE conduits pre-filled with PBS, a solid structure that bridges the two nerve stumps is formed within 3 days. This is much faster than that in a PBS-prefilled silicone chamber, which takes 5–7 days before sufficient material accumulates to form a continuous bridge across a 10 mm gap [18]. It is not unlikely in our case that tube porosity [22] and increased permeability due to tube cracks had allowed the influx of nutrients and growth factors from the surrounding environment and enhanced the constitution of the matrix.

The quality of nerve regeneration within our biodegradable conduits was investigated by morphometric analysis. We have confirmed the regeneration features reported with other types of biodegradable NGCs [4,7,8] and found smaller size and higher density of regenerating axons in comparison with the results from undamaged control nerves. The fiber diameter and G-ratio were similar in the conduits and nerve autografts, indicating comparable degrees of axonal maturation and myelination with the two approaches. The fiber density values in the conduits and nerve autografts were close as well, but much higher than that from the normal control. This indicates that regenerating axons were immature after 3 months both in the conduits and nerve grafts and their axonal sprouting had proceeded to a similar extent. However, when the total number of axons were considered, PPE conduits were not fully equivalent to nerve autografts. A PPE conduit used in the present study is merely a tube without any internal cellular elements while a fresh nerve autograft inherently contains Schwann cells that are very efficient in promoting nerve regeneration. It is well documented that a bioartificial nerve graft comprising Schwann cells that were seeded into the lumen of a synthetic nerve conduit supported extensive nerve regeneration [23]. Performance of a PPE conduit may be improved in the same way.

4.3. Degradation of PPE conduits

PPE polymers degrade in the presence of nucleophiles, such as the hydroxyl ion. Their phosphoester bond is cleaved by hydrolysis at random sites along the polymer chains. Two types of hydrolysis, surface versus bulk, have been described. Surface hydrolysis is characterized by a linear decrease in mass together with little initial decrease in molecular weight while typical bulk erosion shows a rapid decrease in molecular weight before any significant mass loss [24]. The parallel reduction in conduit mass and in Mw as observed in this study suggests that both surface and bulk hydrolysis had occurred in PPE NGCs.

Continuous decrease in conduit mass by leaching out of degradation products will reduce the strength and toughness of a tube, ultimately resulting in a loss of structural integrity of a conduit under the pressure of surrounding structures. If this occurs too early, before a tissue capsule is formed over the conduit and a matrix cable is strongly attached to both stumps, the failure of regeneration would be a most likely consequence. Complete breakdown of the conduit is, however, desirable after the nerve has satisfactorily regenerated, to obviate the painful complications arising from nerve compression by a rigid conduit. While the time needed to have complete in vivo resorption of a PPE NGC in the body is still under investigation, the current study has shown that a NGC made from P(BHET-EOP/TC) is degradable. The difference in degradation rate between Type I and II conduits in our in vitro study can be explained based on the polydispersity indexes or the molecular weight distribution of polymers used. The polymer for fabrication of Type I conduits possessed a higher low molecular weight fraction compared to the one for Type II NGCs. This fact, in addition to its higher porosity, results in a higher degree of swelling for the Type I conduits. As such, the hydrolyzable linkages in the polymer become more accessible to be attacked by water molecules, leading to a higher degradation rate. A slower degradation rate of PPE NGCs in the body, in comparison with that in vitro is probably due to the gradual formation of a fibrous tissue capsule around the implant. This may result in decreased accessibility of the polymer’s ester linkages to water molecules and hence, a lower observed rate of degradation. Nevertheless, it was obvious in view of changes in molecular weights and SEM pictures that degradation of the NGC implants did occur in the body.

The degradation rate of a P(BHET-EOP/TC) polymer is related to the phosphate content of its backbone. The higher the phosphate content, the faster the degradation rate of the polymer [15]. This feature has provided a simple way to control the time frame of degradation of a P(BHET-EOP/TC) conduit. Increase in the BHET-EOP part during polymer synthesis may provide more phosphate content, therefore speeding up degradation. It is also possible to reduce this part to slow down degradation of the polymer when persistence of structural integrity is required. The time required for structural disintegration of a biodegradable NGC is decided by the time needed for nerve regeneration, which is variable with defect length, nerve location, and patient age [12]. P(BHEP-EOP/TC) copolymers with different ratios could be a choice to meet different requirements in obtaining optimal degradation rates of NGCs.

4.4. Tube swelling

Tube swelling is often observed with biodegradable nerve guide conduits. In those tubes with porous wall, the
initial swelling may result from water uptake into the pores, which explains the relatively higher swelling experienced by our Type I NGCs. As tube degradation starts, lower molecular weight degradation products will absorb water and enhance swelling. Swelling may cause problems in the use of a biodegradable nerve guide if it is sufficient enough to distort the tube lumen. Such a deformation had resulted in nerve compression and impeded outgrowth of regenerating nerves in nerve guides constructed of an amorphous copolymer of lactic acid-caprolactone [9]. A correlation between biodegradability and distortion was observed with biodegradable polyester tubes, showing that the more biodegradable a tube, the more likely it was to incur distortion and regeneration failure [25]. Ways to relieve the problem include increasing the internal diameter and reducing the wall thickness of the tube. These may, however, result in growth of fibrous tissue into the lumen or affect the mechanical strength of the tube.

A nerve guide that is less affected by swelling may have increased practical flexibility in clinical application. We have used an amorphous polymer, P(BHET-EOP/TC) to fabricate guides. Neither tubes with 170 μm wall thickness nor those with 240 μm (unpublished observation) showed any significant swelling. Our data are consistent with those obtained from NGCs made from DegraPol, a biodegradable polyester urethane with a low degree of swelling [4]. Thus, materials used could be decisive for the extent of swelling of a biodegradable synthetic tube. In the case of the P(BHET-EOP/TC) series of PPE polymers, changing the relative hydrophobicity by substituting ethylene glycol with other diols may affect swelling [15].

4.5. Crystallinity

Crystallinity has been given attention among the various properties of degradable polymers suitable for NGC fabrication [4,11]. Mixing a crystallizable polymer with an amorphous one may improve the mechanical properties of a tube that allow easy suturing during implantation [4]. Since crystallinity decreases the solubility of polymers, this mixing may slow down tube degradation, which may in turn aggravate foreign-body reaction. Some of the copolymers obtained from ε-lactide and ε-caprolactone show a gradual crystallization at room temperature, resulting in progressive hardening with time [26]. In the P(BHET-EOP/TC) series of PPE polymers, those with a BHET-EOP/TC charging ratio higher than 50/50 are amorphous with no melting point. Only when the ratio is reduced to 50/50, a crystalline phase begins to form in the polymer (T_m = 201 °C) [15]. Consistent with this, the PPE polymer used in the present study in which the BHET-EOP/TC ratio is 80/20 showed no crystallinity. This property would allow for complete biodegradation of a P(BHET-EOP/TC) conduit.

In summary, besides promoting axonal regeneration and eliciting a low inflammatory response, PPE nerve guide conduits have shown negligible swelling, fast degradation and no crystallization. The properties of this copolymer are adjustable with modification of molecular weight or monomer composition. The unique advantage of the phosphorus atom in a PPE polymer will allow for the chemical attachment of bioactive components, such as neurotrophic factors. Modification of the surface chemistry within the lumen of a NGC in this way may help to direct nerve regeneration and/or to support nerve fiber maturation [27]. Further studies are warranted to test the possibility.

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References


