Three-dimensional conductive constructs for nerve regeneration

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Abstract: The unique electrochemical properties of conductive polymers can be utilized to form stand-alone polymeric tubes and arrays of tubes that are suitable for guides to promote peripheral nerve regeneration. Noncomposite, polypyrrole (PPy) tubes ranging in inner diameter from 25 μm to 1.6 mm as well as multichannel tubes were fabricated by electrodeposition. While oxidation of the pyrrole monomer causes growth of the film, brief subsequent reduction allowed mechanical dissociation from the electrode mold, creating a stand-alone, conductive PPy tube. Conductive polymer nerve guides made in this manner were placed in transected rat sciatic nerves and shown to support nerve regeneration over an 8-week time period. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 91A: 519–527, 2009

Key words: biocompatibility; electroactive polymer; nerve guide; nerve regeneration; polypyrrole

INTRODUCTION

The shortcomings of autografts for long segments of peripheral nerve injury have led to the design of tubular nerve guides made from synthetic materials.[1] Nerve guides not only provide mechanical guidance and support but also reduce disruption by surrounding inflammation and connective tissue. Additionally, nerve guides are less surgically invasive than autograft procedures since donor nerve need not be harvested. Unfortunately, current polymeric nerve guides have had limited success in restoring normal function in animal studies.[2] Given that electrical stimulation has been shown to have a beneficial effect on axonal regeneration,[3] conductive polymeric nerve guides hold promise for enhancing restoration of lost nerve function.

Previous attempts at fabricating conductive nerve guides have utilized a composite structure with an additional polymer tubing serving as the template for polypyrrole (PPy) synthesis.[4,5] Devices made entirely of PPy could optimally utilize the polymer's properties, such as controllable charge density, erodability, and wettability, making them well-suited for biomedical applications such as nerve guides.[6–8] Furthermore, such a stand-alone PPy nerve guide would allow greater flexibility in modifying the tube, including the use of biodegradable forms of PPy.

To fabricate stand-alone, three-dimensional (3D) PPy structures, a method is needed to separate PPy from its conductive template. One possibility is to leverage the volume expansion that occurs during redox cycling of the polymer, as demonstrated with PPy actuators.[9–11] By holding PPy at a negative

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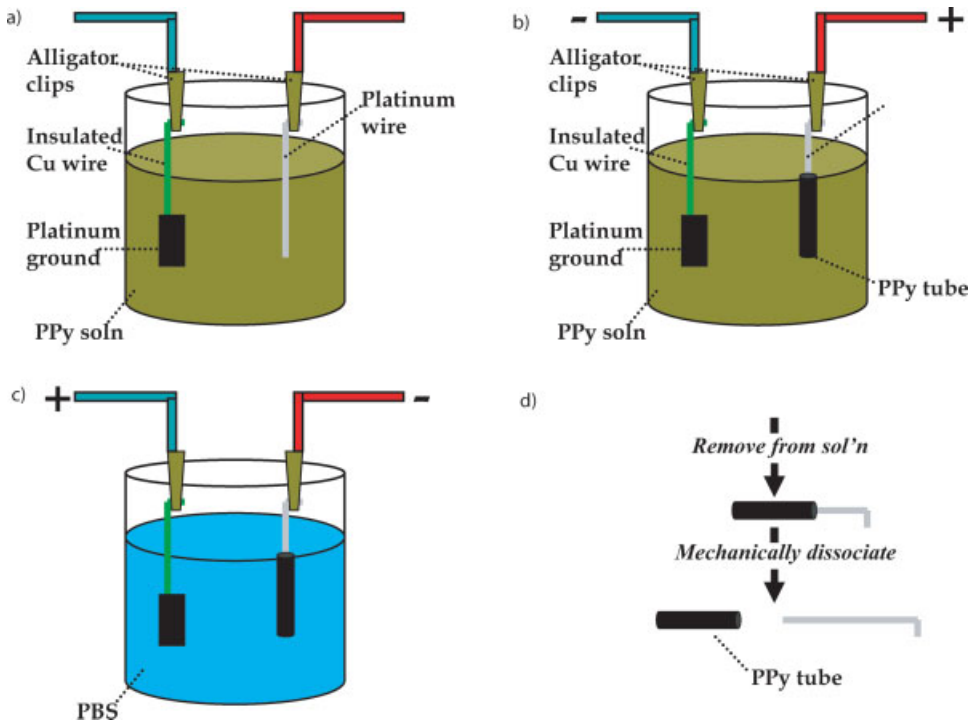

Figure 1. Reverse plating schematic. (a) Platinum wire in PPy solution serves as the plating template. (b) PPy is electrodeposited when a voltage is applied between the platinum wire and reference electrode. (c) Voltage polarity is reversed in a phosphate-buffered saline (PBS) solution to separate the PPy tube from the wire. (d) The tube is formed by separating the PPy from the platinum wire. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

potential relative to an electrolyte solution, the PPy is reduced; and cations from the solution flow into the polymer to neutralize charge, causing volume expansion and facilitating separation of the polymer from the template. Some degree of anion repulsion occurs as well, but cation movement tends to dominate at pH above 7.[12] The reduced 3D PPy structure can then be mechanically removed from the conductive template. Here we use the charge characteristics of PPy and its dopants to form tubes and arrays of tubes and demonstrate their potential application as peripheral nerve guides *in vivo*.

METHODS

Tube formation

Tubes were electroplated in aqueous 0.2*M* PPy/0.2*M* sodium dodecylbenzene sulfonate (NaDBS). Tubes were plated on wires of 25 μm (Al, California Fine Wire), 100 μm, and 200 μm diameters (both Pt, Sigma-Aldrich) using an EG&G Princeton Galvanostat (Model 263A) at a constant current of 0.1 mA for 40 min using a platinum mesh reference at 4 and 24°C (Sigma-Aldrich). Tubes for *in vivo* studies used copper wire (14 AWG, McMaster Carr) submerged 30 mm into the solution with 10 mA applied by a Hewlett Packard 6614 power supply for 60 min at 24°C. Electrodeposition for these tubes occurred under a blanket of N_2 gas. Tubes were imaged by SEM and wall thicknesses were quantitated in Adobe Photoshop CS3.

To remove PPy after plating, −10 V was applied between the wire and a platinum mesh for 2 min via a Hewlett Packard 33120A function generator. The PPy was then removed from the wire. For *in vivo* studies, tubes were cut into 15-mm segments, sterilized in ethanol, and washed three times with PBS.

For impedance measurement, tubes (n = 4) were fabricated at 4, 24, and 37°C with plating conditions described above. One set of tubes was fabricated using platinum wire (14 AWG, ScienceLab) as the plating template instead of copper. In addition to plating tubes at 10 mA for 60 min, one set of tubes was plated on copper wire at 5 mA for 120 min, and another set of tubes was fabricated at 1 mA for 10 h.

Multiarray tubes were formed using cylindrical and square Teflon molds containing evenly spaced 200 or 350 μm stainless steel wires (Malin Company, Cleveland, OH).[13] PPy tubes were formed as described above. A current of 0.5 mA for 14 h was used.

Impedance measurements

Impedance measurements were performed on tubes with the dimensions used in the *in vivo* studies (1.6 mm inner diameter, 15 mm in length). After tube formation, two 10-mm segments of copper wire (14 AWG, McMaster Carr) were inserted 2.5 mm into either end of the PPy tube, leaving a 10-mm space between the copper segments

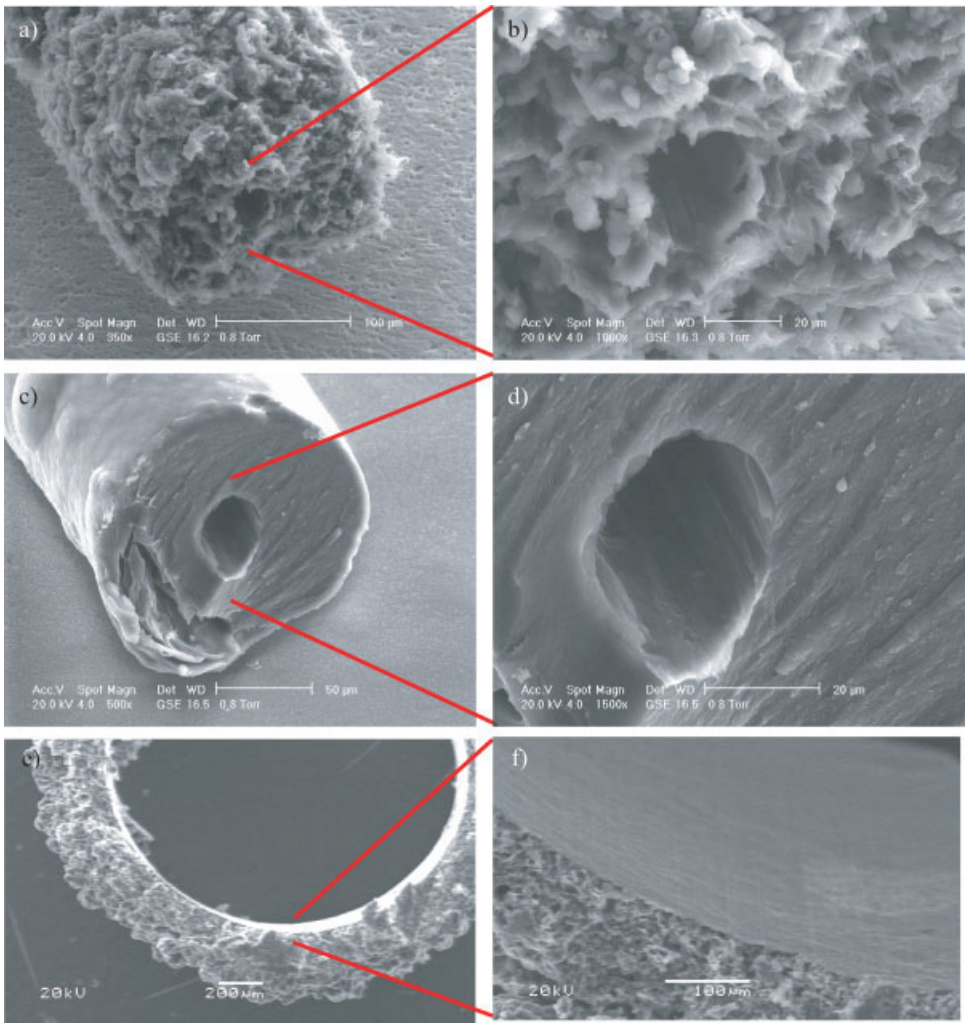

Figure 2. Scanning electron micrographs of transverse sections through the middle of PPy tubes. No wire is left after mechanical removal of the PPy from the wire core. (a) 25 μm inner diameter (i.d.) PPy tube plated at 4°C. Note the rougher texture due to low plating temperatures. (b) Higher magnification of tube lumen. (c) 25 μm i.d. PPy tube plated at 24°C. (d) Higher magnification of tube lumen. (e) 1.6-mm PPy tube plated at 4°C. (e) Higher magnification of tube lumen, showing the smooth lumen compared to textured outer surface. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

connected solely by the PPy tube. The tubes were replated in the pyrrole solution for an additional 5% of the original plating time, applying the same current as used in tube fabrication, to form an electrical connection to the copper wire. Impedances were measured at frequencies ranging from DC to 1 MHz (Solartron, 1255B Frequency Response Analyzer coupled with a 1287 Electrochemical Interface).

Tube implantation

Animal protocols were approved by the Massachusetts Institute of Technology Committee on Animal Care, in conformity with NIH publication No. 85-23, revised 1985. Male Sprague Dawley rats (350–400 g, Charles River) were given buprenorphine 0.1 mg/kg intramuscularly before anesthesia and then anesthetized with 2% isoflurane in balance oxygen. Under sterile conditions with external body warming, a 3-cm incision was made on the right thigh. The sciatic nerve was isolated by retracting the gluteus maximus muscle. Excision of 1 cm of the sciatic nerve was performed proximal to the tibial and peroneal bifurcation. The two ends of the nerve were inserted 2.5 mm into the PPy tube (15 mm in length, 1.6 mm inner diameter) and secured with 9-0 silk suture, (Fine Science Tools),leaving a 10-mm gap between transected nerve ends. Muscle layers were closed with 4-0 silk sutures (Fine Science Tools), and the skin secured with Michel clips. At 4 or 8 weeks after surgery (n = 5 each), rats were euthanized with carbon dioxide gas. Sections of nerve were taken 2 mm proximal to the nerve guide, in the middle of the tube, and 2 mm distal to the nerve guide.

Histology and electron microscopy

H&E stained sections were prepared from formalin-fixed samples using standard techniques. For TEM, samples were fixed for 24 h at 4°C in Karnovsky's KII Solution [2.5% glutaraldehyde, 2.0% paraformaldehyde (Electron

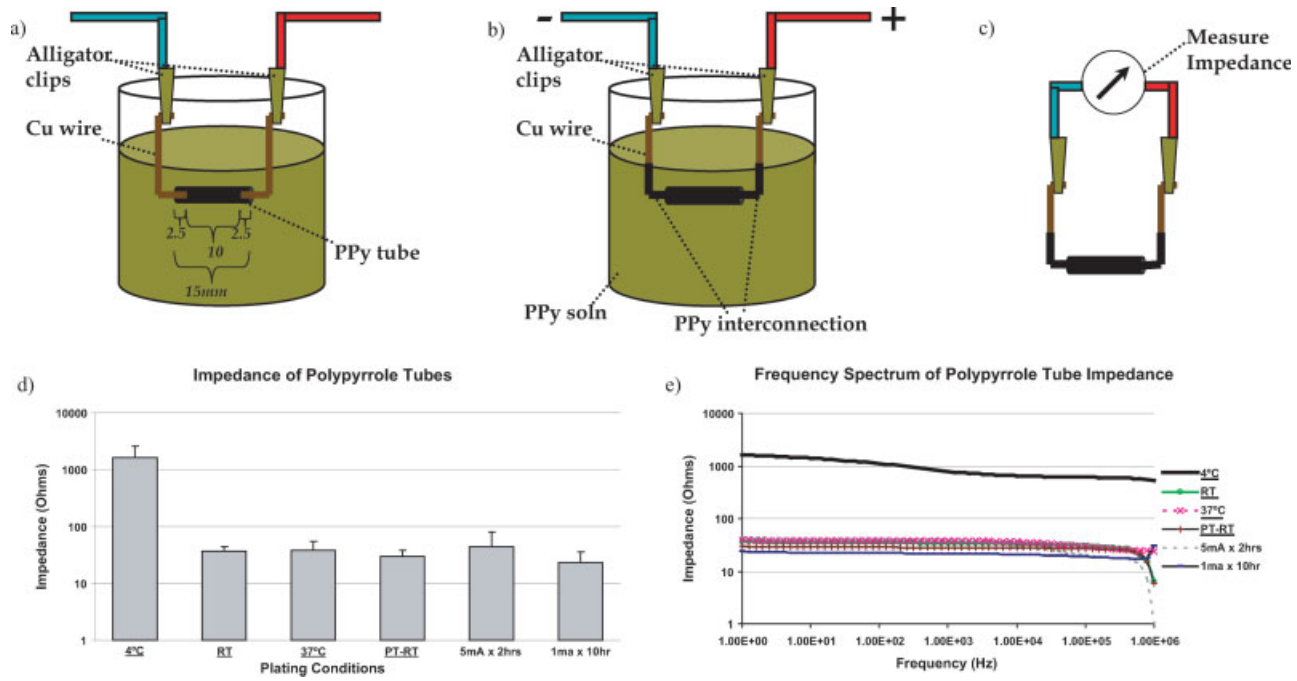

Figure 3. Impedance measurements of PPy tubes. (a) After tube fabrication, pieces of copper wire were placed 2.5 mm into both sides of PPy tube leaving a gap of 10 mm connected by the tube. (b) PPy is electrodeposited to form an electrical connection between the copper wire and the PPy tube. (c) Impedances are measured across the PPy tube. (d) Mean impedance of tubes at 1 Hz (with standard deviations). The impedance of the 4°C group was statistically significantly different from the impedances of all other groups. Underlined groups were plated at a current of 10 mA for 1 h. Groups plated at 4°C, RT (room temperature), and 37°C were plated at the indicated temperature on 1.6-mm copper wire. The PT-RT group was plated on 1.6-mm platinum wires at RT. The 5 mA × 5 h and 10 mA × 5 h groups were plated at the indicated currents and times on 1.6-mm copper wires at RT. (e) Impedance spectrum of PPy tubes. The PPy tubes act primarily resistive up to 1 MHz. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Microscopy Sciences), 0.025% calcium chloride in a 0.1*M* sodium cacodylate buffer (Aldrich) pH 7.4]. Samples were postfixed in osmium tetroxide, stained with 2% uranyl acetate, then dehydrated in graded ethanol solutions and infiltrated with propylene oxide/Epon mixtures. Subsequently, micrometer thick sections were stained with toluidine blue. Representative areas were selected for electron microscopy, and thin sections (0.025 μm) were cut with an LKB 8801 ultramicrotome and stained with Sato's lead. Sections were examined with a Phillips 301 (Eindhoven, Netherlands) transmission electron microscope.

Statistics

Statistics were all performed using a one-way ANOVA with confidence level set to 95% ($\alpha = 0.05$), followed by Bonferroni *post-hoc* analysis. All statistical analyses were done with Excel (Microsoft, Redmond, WA), using the Analyse-It (Analyse-it Software, Leeds, UK) statistical package add-in. Significant differences are shown in the figures.

RESULTS

Production of PPy tubes

The properties of PPy and its dopants allowed the formation of stand-alone, conductive polymeric tubes without an attached template. PPy tubes were formed on metallic wire electrodes as described above. Ensuing reduction at −10 V for 2 min allowed for mechanical dissociation of PPy tubes from the wire mold. The resulting stand-alone PPy tube had an inner diameter equal to the diameter of the wire (Fig. 1). Tubes were fabricated with a range of inner diameters from 25 μm [Fig. 2(a–d)] to 1.6 mm. In the 1.6-mm group, wall thickness averaged 193 μm over all experimental conditions. Total charge deposition was held constant at 36 C, giving a mean wall thickness per charge deposition of 5.4 μm/C. Mean wall thickness ranged from 179 μm for those plated at 37°C to 211 μm for 4°C, but there was no significant difference in thickness between any of the experimental conditions ($n = 4$ per group, $p = 0.76$, ANOVA with Bonferroni *post-hoc* analysis, Supp. Info. Fig. 1). Textured outer tube surfaces were produced by plating tubes at lower temperatures (4°C), which had been described previously for planar surfaces (Fig. 2).[14] Additionally, the tubes fabricated at 4°C had the greatest variability in wall thickness (coefficient of variation = 28%, $n = 4$).

All of the plating conditions used the same charge density and produced tubes with similar impedances except for the tubes plated at 4°C which had a sig-

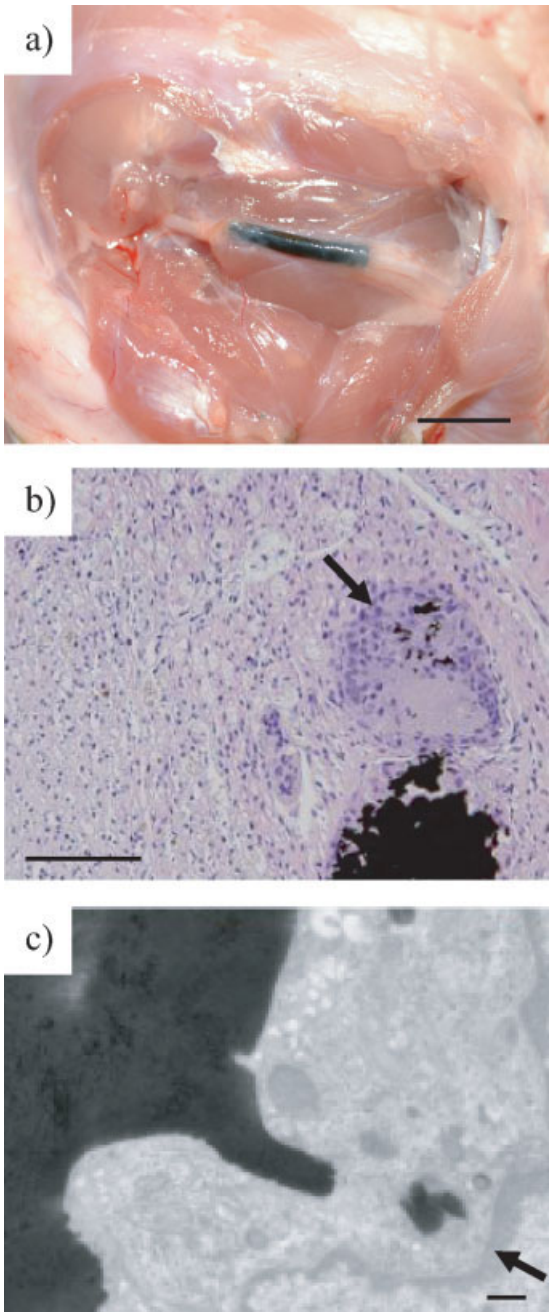

Figure 4. *In vivo* response to PPy tubes. (a) Photograph of 1.6-mm diameter PPy tube joining rat sciatic nerve 8 weeks after implantation. Scale bar: 10 mm. (b) Hematoxylin and eosin stained sections of graft material and nerve at ×400. Scale bar: 100 μm (c) Electron micrographs of osmicated sections of nerve and graft at ×15,000. Scale bar: 0.5 μm. Arrows indicate polymer fragments (black debris) in foreign body giant cell. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

nificantly higher impedance when compared to each of the other groups ($n = 4$ per group, $p < 0.001$, ANOVA with Bonferroni *post-hoc* analysis) (Fig. 3). The different electrodeposition currents did not change the impedance, and there was no statistical difference in impedance between tubes made with a copper or a platinum template. The PPy tubes' impedances were dominated by resistive over reactive components at frequencies less than 1 MHz (Fig. 3).

Sciatic nerve guides

To ascertain the PPy tubes' structural integrity, biocompatibility, and ability to support nerve growth *in vivo*, they were sutured into transected sciatic nerves of Sprague Dawley rats. At both 4 and 8 weeks, the tubes were grossly intact [Fig. 4(a)]. However, on microscopy, focal disruptions were noted in some grafts, through which the cellular reaction was able to enter the lumen, and fragments of the graft material were seen in some phagocytic cells [Fig. 4(b,c)].

Nerves proximal to the transection [Fig. 5(a–c)] appeared uninjured, with myelinated axons, scattered Schwann cell nuclei, and small blood vessels [Fig. 5(a)]. Axons with thick myelin rings, better visualized with osmium tetroxide/toluidine blue staining, were evenly distributed in the nerve, with thicker myelin on larger nerve fibers [Fig. 5(b), 4 weeks; Fig. 5(c), 8 weeks].

The nerves within the grafts [Fig. 5(d–f)] were hypercellular, with focal hemosiderin deposition and a foreign body giant cell (FBGC) reaction present along with histiocytes and fibroblasts. The hypercellularity appeared to be a result of Schwann cell proliferation, as indicated by the presence of small ensheathed axons visible on both light and electron microscopy (Fig. 6). A larger proportion of the fibers appear to be myelinated after 8 weeks, and the myelin sheaths surrounding the fibers were thicker compared to 4 weeks.

Nerves distal to the graft sites were hypercellular but showed no FBGC reaction or hemosiderin deposition. As within the grafts, many of the cells contributing to the hypercellularity were Schwann cells, as demonstrated by their ensheathment of regenerating axons. Four weeks postoperatively, many ensheathed axons were seen, with rare scattered thinly myelinated fibers. At 8 weeks [Fig. 5(g–i)], many myelinated nerve fibers were seen, showing further regeneration and remyelination over this time period.

Electron microscopy performed on tissue inside the grafts at 4 weeks [Fig. 6(a,b)] and 8 weeks [Fig. 6(c,d)] postoperatively clearly identified axons within the ensheathing Schwann cells. At 4 weeks, there were many large axons that were ensheathed but were not yet myelinated. By 8 weeks, most of these displayed some degree of myelination, showing progressive axonal regeneration and remyelination.

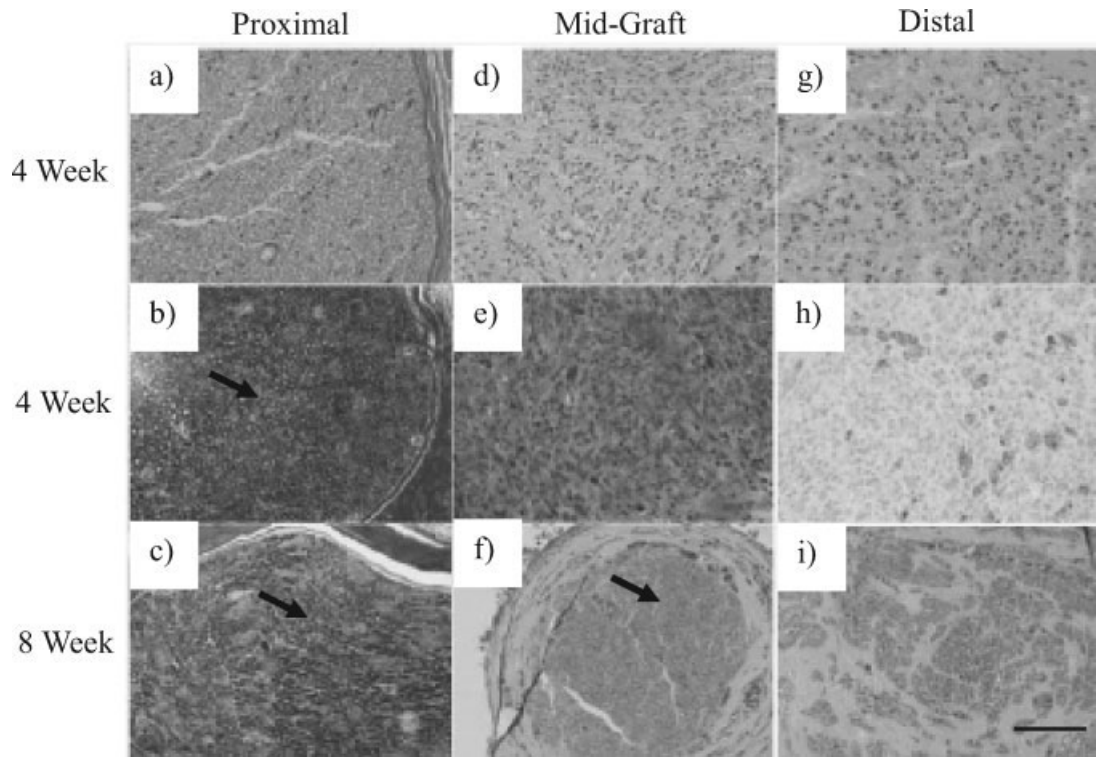

Figure 5. Light microscopy of engrafted nerves at 4 and 8 weeks, postoperatively. Hematoxylin and eosin (a, d, g) and toluidine blue (b, c, e, f, h, i) stained sections of nerves 4 and 8 weeks following graft placement. Arrows point to examples of myelinated axons. Sections were taken proximal to the graft and lesion site, within the graft, and distal to the graft. Scale bar: 100 μm.

Multichannel nerve guides

Although these *in vivo* results appear promising, the ideal dimensions and structure of a conductive nerve guide are unknown. Additionally, other applications may require more finely detailed geometries. To address this and demonstrate the flexibility of the tube fabrication technique, multichannel arrays were formed by spacing multiple wires in a support base [Fig. 7(a)]. PPy electrodeposited onto each of the individual wires grew radially. Once the polymer growing from adjacent wires came into contact, the outermost polymer surfaces continued to grow, while the PPy surface no longer exposed to the solution did not [Fig. 7(b)]. The number of channels and the array dimensions could be controlled by the number of wires and the shape of the template [Fig. 7(c,d)].

DISCUSSION

This wire-plating method precisely controlled PPy tube dimensions and properties, allowing the fabrication of complex 3D geometries such as multichannel tubes. Importantly, due to the electrochemical reduction of the PPy, the tubes were easily detachable from the electrodes on which they were produced, thus obviating the need for a nonconductive template. Electrolysis likely contributed to separation of the tube from template, as bubbling was seen during reduction at −10 V. However, the tubes maintained both mechanical stability and electrical conductivity, as demonstrated above. As expected, the constant charge deposition (36 C) at which all tubes were plated led to no significant difference in wall thickness between tubes of different plating conditions. The loose matrix structure of the tubes plated at 4°C, as seen in SEM images, contributed to the higher variability in wall thickness compared to the other groups.

These 4°C tubes were less conductive than the smoother tubes plated at 24 and 37°C. The less compact matrix structure at lower temperature may lead to a more tortuous electron path and higher impedance. Because of the lower impedance of the tubes produced at 24°C, these tubes were used in the *in vivo* experiments. The inherent rigidity of noncomposite PPy tubes led to cracking when large diameter sutures were used; this problem was eliminated with the use of 9-0 sutures.

Tissue response to PPy *in vivo* consisted primarily of a FBGC reaction, qualitatively similar to what is seen with implantation of biodegradable suture materials and polymeric microspheres or inert substances such as tetrahedral amorphous carbon.[15,16]

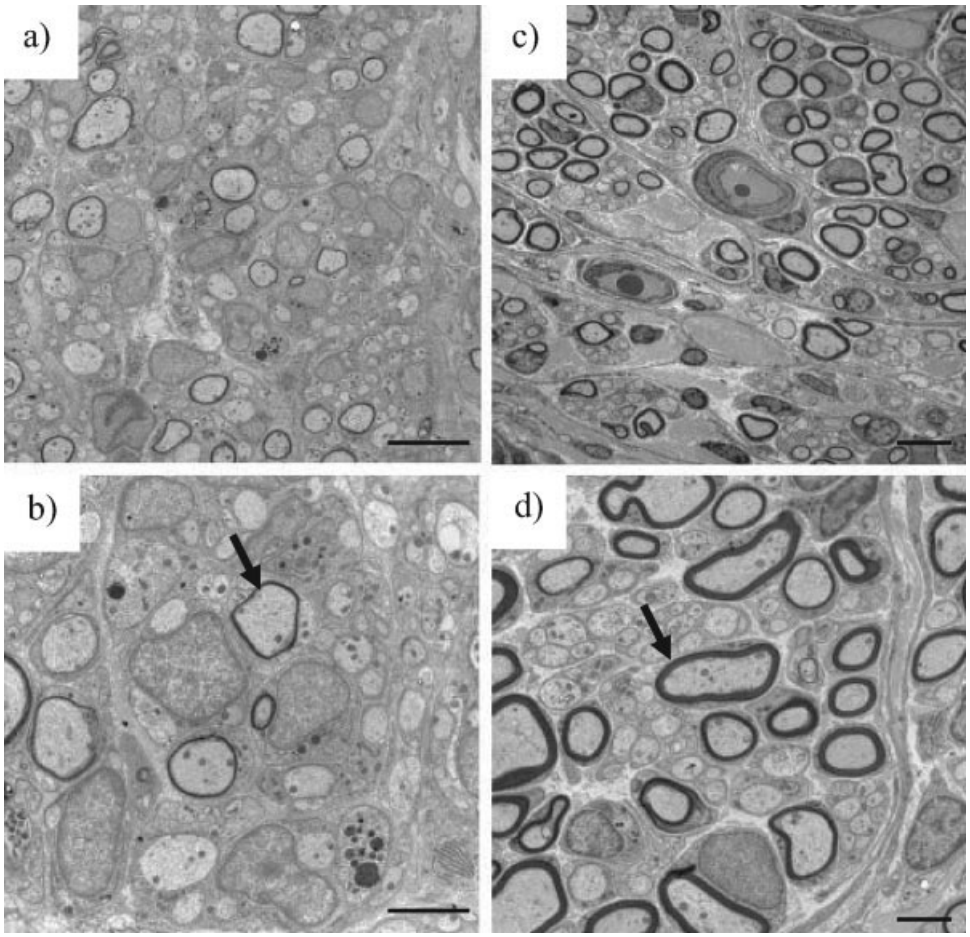

Figure 6. Electron micrographs taken within the nerve graft postoperatively, at 4 weeks (a: ×1900, b: ×4500, scale bar: 10 μm), and 8 weeks (c: ×1900, d: ×4500, scale bar: 2 μm). Arrows point to myelinated axons. Note the increase in numbers of myelinated axons at 8 weeks.

No acute or active chronic inflammatory infiltrate, or tissue damage in the surrounding tissues were present. Conduits made of this material were not only biocompatible *in vivo*, but supported nerve regeneration. Light and electron microscopic evaluation of lesioned and engrafted nerves showed nerve regeneration into and through the graft. There was histologic evidence of axonal regeneration and remyelination 4 weeks following graft placement, with many thinly myelinated fibers seen within the graft and many large unmyelinated fibers seen distal to the graft. Nerves taken at 8 weeks postoperatively displayed an increased proportion of myelinated fibers within and distal to the graft, indicating that remyelination of regenerating axons had taken place during this period. In addition, the fibers were more thickly myelinated than axons of similar caliber at 4 weeks, indicating a progression of myelination to a more normal myelin thickness. These findings demonstrate axonal regeneration and remyelination into and through the graft following transection injury.

Arrays of wires can be used to fabricate more complex nerve guides with smaller channels which provide more axonal contact guidance and could potentially improve nerve regeneration. PPy nerve guides could be further optimized by using the erodable form of PPy which would biodegrade after nerve regeneration was completed.[7] PPy guides could also be used for controlled release of neurotrophic factors, e.g. nerve growth factor (NGF), to encourage nerve regeneration.[17,18] PPy's conductive properties could be utilized to create electric fields across the nerve which have been shown to increase nerve regeneration rates and are believed to play a role in growth cone guidance for growing axons.[3,19,20] The ability to further manipulate PPy characteristics, such as conductivity, erodibility, and the ability to integrate bioactive compounds, may allow for optimization of nerve regeneration.

CONCLUSION

Stand-alone, conductive PPy tubes can be produced by electrodeposition of PPy onto wire tem-

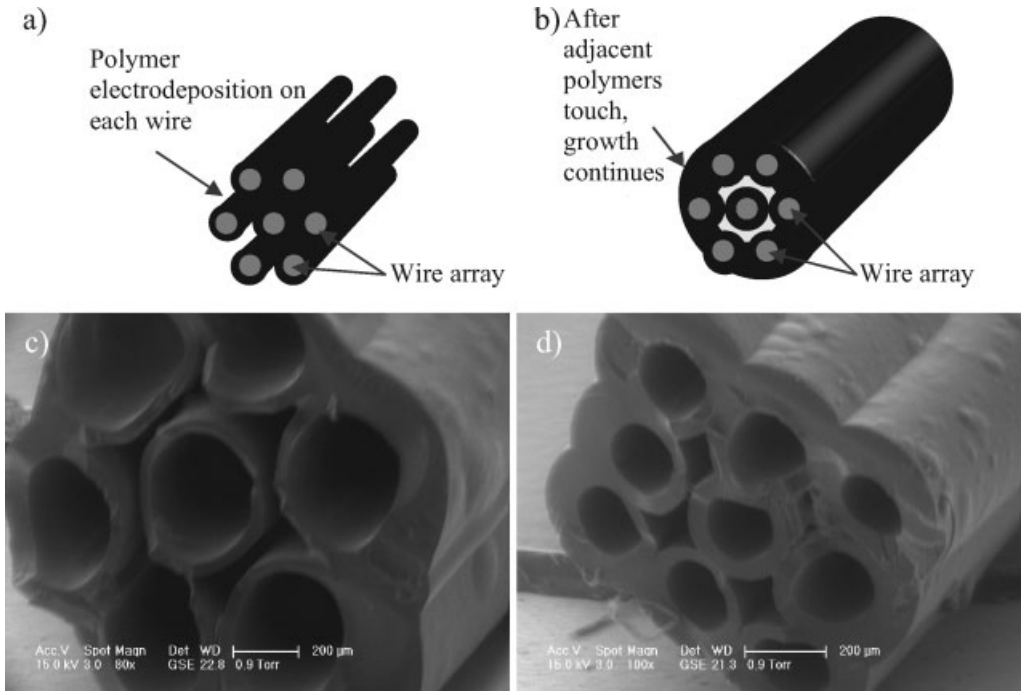

Figure 7. Fabrication of PPy multichannel arrays. (a) Schematic of multichannel tube on wire mold. Initially polymer grows on each individual wire. (b) Adjacent polymers begin to join and electrodeposition continues on the outside surface which is exposed to the solution, forming a single multichannel tube. Scanning electron microscope images of cross-sections of (c) a circular multichannel array made from a 7-wire template with each channel having a 350-μm inner diameter and (d) a square array made from a 9-wire template forming a multichannel tube with each channel having a 200-μm inner diameter.

plates and subsequent separation from the template after electrochemical reduction. Conductive polymeric nerve guides formed in this manner are biocompatible and support nerve regeneration for at least 8 weeks *in vivo*. More complex structures, such as multichannel tubes, can be fabricated through control of the plating template, allowing for future fine tuning of the structure of the conductive nerve guides.

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