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Fabrication and biocompatibility of polypyrrole implants suitable for neural prosthetics

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Abstract

Finding a conductive substrate that promotes neural interactions is an essential step for advancing neural interfaces. The biocompatibility and conductive properties of polypyrrole (PPy) make it an attractive substrate for neural scaffolds, electrodes, and devices. Stand-alone polymer implants also provide the additional advantages of flexibility and biodegradability. To examine PPy biocompatibility, dissociated primary cerebral cortical cells were cultured on PPy samples that had been doped with polystyrene-sulfonate (PSS) or sodium dodecylbenzenesulfonate (NaDBS). Various conditions were used for electrodeposition to produce different surface properties. Neural networks grew on all of the PPy surfaces. PPy implants, consisting of the same dopants and conditions, were surgically implanted in the cerebral cortex of the rat. The results were compared to stab wounds and Teflon implants of the same size. Quantification of the intensity and extent of gliosis at 3- and 6-week time points demonstrated that all versions of PPy were at least as biocompatible as Teflon and in fact performed better in most cases. In all of the PPy implant cases, neurons and glial cells enveloped the implant. In several cases, neural tissue was present in the lumen of the implants, allowing contact of the brain parenchyma through the implants.
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1. Introduction

As neurodegenerative diseases become a more pressing concern in society, the need for effective treatment methods increases. Therapeutic possibilities range from electrical interactions with the damaged neuronal circuits to the use of stem cells to replace injured tissue [1–3]. One challenge is finding materials that effectively interact with neural tissue for these applications. The stability and biocompatibility of different polymers have been studied by examining their effect on the surrounding tissue after implantation [4–8]. A unique subset of these materials, conducting polymers, has been investigated for use in biomedical applications [9–11]. Polypyrrole (PPy) has emerged as a promising candidate material that has been effective as a coating in both in vitro and in vivo neural studies [12–14]. PPy also has shown promise as a scaffold material for nerve regeneration [15].

PPy is an electrodeposited polymer that can be doped with various agents to alter its physical, chemical and

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electrical properties [16–19]. The ability to control PPy's surface properties such as wettability and charge density creates the potential for modifying neural interactions with the polymer [20]. Two of the most common dopants that are co-deposited with PPy are polystyrene-sulfonate (PSS) or sodium dodecylbenzenesulfonate (NaDBS). PSS/PPy and NaDBS/PPy polymers have been used in many applications ranging from actuators to neural electrode coatings to neural substrates [12,18,21]. Another strength of PPy is that erodible forms have been developed which increase the scope of biomedical applications including polymeric devices and neural scaffolds [10,12]. The ease of deposition and the ability to control growth in both the horizontal and vertical dimensions [22] enables flexibility in the three-dimensional design of polymer implants.

The following in vitro and in vivo studies show the ability of PPy to interact with neural tissue from the mammalian cerebral cortex. The biocompatibility of the PPy implants is compared to stab wounds (where an implant-sized incision is made with no implant left behind) and Teflon implants with similar size and features, and these results demonstrate the positive surface interactions at the PPy implant-cortical interface.

2. Methods

2.1. Template design

Our research presents a novel method for fabricating stand-alone PPy neural implants. The implants were designed with several apertures to permit potential neural growth through the implant windows. AutoCad software was used to create the designs for the PPy templates used in electrodeposition. These files were converted to DXF format to fabricate chrome-on-glass masks (International Phototool Company). Two designs were produced, one for the dissociated primary cerebral cortical cell studies and one for the in vivo implants. The following steps were the same for both designs. A 3000 Å silicon nitride layer was grown using low-pressure chemical vapor deposition (LPCVD, MRL Industries Model 718 System) on 4-inch (10 cm) silicon wafers (Silicon Quest International) to provide insulation. Standard lift-off was used to pattern the gold template from the mask onto the wafer. In short, photoresist (OCG 825 35 CS G-line photoresist) was patterned on the wafer followed by e-beam deposition of 200 Å of titanium for adhesion and 3000 Å gold. Removal of the photoresist left the patterned gold that was deposited directly on the insulating silicon nitride. The wafers were then diced using a flood-cooled die saw (Disco DAD-2H/6 T) with a layer of photoresist to protect the gold. The wafers were cleaned after dicing using acetone, ethanol, and water.

2.2. Electrodeposition

After the templates were cleaned, various forms of PPy (Aldrich Chemicals) were electrodeposited onto the gold surface using a constant-current power supply (HP 6614C). A current density of 1 mA/cm^2 was applied between the gold template and a platinum wire mesh reference electrode. The electrodeposition chamber was perfused with N_2 5 min prior to the start of deposition as well as throughout the electrodeposition process. By varying dopant composition and electroplating temperature, four types of culture substrates were made for in vitro studies, and five types of implants were made for in vivo studies. Electrodeposition solutions were aqueous solutions of 0.2 M PPy plus 0.2 M PSS (Aldrich), and 0.2 M PPy plus 0.2 M NaDBS (Aldrich). We attempted to control surface textures by varying the temperature during electrodeposition: 4 °C was intended to create a more macroscopic/coarse surface, while 25 °C was intended to create a fine-textured surface. Finally, a fifth formulation, 0.2 M PPy plus 0.2 M PSS in PBS, was electrodeposited at 25 °C to create the fifth type of implant to evaluate solvent conditions on the electrodeposition product (Table 1).

The PPy remained on the silicon die for the in vitro experiments, but for the in vivo work, the implants were released using a variety of methods depending on the PPy dopant. The PSS/PPy implants of both temperatures could be removed from the gold template by a gentle mechanical force. The removal of the NaDBS/PPy implants required chemical etching. The silicon nitride was etched by a 6:100 mixture of fluoroboric acid:phoshoric acid at 105 °C (US patent number 3,859,222) for 12 h. Upon removal of the silicon nitrogen layer (color change from purple of silicon nitride to gray of silicon), the PPy implants and the template die were placed in KOH. After approximately 1 h the PPy implants would float off of the template or could be removed by a gentle mechanical force. After removal from the template, the implants were separated by a razor into individual implants and soaked in 4 separate baths of filtered deionized water for 1 h each.

Table 1
Implants for in vivo experiments

Implant type	3 weeks	6 weeks
Stab	4	4
Teflon	4	4
4 °C PSS/PPy	4	4
24 °C PSS/PPy	4	2
4 °C NaDBS/PPy	4	
24 °C NaDBS/PPy	4	2
24 °C PSS/PPy in PBS		4

2.3. In vitro experiments

2.3.1. Cell harvesting

All animal procedures were performed in accordance with protocols approved by the MIT Committee on Animal Care and conformed to NIH guidelines. All reagents are from Sigma-Aldrich (unless noted otherwise). Dissociated cortical neurons were placed on 1×2.5 cm squares of the various types of PPy with four samples of each substrate type being tested. Brains were removed from 1–3 day old Sprague–Dawley rat pups (Charles River). The cerebral cortices were dissected out in 80% Ca/Mg-free Hank's balanced salt solution (HBSS) containing $NaHCO_3$, (4 mM), HEPES (5 mM) and 20% fetal bovine serum (FBS). The meninges were removed, and the cortex was cut into millimeter sections with a scalpel. The cortex was washed 3 times in HBSS and incubated for 5 min at 37 °C in digestion medium (trypsin type XI (5 mg/ml), DNase type IV (0.5 mg/ml), 137 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_4, and 25 mM HEPES, pH 7.2). Trypsin was neutralized with FBS. The cells were chemically dissociated in 12 mM $MgSO_4 \cdot 7H_2O$ in HBSS containing DNase type IV (0.5 mg/ml) and then physically dissociated by triturating through 2 glass pipettes of decreasing size. Cells were harvested by centrifugation (1000 rpm, 4 °C, 10 min); plated onto the various PPy substrates in plating medium (90% of 28 mM Glucose 2.5 mM $NaHCO_3$, 1 mg/10 ml of transferrin (Calbiochem), 30 mM glutamine, 0.73 mM HCl, and 2.5 mg/100 ml in Minimum Essential Medium with Earle's salts, without L-glutamine or phenol red (MEM) (Gibco) and 10% FBS); and, cultured at 37 °C with 5% CO_2. The PPy culture substrates were sterilized using ultraviolet light exposure for 12 h prior to the addition of cells. After 24 h, plating medium was replaced with feeding medium (28 mM Glucose, 2.5 mM $NaHCO_3$, 1 mg/10 ml of transferrin (Calbiochem), 30 mM glutamine, 1 ml/100 ml B27 50x supplement (Gibco), and 0.84 mM cytosine arabinoside in MEM). Cultures were fed weekly. After 21 days in vitro, culture were fixed and prepared for immunofluorescence.

2.3.2. Immunofluorescence

The cells were fixed in 4% paraformaldehyde in PBS for 20 min; washed 3 times in PBS; permeabilized in 0.2% Triton X in PBS; and, then the cells were again rinsed in PBS. A blocking solution of 5% goat serum (Vector Labs) in PBS was applied for 1 h. The primary antibodies (guinea pig anti-vesicular glutamate transporter 1 (VGLUT1) 1:500 (Chemicon); mouse monocolonal anti-glial fibrillary acidic protein (GFAP) 1:100 (Sigma); and, rabbit anti-neuronal class β3-tubulin 1:250 (Covance)) were diluted in PBS with 5% goat serum and left at 4 °C overnight. The cells were rinsed three times for 5 min each in PBS and the secondary antibodies (Alexafluor 647 goat anti-mouse IgG 1:500; Alexafluor 546 goat anti-guinea pig IgG 1:400; and, Alexafluor 488 goat anti-rabbit IgG 1:500 (all Molecular Probes)) were diluted in PBS with 5% goat serum, and applied for 30 min. DAPI was added to label cell nuclei. After thorough rinsing, the samples were mounted on slides for imaging.

2.4. In vivo experiments

2.4.1. Surgical implantation

We fabricated specially shaped PPy implants with dimensions of approximately $2 \times 3 \times 0.25$ mm (sufficiently long to span the cerebral cortex and sufficiently thick to be manipulated during surgery) using the same dopants from the in vitro studies and surgically implanted them into rat cerebral cortex (Fig. 1). Implants also bore three, 500 μm diameter apertures to permit neural tissue to cross the lesion through the implant. Each side of the cerebral cortex received one implant. One of the implants was fabricated to determine if the monomer solvent (H_2O or PBS) had any affect on the implant properties. Teflon (McMasterCarr) implants with this same design (including the apertures) were also implanted into the rat cerebral cortex. Finally, stab wounds were performed as a further control in lieu of inserting an implant.

All procedures were performed on 150 g male Sprague–Dawley rats (Charles River) with sterile technique under a surgical microscope. Implants were sterilized with ethylene oxide. Anesthesia was induced with 3% isoflurane with oxygen (1l/min) for 10 min and maintained with 1% isoflurane with oxygen for the duration of the procedure (20 min). The anterior scalp was incised along the midline. A 1.5-mm diameter craniotomy was made 2 mm posterior to bregma and 4 mm from the midline on each side. Implants were inserted with fine forceps into the cerebral cortex. The scalp was closed with Ethilon suture. Once fully alert, rats were returned to home cages. Buprenex (0.1 mg/kg, i.m.) was given for analgesia 2 times daily for 2 days post-implantation.

2.4.1.1. Immunofluorescence. At the 3- or 6-week timepoints, rats were terminally anesthetized and perfused with PBS and 4% paraformaldehyde. Brains were dissected from the skull, postfixed in 4% paraformaldehyde overnight at 4 °C, and then transferred to (30%) sucrose in PBS until they sunk. Brains were frozen with dry ice and sectioned at 50 μm.

Sections were processed free-floating for immunofluorescence. Blocking and antibody incubation steps were performed in 0.5% triton X-100 in PBS with 5% donkey serum. The sections were incubated overnight in the primary antibody (rabbit anti-GFAP 1:100, mouse monoclonal anti-microtubule associated protein

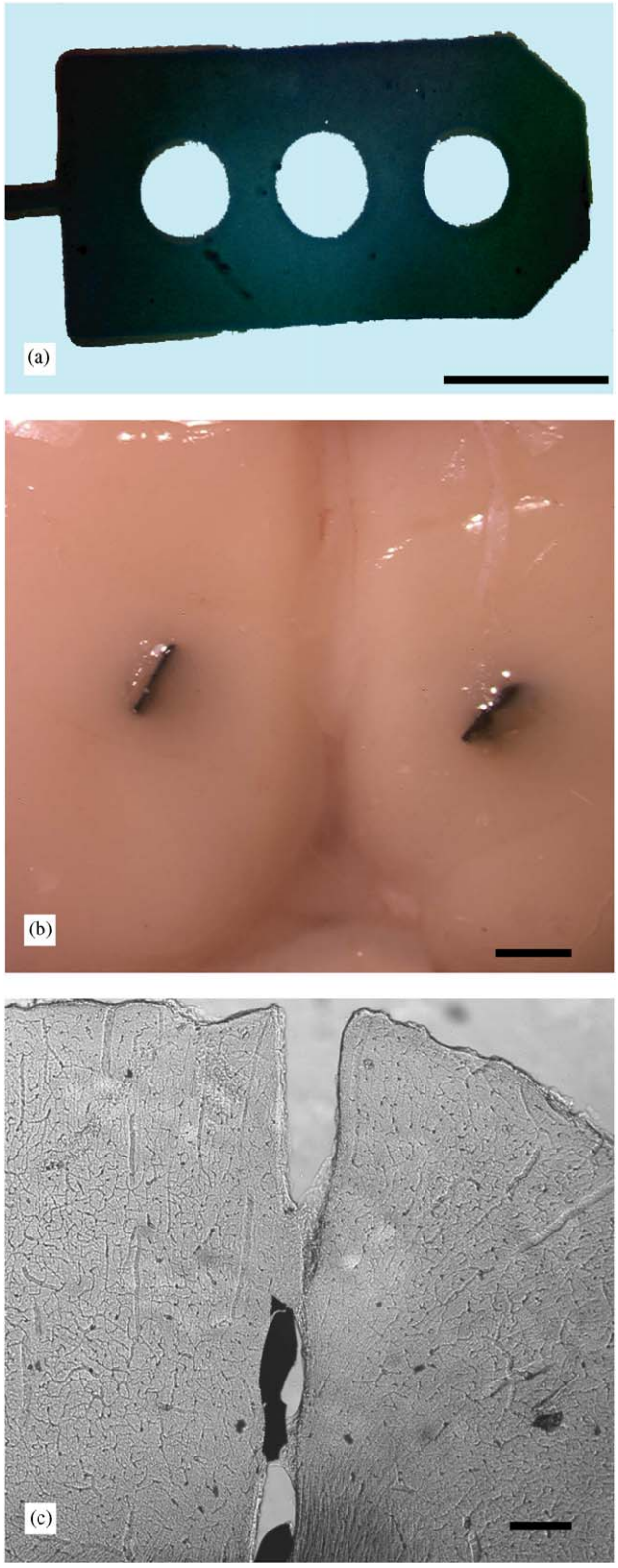

Fig. 1. PPy implants (a) an example of a typical PPy implant. Scale bar: 1 mm (b) Two PPy implants placed in the rat's cortex, scale bar: 2 mm (c) a histological slice at 6 weeks post-implantation with the remnants of the PPy implant, scale bar: 200 μm.

2 (MAP2) 1:1500, mouse anti-macrophage 1:1500, mouse anti-laminin 1:1500) solution at 4 °C. After three washes in PBS for 10 min each, the secondary antibodies (Alexafluor 594 donkey anti-rabbit IgG 1:400 and Alexafluor 488 donkey anti-mouse IgG 1:400) were added to the sections. After incubation of 1 h in the secondary solution, the sections were rinsed three times in PBS and mounted on slides for imaging.

3. Results and discussion

3.1. Dissociated cortical neurons

To examine biocompatibility with standard PPy (doped with NaDBS or PSS), in vitro studies were performed with dissociated cortical neurons. Different plating conditions were used to produce differing surface characteristics of the PPy films. We attempted to modify the texture of the PPy surfaces by varying the electrodeposition temperature. The surface texture of PPy/NaDBS electrodeposited at 4 °C appears course and irregular, while at 25 °C, a smoother surface was obtained (Fig. 2a,b). There was a similar but less dramatic effect of temperature on the surface texture of the PPy/PSS samples, but both 4 °C- and 25 °C-samples appeared relatively smooth and were comparable to the PPy/NaDBS-25 °C sample (not shown, see Fig. 2a). Using four-point impedance measurements, the resistivity of the different PPy samples on their gold templates was measured (Table 2). NaDBS/PPy had lower resistivity than the PSS/PPy samples ($p < 0.05$, $n = 4$) at the respective temperatures, possibly indicating greater surface elaboration and/or greater intrinsic conductivity of the PPy polymer doped with NaDBS.

In vitro tests were performed to first study the response of cortical cells to interaction with PPy. Complex neural networks, whose cellular components (glia, axons, dendrites, synapses) were identified by immunofluorescence, formed on both the PSS/PPy and NaDBS/PPy culture substrates at both temperatures. Fig. 2c shows a culture that is representative of what was seen on all four of the PPy culture substrates that we used. Similar neural networks grew on all of the PPy substrate regardless of its surface structure or dopant. Thus, neurons are capable of extending axonal and dendritic processes, and of forming putatively functional synapses, on all four of the PPy substrates examined in vitro. Based upon these positive PPy/neuronal interactions, in vivo studies began.

3.2. Implants

We examined the implants, stab wounds and the surrounding cerebral cortical tissues at 3- and 6-week time points to determine the extent of rejection or

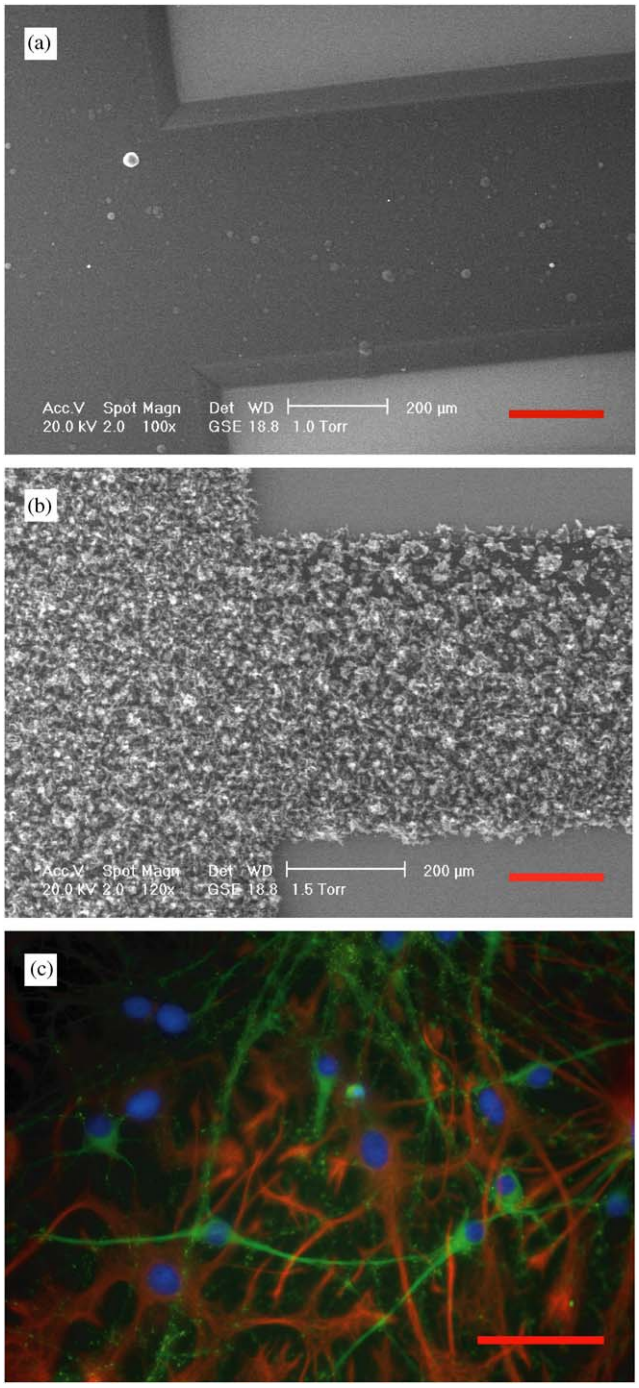

Fig. 2. PPy surfaces. The surface texture of the PPy can be controlled through plating conditions from (a) smooth, PPy/NaDBS plated at 24 °C (b) to rough, PPy/NaDBS plated at 4 °C, scale bars: 200 mm, (c) fluorescently labeled explanted cortical neurons growing and forming networks on a PPy/NaDBS surface after 21 days, scale bar: 50 mm. green: neurons, red: glia, blue: nuclei.

integration between the chips and surrounding neural tissue, as well as the effect of insertion per se (via stab wound). Immunofluorescence of neuronal cell bodies and synapses surrounding the implant site revealed that surrounding cortex tended to envelope the implants.

Table 2
Average resistivity of PPy samples ($n=4$)

Dopant	Temperature (°C)	Resistivity (Ω cm)
NaDBS	4	15.5
NaDBS	25	22.4
PSS	4	22.6
PSS	25	38.6

Fig. 3 shows a typical stab wound, Teflon, and PPy implant site for the 3- and 6-week time points. Staining for macrophages showed the expected increased presence of this cell type around the implant site at the 3-week time point (Fig. 3). At the 6-week time point all of the implants had little macrophage activity, and in most cases, the implant was clear of macrophage activity after 6 weeks. Overall, the neural response to all three types of surgeries appeared qualitatively similar. The neural tissue tended to reform after the stab wounds leaving a scar demarcated by laminin immunofluorescence. Our histological and immunofluorescence studies show that the neural parenchyma completely enveloped the implants and was intimately associated with the surface of the PPy implants, including the implant lumens, demonstrating a very high degree of tolerance of neural tissue for PPy. The neural tissue tended to bridge more completely the PPy lumen than the lumen of the Teflon implants (Fig. 4).

3.3. Quantitative analysis

To quantify differences in the degree of tissue reactions to the various implants, ImageJ software was used to create a single-pixel column region-of-interest (ROI) running parallel to the lesion site. Columns varied in height according to the length of the lesion in a given section. We plotted the average intensities of all ROIs moving perpendicularly away from the lesion site versus distance from the lesion, until the average intensity reached background (Fig. 5a). Gliosis, as measured from ROIs using GFAP-immunofluorescence, was expressed as the peak intensities of the averaged column ROIs and as the slope of the logarithmic regression of the ROI versus distance plots. All intensity values were normalized to a background value that was obtained from an area of tissue positioned away from the implant site.

Fig. 5 shows a comparison of gliosis intensity and slope for the various implants. Stab wounds had the least amount of gliosis and the sharpest decrease in gliosis extending from the implant site in all cases, except for the gliosis gradient of PPy/PSS electrodeposited in PBS at 25 °C at 6 weeks and the peak gliosis of PPy/NaDBS at 25 °C at 6 weeks ($p<0.05$) which were as biocompatible as the stab wounds at these time points.

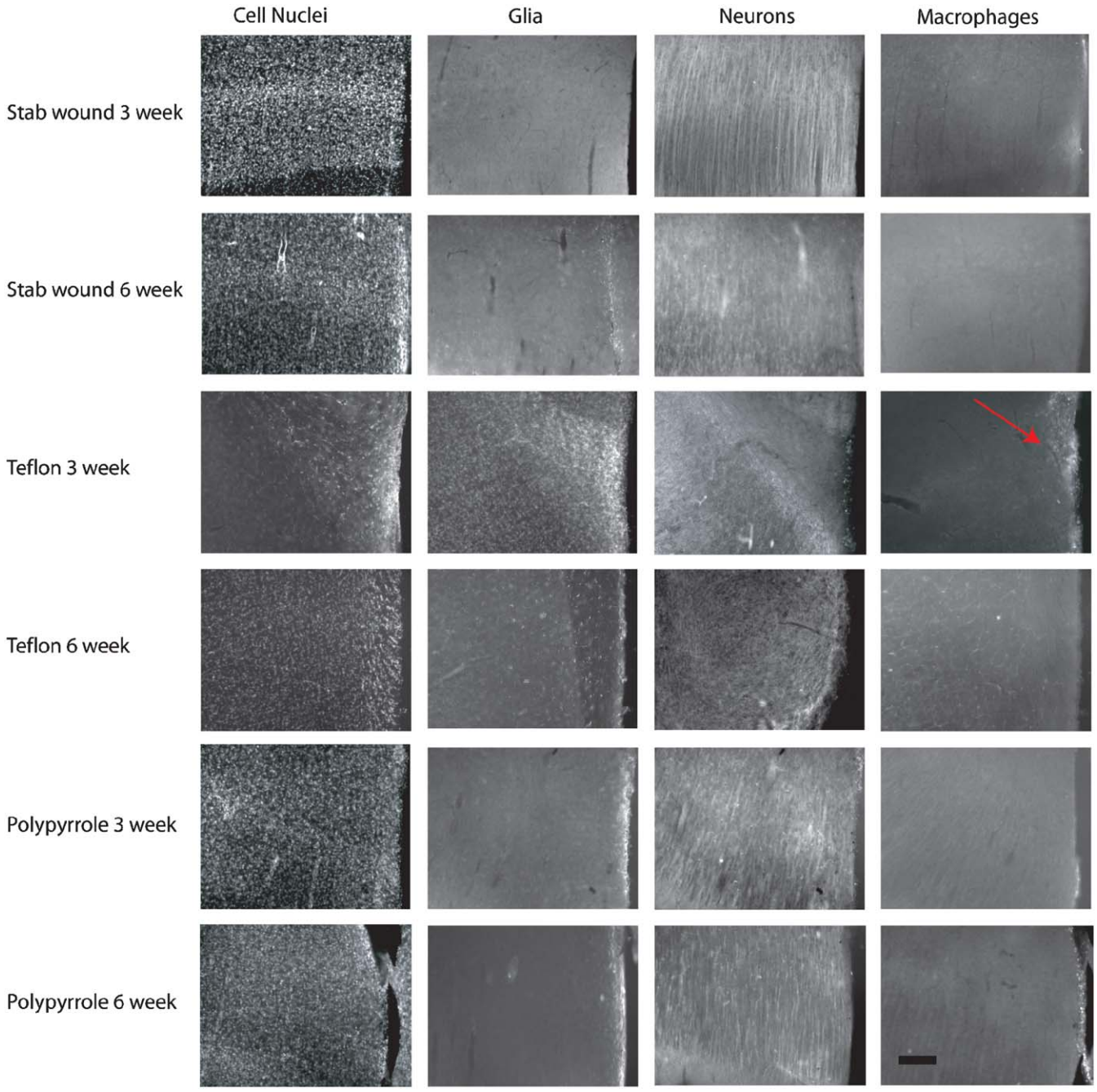

Fig. 3. Representative fluorescently labeled sections of 3- and 6-week time points with the various types of implants. All of the implants are on the right side of the images. The arrow indicates increased macrophage presence in the 3-week Teflon implant, scale bar: 200 μm.

All of the PPy implants at the 3-week time point had less peak gliosis than the 3-week Teflon implant ($p<0.05$). At the 6-week time point the differences had lessened, except for PPy/PSS at 4 °C, which had a sharper decrease in gliosis than Teflon ($p<0.05$), and PPy/NaDBS at 25 °C had less peak gliosis than Teflon ($p<0.05$). Also, PPy/PSS electrodeposited in PBS at 25 °C had less peak gliosis as well as a sharper decrease in gliosis than the Teflon implant ($p<0.05$) at the 6-week time point. The PPy/PSS 6-week sample electrodeposited at 25 °C in PBS has a greater decrease in gliosis than the PPy/PSS and the PPy/NaDBS at 25 °C electrodeposited in deionized water and less peak gliosis than the PPy/PSS sample electrodeposited at 4 °C in deionized water (all $p<0.05$). The PPy/NaDBS sample electrodeposited at 4 °C had less peak gliosis at 3 weeks than the same sample type at 25 °C ($p<0.05$) suggesting that the rougher surface fosters greater implant integration with the surrounding tissue, and less gliosis.

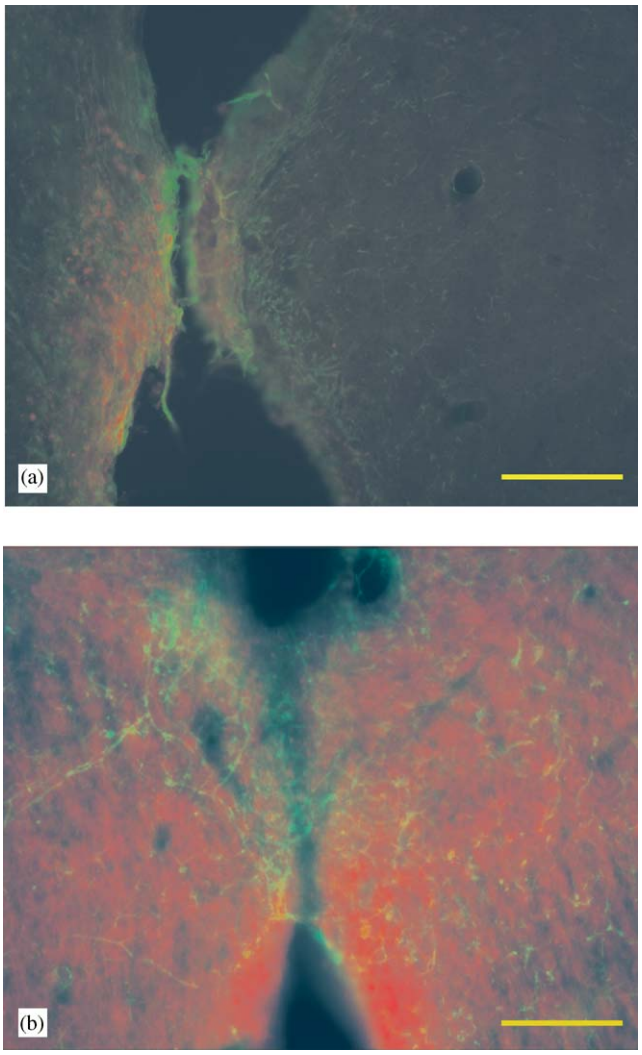

Fig. 4. A fluorescently labeled section of neural tissue in an implant lumen: (a) Neural tissue in the lumen of the Teflon implants, (b) neural tissue in the PPy lumen where the glia has reformed and neurons are present, scale bar: 100 μm, green: glia, red: neurons.

These data taken together tend to indicate that surfaces that are highly inert and relatively unreactive for the host parenchyma (e.g., Teflon) may achieve less physical integration and more inflammatory response (e.g., gliosis) than surfaces (e.g., PPy) that appear to be well tolerated by the host parenchyma and that foster intimate physical interaction between substrate and tissue elements. Because PPy is conductive, bio-electrical circuits could be fabricated that integrate electrical and neural signals. PPy could also serve as a tissue scaffold to support neural cells for placement into areas of neuronal loss in injuries such as stroke and Parkinson's disease [23]. Reducing the amount of gliosis surrounding the implant could enhance the polymer's ability to interact with the normal brain parenchyma.

Pilot studies have been performed to incorporate neurotrophic factors molecules such as nerve growth

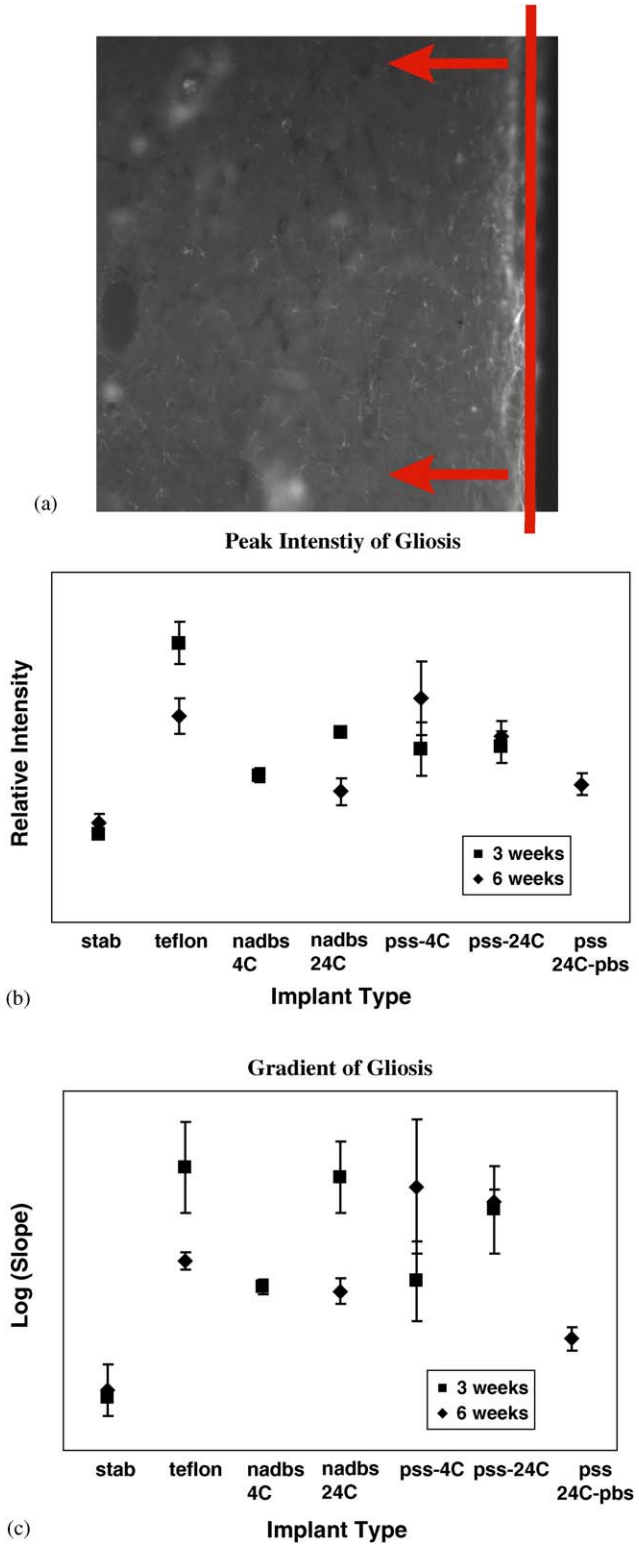

Fig. 5. Quantification of gliosis from implant site: (a) Typical slice with red line indicating a column (width: 1 pixel) where the pixel intensities would be summed to obtain an intensity value for that column. The red line parallels the lesion site and moves away from the implant site (in the direction of the arrows), an intensity value was obtained for each column. (b) Peak gliosis intensity values and (c) a gradient of the intensity values were obtained from these intensity values at 3- and 6-week time points with indicated standard errors.

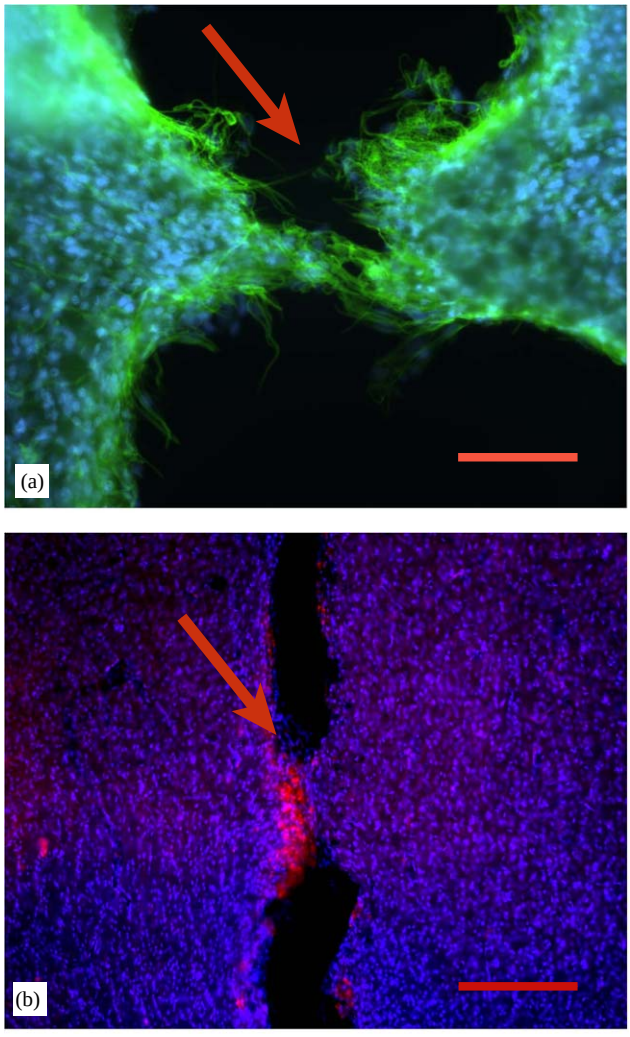

Fig. 6. NGF implants: (a) a stained slice at 6 weeks post-surgery with neurons (blue) and glia (green), scale bar: 100 μm, (b) a fluorescently stained slice at 6 weeks around the implant site showing glial and neuronal cell bodies (blue) with the neurons highlighted (red). No severe gliosis is seen surrounding the implant, scale bar: 200 μm. Arrows indicate region where cells are extending into the PPy lumen and black is the space occupied by PPy implant.

factor (NGF) and brain-derived neurotrophic factor (BDNF) into the polymer matrix (Fig. 6). Compared to Fig. 4, more neural tissue is present in the lumen of the PPy implants containing NGF than those with just PPy. Future studies will elucidate whether the bioactive molecules promote neuronal adhesion and interactions with the PPy implants.

4. Conclusion

Here, we demonstrate the manufacture of three-dimensional, stand alone PPy substrates that can have a progressively positive biocompatibility profile with CNS parenchyma in vivo. These results support future investigations aimed at using PPy in the design and manufacture of neural prosthetics that are capable of integrating with CNS tissues based on specific chemical and physical properties of the PPy polymer. Such prosthetics should enable reliable transmission of external and internal electrical signals for significant postoperative periods. Moreover, they may, if properly formulated, stimulate damaged neural tissues to repair and reconnect.

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