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***In vivo* polymerization of poly(3,4-ethylenedioxythiophene) (PEDOT) in living rat hippocampus does not cause a significant loss of performance in a delayed alternation (DA) task**

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Abstract

After extended implantation times, traditional intracortical neural probes exhibit a foreign body reaction characterized by a reactive glial sheath that has been associated with increased system impedance and signal deterioration. Previously, we have proposed that the local *in vivo* polymerization of an electronically and ionically conducting polymer, poly(3,4 ethylene dioxythiophene) (PEDOT), might help to rebuild charge transport pathways across the glial scar between the device and surrounding parenchyma (Richardson-Burns, Hendricks, & Martin, 2007). The EDOT monomer can be delivered *via* a microcannula/electrode system into the brain tissue of living animals followed by direct electrochemical polymerization, using the electrode itself as a source of oxidative current. In this study we investigated the long-term effect of local *in vivo* PEDOT deposition on hippocampal neural function and histology. Rodent subjects were trained on a hippocampus-dependent task, Delayed Alternation (DA), and implanted with the microcannula/electrode system in the hippocampus. The animals were divided into four groups with different delay times between the initial surgery and the electrochemical polymerization: (1) Control (no polymerization), (2) Immediate (polymerization within 5 minutes of device implantation), (3) Early (polymerization within 3–4 weeks after implantation), and (4) Late (polymerization 7–8 weeks after polymerization). System impedance at 1 kHz was recorded and the tissue reactions were evaluated by immunohistochemistry. We found that under our deposition conditions, PEDOT typically grew at the tip of the electrode, forming a ~500 μm cloud into the tissue. This is much larger than the typical width of the glial scar (~150 μm). After polymerization, the impedance amplitude near the neurologically important frequency of 1 kHz dropped for all the groups, however, there was a time window of 3–4 weeks for optimal decrease in impedance. For all surgery-polymerization time intervals, the polymerization did not cause significant deficits in performance of the DA task, suggesting that hippocampal function was not impaired by PEDOT deposition. However, GFAP+ and ED-1+ cells were also found at the deposition 2 weeks after the polymerization, suggesting potential secondary scarring. Therefore less extensive deposition or milder deposition conditions may be desirable to minimize this scarring while maintaining decreased system impedance.

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1. Introduction

Intracortical neural probes enable the researcher to interface single or a group of neurons in the brain with machines, providing means to record neural activities and stimulate the brain (Ryu & Shenoy, 2009; Taylor, Tillery, & Schwartz, 2003). The method is widely used in applications such as neurophysiology, neural prosthesis and deep brain stimulation (Cogan, 2008; Hatsopoulos & Donoghue, 2009; Schwartz, 2004). However, the long-term functionality and reliability of devices implanted in the central nervous system varies with different animal models (Nicolelis *et al* 2003), electrode designs (Liu *et al* 2006; Vetter *et al* 2004), and experimental protocols (Ludwig *et al* 2006). One of the concerns associated with intracortical implants is the chronic foreign body reaction characterized by an encapsulating layer of glial cells (Turner *et al* 1999) and the associated decrease of neuronal cell density around the implant (Biran *et al* 2005). This glial encapsulation is often associated with an increased system impedance and decreased signal-to-noise ratio for both recording and stimulating devices. Strategies to address this problem include using a two-photon microscope assisted surgery protocol to reduce neurovascular damage (Kozai *et al* 2010), changing device geometry (Seymour & Kipke 2007), coating the probe surface with anti-inflammatory layers (Azemi *et al* 2011; Kim & Martin 2006; Zhong & Bellamkonda 2005), and reducing the probe/tissue mechanical mismatch (Harris *et al.*, 2011; Lind *et al.*, 2010) (Harris, Capadona *et al.*, 2011). There have also been efforts to rejuvenate interface properties by applying voltage pulses (Otto *et al* 2006). However, none of these approaches has yet led to a comprehensive solution to the difficulties encountered by chronically implanted devices.

Previously, we proposed that the *in vivo* polymerization of a conducting polymer, such as poly (3,4-ethylene-dioxythiophene) (PEDOT), could potentially create electronic and ionic conducting pathways through the reactive layer from the metal electrode to the neurons. From *in vitro* neural cell culture tests we found that the cells tolerated the monomer, 3,4-ethylene-dioxythiophene (EDOT) well. At a moderate concentration (0.01M), the cell viability was not negatively affected during 12 hours of exposure to EDOT. Deposited PEDOT polymer was predominately found in the extracellular space (ECS). Removing the cells results in PEDOT negative “cell templates” that preserved the original morphology of the cells (Richardson-Burns *et al* 2007). This finding was further verified by polymerizing PEDOT in rat brain slices that had been incubated with EDOT monomer solution. We found that PEDOT was accommodated into the brain ECS, microscopically assuming a micro-fibrous like morphology. The amount of deposited polymer was directly related to charge applied and thus could be controlled (Richardson-Burns *et al* 2007).

Recently, we developed a method of localized PEDOT polymerization in brain tissue *via* a delivery/micro-electrode system (Ouyang *et al* 2011). The system consists of a microcannula and two electrodes. The EDOT monomer was delivered *via* a microcannula and then polymerized with electric current *in situ*, creating a pathway for charge transport into the tissue. Using fixed rodent brains as a tissue model, we found that PEDOT could grow on the tip of the electrode and into the tissue after 6 μ L of 0.01 M EDOT solution delivery followed by electrochemical deposition. The resultant PEDOT adopted a cloud-like

shape in the tissue. Similar to the previous results, PEDOT was found to wrap around the cells, consistent with deposition through the ECS. *In vivo* animal tests showed that PEDOT deposition decreased the system impedance (Wilks *et al.*, 2011). The decrease was sustained over a period of 4 weeks. As was seen in the *in vitro* model studies, the amount of PEDOT deposition was proportional to the charge delivered.

We have now examined this polymerization method in a rodent model to determine the effects of *in-vivo* deposition of PEDOT on neural function by training rats in the hippocampal-dependent task of delayed alternation (DA) (Ainge, *et al.*, 2007; Hallock, Arreola, *et al.*, 2013) and assessing the retention of the task after the *in vivo* polymerization of PEDOT. One goal here was to establish whether the deposition of PEDOT in hippocampus would significantly disrupt the ability of the rat to perform the DA task, a task that is known to be dependent upon hippocampal function. In order to investigate the effects of *in vivo* deposition on the system impedance under the presence of glial scar, the depositions was performed after a certain time period after cannula/electrode implantation. The impedance of the electrodes after polymerization was monitored and compared with bare electrodes. We also examined the corresponding immune responses of the tissue around the electrode using immunohistology.

2. Methods

2.1. In vivo PEDOT deposition and electrochemical impedance spectroscopy (EIS) measurements

All procedures were carried out in accordance with the University of Delaware Institutional Animal Care and Use Committee. Bilateral cannula-electrodes (Plastics One) (26 gauge cannula between 2 stainless steel wires (~2 mm apart)) were lowered 1.8 mm below dura targeting dorsal hippocampus of all the rodent subjects ($n = 10$). Electrode placements were verified by identifying the placements using atlas plates from Paxinos and Watson (2004). Prior to polymerization, an infusion of 3 μ l of 0.01M EDOT (Sigma) in pH=7.4 phosphate buffered saline (PBS) solution with 0.1% poly (styrene sulfonate) sodium salt (PSS, Mn ~70,000 g/mol, Sigma) at 0.25 μ l/min was infused *via* a cannula. The infusion was immediately followed by galvanostatic deposition at a charge density of 8.4 C/cm² generated by a Gamry Potentiostatic Reference 600™. The counter electrodes were set on the same implant, 2 mm away from the working electrode. Electrochemical impedance spectroscopy (EIS) measurements were determined by a frequency sweep between 1Hz–100 kHz and analyzed.

To evaluate the effects of polymerization at different scarring stages on the system impedance, the rats were divided into four groups: (1) the control group: (working electrode sample size $n_e = 4$), only infusion of EDOT monomer on the 30th day post device implantation, no polymerization was conducted; (2) the immediate group ($n_e = 4$): the polymerization was conducted immediately after device implantation; (3) the early group ($n_e = 6$) consisted of rats that underwent the polymerization within 3 to 4 weeks post device implantation; (4) the late group ($n_e = 6$) consisted of rats that underwent the polymerization between 7 to 8 weeks after initial device implantation (late group). For all the groups, impedance was recorded on the 1st, 2nd, 7th and 14th days post polymerization/EDOT

infusion. The immediate group was not subject to impedance measurement for the first two days post polymerization due to surgery recovery. All the animals were sacrificed two weeks after treatment (table 1 for the details). The brains were harvested for histology.

2.2. Delayed alternation

The effects of polymerization on hippocampal functions were evaluated with the delayed alternation (DA) task. DA task requires the rat to alternate between right and left goal arms of a T-maze on each trial with a delay separating the trials and is known to be dependent on hippocampal function (Hallock *et al.*, 2013). On the first trial of each DA training session, the rat was rewarded for choosing either goal arm on the first trial of the session. For the subsequent trials, the alternate goal arm was baited. If the rats chose an incorrect goal arm then they were not rewarded (Fig. 1, above). The rats completed 12 trials per session with a 30 second delay period between each trial. Once the rats hit a performance criterion of 3 consecutive days of 75% correct or more they underwent surgery to implant the cannula/electrode device. After recovery from the surgery, rats were trained until performance levels again reached criterion and were then given intracranial infusions prior to behavioral sessions as described in section 2.1 (Fig. 1 below). The rats from the early and late groups were trained on DA task. Due to condition limits, the immediate group was not involved in DA training.

2.3. Histology

Rats for the representative immunohistological examples that were shown in this paper were deeply anesthetized with isoflurane and given an overdose of sodium pentobarbital (200 mg/kg, ip) and perfused using 0.9% saline followed by a home-made PLP (Paraformaldehyde/Lysine/Periodate) fixative (Oliver & Jamur 2010). The brains were then removed and placed PLP for at least 24 hours. The rest of rats were anesthetized with isoflurane and decapitated. The brains were removed and fixed in PLP. After fixing, all the brains were transferred to a 30% buffered sucrose solution (30 g sucrose/100 ml PBS). After 2 days, the brains were frozen and sectioned (40 μ m for cresyl violet staining and 100 μ m for immunohistology) using a cryostat. Slices with PEDOT deposition were mounted on glass slides, visualized under UV light and photographed by a Canon EOS T3i camera. The mounted sections were soaked in formaldehyde and then stained using cresyl violet and photographed. Electrode placements were verified by visualizing the sections under a microscope and identifying the placements using atlas plates from Paxinos and Watson (2004). Converged images were made in Adobe Illustrator. This histological examinations were performed on all the subjects (n = 10).

For immunohistochemistry the tissue sections were treated with 0.1 mg/ml protease XXV (Thermo Scientific) in 0.01M PBS for 2 minutes prior to blocking. All the sections were then blocked with blocking medium (containing 5% normal serum from the same species of the secondary antibodies (JacksonImmuno), 0.05% Tween 20 (Fisher), 0.05% sodium azide (Sigma-Aldrich) in 0.01M PBS under room temperature for 30–40 min, followed by washing for 3 times (3*15min) with 0.01M PBS. After washing, a cocktail of diluted primary antibodies (1:100–1:200, in 15 mg/mL bovine serum albumin (BSA) in 0.01 M PBS solution containing 0.05% sodium azide) were added onto the sections and was incubated at

4 °C for overnight. A list of antibody used is shown in table 1. Briefly, two kinds of primary antibody cocktails were used: 1, glial fibrillary acidic protein (GFAP) for astrocytes, neurofilament (NF) 70 kDa for axon cytoskeleton (neurofilaments) and NeuN for neuronal nuclei; 2, GFAP, CD11b (clone OX-42) and CD68 (clone ED-1) for pathological microglia and macrophage, and NeuN. After staining, the sections were washed with 0.01 M PBS for 5*20 min to remove unbound antibodies. A cocktail of appropriate fluorescence-conjugated secondary antibodies were then added and the section was allowed to incubate at 4 °C for overnight. The unbound secondary antibodies were then washed with 0.01 M PBS for 3*60 min at 4 °C. During staining and washing, the sections were kept from light.

After secondary antibody staining, the sections were mounted onto 90% glycerol in 0.01M PBS solutions with 0.1% p-phenylenediamine as anti-fading agent. It was covered with coverslip and sealed with nail polish.

2.4 image processing

The fluorescence images were taken on a Zeiss LSM 510 equipped with High Speed DUO detector. On the same tissue section, a 10-slice vertical Z-stack at 2.25 µm interval and 6*6 horizontal tile scan was imaged. The series of Z-stack images was projected onto a single image at the maximum fluorescence intensity by Zeiss ZEN 2011 software. This image was loaded into FIJI (Schindelin et al., 2012). A line along the anti-GFAP signal was carefully drawn to contour the deposition associated area using the freehand selection tool. With the line as the center, an area of 750 µm was selected and a straightened image of that area was generated (Fig. 2). 50 random straight lines were drawn and the plot profile on each line was measured (Fig. 2 b). The gray scale *versus* distance was averaged to generate the overall plot profile along the deposition.

3. Results

3.1. The deposition of PEDOT in living hippocampus

All rats (n = 10) included in the study were implanted with cannula-electrode devices strictly in dorsal hippocampus. The position of all implants and depositions (early, late and control groups, n=8) were verified and correlated with the Paxinos and Watson atlas (Fig. 3 left, see the black squares) in order to determine the effects of PEDOT on local hippocampal function. Cresyl violet stained tissue slices visualized under UV illumination confirmed that PEDOT was locally polymerized in the hippocampus for all the experimental groups (Fig. 3 right). Typically, the dark PEDOT depositions were found near the end of electrode insertion track. The clouds assumed nominally spherical shapes, although there were some subtle asymmetrical shapes observed, presumably due to local anisotropic variations in the charge transport pathways in the tissue.

3.2 Impedance

Fig. 4 shows impedance amplitudes at 1 kHz from the different groups. The deposition was performed at different days post cannula/electrode implantation. Due to the dynamic impedance change after implantation (K. a Ludwig et al., 2006; Prasad & Sanchez, 2012; Williams, Hippensteel, Dilgen, Shain, & Kipke, 2007), the average impedance amplitude for

each group started at different value right before polymerization. The lowest (33.6 ± 1.4 kOhm) was recorded from the immediate group (day 0) while the highest (233.6 ± 47.3 kOhm) the early group (21~28 days post surgery). In all the groups except for the control group, for which only EDOT monomer was infused, the *in vivo* deposition was found to decrease the system impedance to about half of its original value regardless of the stage of scarring. We found that by delivering EDOT monomer solution, the impedance also slightly dropped from 168.9 ± 36.1 kOhm to 106.4 ± 29.4 kOhm). However, the impedance increased back to 201.2 ± 47.0 kOhm the next day. This may be because that the PBS solution introduced more ions into the system, thus temporarily decreased the solution resistive. Compared to the control group, the decrease of the impedance amplitude at 1 kHz from the early and late groups was sustained days after polymerization. After the first week post treatment, there was an increase of impedance in both the immediate and late groups. In order to compare, the impedance amplitudes were normalized by taking the percentage of the impedance at each time point for each subject *versus* that right before polymerization (Fig. 4 b and Fig. 5). We found that, percentage-wise, the decrease of impedance sustained for more than two weeks post treatment.

A 4 (group) by 3 (day) ANOVA on the percentage change in impedance amplitude at 1 kHz from baseline revealed a main effect of day ($F(2, 20) = 90.272$, $p < 0.001$), a significant group-by-day interaction ($F(6, 20) = 22.025$, $p < 0.001$), and a significant main effect of group ($F(3, 10) = 14.278$, $p = 0.001$). Post-hoc comparisons using Bonferroni tests revealed that the groups did not differ significantly at the time point immediately following polymerization (or EDOT:PSS infusion in controls) or at the 1-week post-polymerization timepoint. However, at 2-weeks post-polymerization, the impedance was significantly higher in the immediate group as compared to the control group ($p = 0.002$) and compared to the early group ($p = 0.001$) (Fig. 5 c).

Figure 5a depicts the immediate decrease in impedance due to polymerization in the immediate, early, and late groups. A between subjects One Way ANOVA reveals that there was no statistically significant difference between groups ($F(3, 18) = 0.331$, $p = 0.803$). The impedance drop was then compared across the four groups (control (infusion only), immediate, early, and late groups) a week following polymerization. As seen in figure 5b, the control and early groups were not changed from their baseline measures and the immediate and late groups had an increased impedance (~300%); a between subjects One Way ANOVA reveals that this difference in impedance was not significantly different ($F(3, 18) = 2.903$, $p = 0.067$). Finally after 2 weeks post treatment (Fig. 5c.) there was a significant difference between groups ($F(3, 18) = 15.296$, $p = 0.000$). Post hoc bonferroni multiple comparisons show that the controls significantly differed from the immediate and late groups ($p = 0.001$ and $p = 0.030$ respectively) and the early group also differed from the immediate and late groups ($p = 0.001$ and $p = 0.013$ respectively) as indicated by an asterisk. However, the early and control groups did not significantly differ from one another.

3.3 Tissue reactions to chronically deposited PEDOT

Representative confocal images of *in vivo* deposited PEDOT from the early group and the late group are shown in Fig. 6. These samples were stained with anti-GFAP (colored in red),

anti-NeuN (colored in blue) and anti-neurofilament 70 kDa (colored green). NeuN staining confirmed again that the deposition was in the neuronal nuclei-rich hippocampal CA1 pyramidal cell layer (Elkin, Azeloglu, Costa, & Morrison, 2007). At a deposition charge of 2.64 mC, PEDOT typically covered a span in the tissue of about 500 μm , which is substantially larger than the glial sheath (Biran et al, 2005). However, for both of the groups, a $\sim 100 \mu\text{m}$ layer of GFAP+ signals was found adjacent to the PEDOT deposition. Anti-NeuN and anti-neurofilament 70 kDa positive signals were found outside the peak of GFAP + immunoactivity (Fig. 6 e and f). A similar trend was also found in the immediate group, where a layer of astrocytes was found both along the insertion track and PEDOT deposition (Fig. 7).

A depiction of the interaction of PEDOT with glial cells is shown in Fig. 8. As seen in the figure, in the PEDOT deposition area (Figure 8b, inverted into white color) there was a population of ED-1 and OX-46 positive cells (Fig. 8a, colored in green). The cells adopted a rounded morphology, suggesting macrophage behavior (Ladeby *et al* 2005; Lehrmann *et al* 1997). The origins of this ED-1 and OX-46 immunoactivity are not yet clear. We propose that it may come from the blood-brain-barrier leakage (Winslow & Tresco 2010) caused during the surgical implantation. Similar immune activities were also found in the immediate group (Fig. 7). Hence, this study clearly shows that the *in vivo* deposition of PEDOT, at least using the conditions we have employed to date, still elicits a foreign-body response. This response, especially the microglia accumulation, is likely responsible for the impedance increase.

3.4 Behavior

The DA task was employed to evaluate the effects of infusion volume, monomer, dopant, and importantly, the polymerization on hippocampal function. The subjects that would receive *in vivo* polymerization were firstly trained to perform the DA task until choice accuracy was above 75% correct for 3 consecutive days (table 1). Following task acquisition, a series of infusions were given. The task schematic and experimental procedure are shown in Fig. 1. First, to verify that inactivation of the dorsal hippocampus would cause a significant impairment in task performance, an injection of muscimol that would typically inactivate a brain region of $\sim 1 \text{ mm}^2$ was infused (Hallock, Wang *et al*, 2013; Hallock, Wang *et al*, in press), and the results were compared with 0.9% saline injection at the same volume. As shown in Fig. 9 Left, the percentage of correct trials ($46\% \pm 14\%$) was significantly lower on the muscimol-infusion session than the saline-infusion session ($73 \pm 8\%$, $p < 0.05$), which proved that the function of this brain region is essential for completing the DA task. An infusion of EDOT + PSS dopant solution and an infusion of saline + PSS dopant solution both at the same volume were then infused on the day 2 and 4 post criterion day, respectively, to exclude the potential influences of excess monomer and dopant on DA performance. On day 5 post criterion day, another EDOT + PSS solution was infused immediately followed by polymerization. Fig. 9 right depicts the effects of the polymerization procedures on DA task performance, including two consecutive days post polymerization. A One-Way ANOVA confirmed that there were no differences in performance of the task across these treatments ($F(2,228,27) = 1.695$, $p = 0.227$). Therefore the only significant deficit in performance of the task was caused by an infusion of

muscimol, our positive control condition. These results show that the polymerization procedure is not disruptive to the overall function of the hippocampus.

4. Discussion

This paper investigated the influence of the *in vivo* polymerization of conducting polymers in hippocampus on the ability of rats to perform a delayed alternation (DA) memory task. The PEDOT polymerization conditions were refined and the PEDOT depositions in neural tissue were visualized to verify the localized polymerization in DA related hippocampal CA1 region. The system impedance after polymerization was recorded. We performed immunohistochemistry to study the immune responses to the chronically deposited PEDOT. The effects of the infusion of the monomer solution as well as the PEDOT deposition on the rat memory were compared with muscimol and saline controls.

One of the purposes of this study was to create a conducting pathway between the neurons and the probe after device implantation. The variability in recording quality and the device failure for chronic neural implants was found to be correlated to complex and dynamic foreign body reactions (Prasad & Sanchez, 2012)(Prasad *et al.*, 2012)(Streit *et al.*, 2012) (Potter, Buck, Self, & Capadona, 2012). A stereotype of the tissue reactions caused by the constant presence of probe includes the migration and proliferation of astrocytes and pathological microglia within ~150 μm to the probe. Meanwhile, neuron degeneration was also found adjacent to the implants (Biran *et al.*, 2005; Harris, Capadona, *et al.*, 2011; Polikov, Tresco, & Reichert, 2005; Potter *et al.*, 2012; Winslow *et al.*, 2010). Since it is essential to have neurons within the probe “listening radius” (Buzsáki, 2004), the *in vivo* polymerization of conducting polymer in the tissue allows an effective “extension” of the probe. The extent of polymerization can be controlled by the deposition charge (Richardson-Burns, Hendricks, & Martin, 2007; Wilks *et al.*, 2011). Using our current procedures, we found that the *in vivo* polymerization in hippocampus typically formed a ~500 μm spread of PEDOT cloud near the end of the working electrode. The size of the cloud was considerably larger than the ~150 μm dimensions of a typical glial scar.

With both ionic and electronic conductivity, *in vitro* PEDOT coatings on metal electrodes can decrease the system impedance by up to 3 orders of magnitude (Ludwig *et al* 2006). Previously we found that the *in vivo* deposition of PEDOT in brain slices and living cortex could also decrease the impedance (Richardson-Burns, Hendricks, & Martin, 2007)(Wilks *et al.*, 2011); however, in these *in vitro* or acute studies, there were no scar tissue formation around the implants. On the other hand, the sustainability of the impedance drop in long term also needs to be investigated. Gliosis is a complex and dynamic process. For example, between 2 and 4 weeks post implantation, Turner *et al* found that the glial sheath was less compact, as it was observed to remain adherent on the probes when they were extracted from the brain. By 6 and 12 weeks, the glial sheath was relatively more coherent, with the extractions showing much less disruption to the astrocyte layers (Turner *et al.*, 1999). It has also been found that the glial scar reached a mature stage after 1 month in rodents (Winslow & Tresco 2010). The gliosis was found to accompanied by neuronal density decrease (Biran *et al.*, 2005). Potter *et al* found a biphasic pattern which consisted of the initial neuronal denegation in 2 weeks followed by a recovery from 4 to 8 weeks and relapse in 16 weeks.

Activated microglia was found to be adjacent to the implants for all the time points (Harris, Capadona, *et al.*, 2011; Potter *et al.*, 2012). In terms of neural recording, it was found that during the first 2 weeks, there was a decrease of functional unit recording yield as well as signal-to-noise ratio, both of which recovered after 4 weeks (K. a Ludwig *et al.*, 2006; K. A. Ludwig *et al.*, 2009). The change in tissue composition can also be correlated to the system impedance (McConnell, Butera, & Bellamkonda, 2009; Williams *et al.*, 2007). Prasad *et al* found from *in vivo* impedance recording that the impedance magnitude generally increased in the first 3 weeks post implantation and then plateaued after 3 to 4 weeks with daily fluctuations (total recording time = 6 weeks) (Prasad & Sanchez, 2012). When the impedance entered this chronic phase, they found the unit recording yield increased.

In this paper, we divided the subjects into 4 groups: a control group (with no polymerization), an immediate group (where *in vivo* polymerization was performed immediately after implantation, with as little glial scar as possible), an early group (21~28 days, i.e. 3 to 4 weeks post surgery, before the stabilization of gliosis) and a late group (47~52 days, i.e. 7 to 8 weeks post surgery, after the scar stabilization). PEDOT was able to be deposited in all the groups. Similar to the *in vitro* and acute surgeries (Richardson-Burns, Hendricks, & Martin, 2007; Richardson-Burns, Hendricks, Foster, *et al.*, 2007; Wilks *et al.*, 2011), the presence of *in vivo* polymerization dramatically decreased the system impedance (Figure 4). However, the sustainability of this decrease was a function of the age of the scar. For the late group with mature scarring, the impedance at 1 kHz was decreased by less than 50% of its original value. It continued to be lower two days following polymerization but started to increase one week after treatment. A similar pattern was also found in the immediate group, with limited scarring. On the other hand, the early group, with presumably some limited scarring, had a different response. Specifically, the impedance drop was maintained 2 days post polymerization and remained significantly lower than that before treatment for more than 2 weeks. In contrast, the impedance of the control group continued to rise following implantation. From *in vitro* cell culture tests, we found that PEDOT mainly deposits in the extracellular spaces between cells, which may induce cell apoptosis after several days (Richardson-Burns, Hendricks, Foster, *et al.*, 2007). With a mature and denser tissue composition, the polymerization of PEDOT in the late group may interface with more cells. The disruption may cause a reactive tissue response that may contribute to the impedance increase, while in the early group, with less compact structure, the response could be somewhat less intense.

We verified the cell types near the electrodes and depositions two weeks after the *in vivo* polymerization with immunohistochemistry methods. In all of the groups we found a layer of reactive astrocytes along the insertion track and around the deposited PEDOT. Neuronal nuclei and neurofilament were found adjacent to the deposition region. Anti-CD11 b/c and anti-CD68 immunoactivity was found inside the PEDOT deposition area (Fig. 7 and 8). These cells typically assumed a rounded morphology suggesting that activated microglia and macrophages may have been involved in the chronic process. *In vitro* cell culture suggests that the aggregation of glial cells on the interface increases the system impedance (Frampton, Hynd, Shuler, & Shain, 2010). *In vivo*, the astrocytial encapsulation has been strongly correlated to the impedance increase (McConnell *et al.*, 2009; Williams *et al.*,

2007), as the compact structure blocks the ion diffusion (Sykova & Nicholson, 2008). Although not tested in this study, it was demonstrated that the chronic immunoreaction is also associated with a high concentration of reactive oxygen species (ROS) (Potter *et al.*, 2013). In some cases, the ROS may cause electrode corrosion (Prasad *et al.*, 2012). All these reactions may cause the PEDOT rearrangement or degradation and may be responsible for impedance increases after deposition. Across the groups, we found that *in vivo* PEDOT deposition was not free from secondary scarring. Inflammation was found both in the area of deposition itself as well in the glial scar around the deposition.

Future improvements in the process would likely be obtained by decreasing the total amount of PEDOT material deposited. An alternative would be to deposit the polymer at a slower rate, which might cause less damage to the tissue due to the lower average current densities in the polymerization zone. To overcome potential issues that are likely to arise from the secondary glial scarring, it may be necessary to perform repeated small *in vivo* PEDOT depositions on the same electrode during the process. Another option is to consider the use of functionalized EDOT monomers or co-dopants that have biologically specific groups such as peptide sequences from cell-adhesion mediating proteins such as fibronectin, laminin, or L1, or from neurotrophic proteins such as NGF or BDNF to improve neuronal proximity around the deposition. Recently, an EDOT-carboxylic acid monomer has been developed that can be used to covalent attach functional peptides, such as the RGD integrin binding sequence from fibronectin (Povlich, 2012). Lastly, with the microcannula, this method allows administration of anti-inflammatory drugs or anti-oxidants (Potter *et al.*, 2013) to reduce the scarring as well as to promote the neuroregeneration.

We utilized a delayed alternation behavioral task to determine the effects of *in vivo* polymerization of PEDOT on hippocampal function. It has previously been shown that the DA is hippocampal-dependent. We included in the design a positive control to ensure that the implanted microcannulae targeted the dependent structure. As shown in Fig. 9 left, compared to the negative control (saline), an infusion of muscimol caused a deficit in the performance of the task; this further verifies that the successful performance of the DA task relies on hippocampal integrity, consistent with previous findings (Hallock *et al.*, 2013). Interestingly, although muscimol infusion caused a deficit in the performance of the DA task, at the same volume of material infusion, there was no deficit caused by PEDOT deposition or any of the other treatment groups that controlled for volume, EDOT:PSS, and the dopant alone (Fig. 9 right). These data demonstrate that our experimental protocol did not cause significant damage to overall hippocampal function.

5. Conclusions

Immunohistochemistry confirmed that PEDOT could be *in vivo* polymerized to form a ~500 μm cloud in neural tissue using 200 μm stainless steel electrodes, with a 8.2 C/cm² total charge density, delivered over a 2 minute deposition time. The electrochemically deposited PEDOT decreased the system impedance at 1 kHz immediately after deposition and the decrease was sustained. We found that there was a dependence of the impedance on the time post surgery when the polymerization took place. The decreased impedance was not sustained for more than a few days post polymerization when it was performed immediately

after device implantation or after mature scar formation. The impedance data suggest that there is an optimal window of time within which PEDOT should be deposited (3 to 4 weeks) to best lower the impedance of the implanted cortical devices. By using a hippocampus-dependent delayed alternation (DA) task, we found that there were no significant deficits in performance after PEDOT deposition in dorsal hippocampus. Our histological data showed a secondary scarring effect consistent with a reactive response to the electrochemically deposited PEDOT.

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	Treatment					
Treatment day	1	2	3	4	5	6
Infusions	muscimol	PBS	EDOT:PSS	N/A	PSS	Polymerization

Figure 1.

Above: A diagram of the T-maze that the rats were trained on to perform the hippocampal-dependent delayed alternation task. This task required the rat to alternate between right and left goal arms on each trial with a 30-second delay separating the trials. Below: Schematic of the treatment days for control and experimental rats. The start day for the treatments began the day after the rats hit criterion on the task, therefore there is some variability.

Figure 2.

Plot profile survey for GFAP signal around the deposition. (a) A projection of 10 slices of 2.25 μm axial images were created and a 750 μm area centered along the GFAP signal (red) was selected. (b) (a) was straightened and 50 random lines were drawn. The image intensity along each line was measured and averaged to generate an overall plot profile as shown in Fig. 5 e and f.

Figure 3.

(Left) Locations of the implanted electrode tips for rats included in the hippocampal-dependent behavioral task. Coronal sections -3.24 to -3.72 mm from bregma, adopted from Paxinos and Watson (2004). (Right) Electrode tracks and the typical spread of the PEDOT cloud are visualized. (a) Cresyl violet-stained section showing cannula-electrode tracks, PEDOT clouds are found at the working electrode tip. (b) The same section under UV light (PEDOT is indicated with arrows). (c) PEDOT cloud at a higher magnification.

Figure 4.

Impedance amplitude at 1 kHz after either polymerization for Immediate (polymerization immediately after implantation), Early (3~4 weeks post surgery) and late (7~8 weeks post surgery) groups or an infusion of EDOT:PSS for the control group (performed at 30 days), a. Impedance amplitude at 1 kHz for each group; b. Percentage change of $|Z|$ at 1 kHz for each group. The error bar represents the standard deviation.

Figure 5.

Impedance results for controls (an infusion of EDOT:PSS only), immediate polymerization group, early (21–28 days post surgery) and late (47–52 days post surgery) polymerization groups. (a) Impedance change due to the polymerization (taken immediately following polymerization) for immediate, early, and late groups. (b) Impedance change 1 week following treatment (polymerization for immediate, early, and late groups and EDOT:PSS infusion for controls). (c) Impedance change 2 weeks following treatment. Asterisks indicate a significant difference between groups ($p < 0.05$). The error bar represents standard deviation.

Figure 6.

Histology of PEDOT electrochemically deposited in rat hippocampus CA1 region *in vivo*.

(a) Confocal micrograph of an immunostained brain section with "early deposition" (PEDOT polymerized 21 days post surgery) shows that PEDOT (white arrows) grows from the electrode tip out into the hippocampus. (b) Confocal image from "late deposition" (PEDOT deposited after 42 days post surgery). PEDOT was encapsulated by a layer of reactive GFAP+ astrocytes. (c) transmitted light image of (a) (yellow dashed line shows the center of sampling area in C (the 350 μm position)). (d) Transmitted light image of (b). (e)

Normalized average profile of GFAP, NeuN and NF 70 kDa signals from (a). (f), normalized profile of GFAP, NeuN and NF 70 kDa from (b). The sampling method is described in section 2.4.

The white boxes in (a) and (b) are zoomed in image of the area around the deposited. White dashed line depicts the insertion tract. Scale bars in (a) and (b) represent 500 μm and 100 μm in the white box. *Primary antibodies: Green: anti-Neurofilament 70 KDa; Red: anti-GFAP; Blue: anti-NeuN.*

Figure 7.

A representative confocal image shows the onset of glial scar (red, GFAP+) formation both on the insertion track and near the PEDOT deposition area (the dashed shape indicates the position of the original electrode). The sample is from immediate group. ED-1 and OX-46 positive granule cells (green) are found directly at the tip of the electrode. There are populations of ED-1 and OX-46 positive cells in the PEDOT deposition. *Primary antibodies: Blue: NeuN; Red: GFAP; Green: anti-CD68(ED-1) and anti-CD11b/c (OX-46).*

Figure 8.

Activated microglia interacting with PEDOT. (a) a representative confocal micrograph of immunostained rat brain section with late PEDOT deposition shows dense ED1+ and CD11b/c+ cell infiltration. White arrows indicate PEDOT deposition site. (b) Higher magnification of the same deposition site with superimposed with a transmitted light image (color inverted, PEDOT shows as white) indicates macrophage (green) attack on PEDOT (white particles). *Primary antibodies: Green: anti-CD11b/c and anti-CD68 (ED1); Red: anti-GFAP; Blue: anti-NeuN*

Figure 9.

Left: Hippocampal inactivation impairs DA task performance ($p < 0.05$). Right: Behavioral results for polymerization controls and treatments. Blue line represents the 75% criterion. Polymerization procedure and control infusions do not cause a deficit in the performance of the DA task.

Table 1

Animal groups involved in impedance recording and DA task

Group name	n ^a	n ^b	treatment time ^c	sacrifice time ^d	involved in DA task
Control	2	4	30 days	14 days	no
immediate	2	4	0 days	14 days	no
early	3	6	21~28 days	14 days	yes
late	3	6	47~52 days	14 days	yes

^a : the number of animals involved in each group
^b : the total number of working electrodes measured in the group
^c : the time point post initial device implantation when the animal receive treatment (polymerization or infusion of monomer only)
^d : the time point post polymerization or monomer infusion when the animal was sacrificed

Table 2

List of antibodies

Primary antibodies	Host	Dilution	Company	Secondary antibodies	Dye conjugation	Company
GFAP	Chicken	1:200	Millipore	Goat-anti-rabbit IgG	Alexa Fluor 405	JacksonImmuno
CD11b (OX-42)	Mouse	1:100	Millipore	Goat-anti-mouse IgG	Alexa Fluor 488	JacksonImmuno
CD68 (ED-1)	Mouse	1:100	Millipore	Donkey-anti-chicken IgG	Alexa Fluor 565	JacksonImmuno
NeuN	Rabbit	1:200	Millipore			
NF 70 Kda	Mouse	1:200	Millipore			