Conductive polymer nanofibers as a platform for both stimulating and measuring differentiation in oligodendrocyte progenitor cells

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Conductive polymer nanofibers as a platform for both stimulating and measuring differentiation in oligodendrocyte progenitor cells

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April 2017

This material is submitted as partial fulfillment of a B.A. degree with honors in Neuroscience.

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Acknowledgements

I would first like to thank Monia Joblin and the Buegeleisen Family MS Research Fellowship for selecting me to be their first undergraduate research fellow in 2015. It was their fellowship which granted me the opportunity to join the Van Vliet Laboratory and begin my research career in the field of neuroscience. For additional funding, I am grateful to the Jerome A. Schiff Charitable Trust and their fellowship program. I would also like to thank Professor Krystyn J. Van Vliet for offering me this research position, and I would like to thank the members of her laboratory who have helped me to grow so much along the way: Jess, Daniela, Frances, and especially Dr. Anna Jagielska who has been an irreplaceable mentor to me.

I would like to thank those who helped me during this project by providing me with access to equipment outside of my laboratory. I owe thanks to Wenda Tian and the Rutledge group for access to the electrospinning apparatus, and thanks to Liz Canovic and the Anikeeva group for granting me access to the confocal microscope used for fluorescent image collection.

I would also like to use this space to dedicate this work to my family to whom I attribute all my success. I thank my mom for letting me make it on my own. I thank my dad for always believing in me and pushing me to do my best. I thank Grandma Debbie for instilling in me a respect for the unknown in this world. I thank my grandparents Tony and Janet for reading Nancy Drew books with me as a child and playing Barbie detective games. Thanks to you I have grown up to become a sleuth like I always wanted to be, passionate now about solving mysteries of science.
Abstract

Oligodendrocytes are specialized glial cells that insulate neuron axons in the central nervous system with a vital proteolipid sheath called myelin. Oligodendrocytes in the adult brain are derived from oligodendrocyte progenitor cells (OPCs), and their differentiation into mature oligodendrocytes can be observed in vitro; however, this process is incomplete as myelin wrapping cannot be observed when there are no physical structures in culture with which developing OPCs can interact. The development of artificial axon surrogates aims to address this problem by developing geometric and mechanical mimics of axons from materials. The work presented here describes the development of a new axon surrogate that incorporates electrical activity into the in vitro platform. Axon surrogates were developed as conductive polymer nanofibers composed of a polyaniline composite, PANI/PG. PANI/PG nanofiber mats fabricated by electrospinning demonstrate unique and dynamic electrical properties that change over the course of the OPC culture. These changes provide evidence that conductive fiber substrata in the future might be used as an indirect method for measuring cell behavior on the mats. Moreover, this myelination detection method could be adjusted for quick and high-throughput readouts, providing an efficient new platform for the drug discovery of remyelination therapies. In addition, when an electrical potential was applied to PANI/PG fiber mats during OPC differentiation, cells showed an upregulation of differentiation and myelination transcripts \textit{Hdac11}, \textit{Cnp}, and \textit{Mbp} 2-4-fold compared to OPCs which did not receive electrical stimulation. These data suggest that developing OPCs are strongly influenced by electric fields.
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Introduction

The Important Role of Myelin

The neural network of the central nervous system (CNS) is heavily dependent on the support of glial cells known as oligodendrocytes. Oligodendrocytes are unique cells which produce a myelin sheath, an insulating layer composed of proteins and lipids that wraps around neuron axons to assure proper transmission of action potentials. In human development, oligodendrocytes mature and begin myelination during the third trimester, deriving themselves from oligodendrocyte progenitor cells (OPCs) that are abundant throughout the CNS (Dawson et al., 2003). A stable pool of OPCs are maintained in the adult CNS and may differentiate into mature oligodendrocytes if they are triggered by a demyelinating event, such as those that occur in patients with multiple sclerosis (MS) (Franklin et al., 1997; Levine and Reynolds, 1999). While adult OPCs express the same genetic markers as those seen early in development, the new myelin sheath generated by adult OPCs as a repair mechanism is not as thick as the original myelin sheath before it (Franklin and Ffrench-Constant, 2008; Jepson et al., 2012). This means that successful axon conduction of action potentials in demyelinated lesions cannot be fully recovered through the endogenous process of remyelination alone, and there are currently no FDA approved drugs to promote more efficient remyelination in MS patients.

Oligodendrocyte progenitors in the human brain live in a highly-specialized environment and are therefore very sensitive to deviations. Research has shown that OPCs respond to mechanical, chemical, and electrical cues from their environment which ultimately affect their potential to become fully myelinating cells (Colognato et al., 2004; Jagielska et al., 2012; Relucio et al., 2012; Cheli et al., 2015). The environmental sensitivity of OPCs indicates that OPC cultures in vitro (such as those used for preliminary drug screens) will exhibit significantly
different behavior from what is expected in vivo. This thesis aims to build onto the body of work which argues that a truly controlled observation of OPC development in vitro will be performed on a platform which accurately mimics the in vivo environment. Such platforms will not only improve the quality of observations that investigators can make about OPCs developing in monoculture, it will also improve the accuracy with which pharmaceutical companies can identify truly viable drug candidates during the early stages of in vitro screens of drug libraries.

**Oligodendrogenesis**

Studying the complete process of OPC differentiation, also known as oligodendrogenesis, is important for developing enhanced remyelination therapies. The transition from OPC to mature oligodendrocyte is noted when a cell has begun to express genes that are necessary for the construction of myelin: myelin basic protein (Mbp), 2’,3’-cyclic nucleotide 3’-phosphodiesterase (Cnp), proteolipid proteins 1 and 2 (Plp1/2), myelin associated glycoprotein (Mag), etc. (Gobert et al., 2009). An immature OPC can be identified by its high level of expression of inhibitors of DNA binding Id2 and Id4, the repressor Hes5, and other genes which suppress the expression of myelin-associated genes (Hernandez and Casaccia, 2015). The expression of transcriptional inhibitors is down-regulated by the recruitment of histone deacetylases (HDACs) during differentiation, especially HDAC11, to their promoter regions, the process of which becomes more inefficient with age (Shen et al., 2008; Liu et al., 2009).

Epigenetic modifications made by HDACs as well as micro RNAs are critical in oligodendrogenesis (Siebzehnrubl et al., 2007; Foti et al., 2013; Jovicic et al., 2013). The OPC nucleus is characterized as containing mostly euchromatin, while the mature oligodendrocyte has a smaller, rounder, heterochromatin-filled nucleus (Hernandez and Casaccia, 2015).
structural differences indicate a much lower level of transcriptional activity in fully matured oligodendrocytes despite a higher level of expression of myelin-associated genes (Fig 1). Observations of overall nucleus acetylation (levels being lower in oligodendrocytes than in OPCs) as well as levels of gene transcription aid in confirming OPC differentiation when cell microscopy results are too vague. It is impossible to determine the quality of myelin wrapping by cells when they are cultured on the flat surfaces of tissue culture polystyrene dishes; therefore, RT-qPCR and other mRNA analysis techniques currently provide the strongest evidence when proof of oligodendrogenesis is necessary.

![Figure 1. Oligodendrocyte differentiation.](image)

Oligodendrocyte progenitor cells (OPCs) are bipolar or tripolar cells in the CNS. Initially they have a high level of transcription for regulatory factors such as Hes5 and Id2/Id4 as well as platelet-derived growth factor (PDGF) receptors and (in rats) neural/glial antigen 2 (NG2) proteoglycan. After differentiation, which can be induced by mechanical or chemical cues, OPCs begin to express phenotypic markers for oligodendrocytes. The nucleus of an oligodendrocyte is smaller with a higher level of acetylated lysine residues on histones (shown as acetylated lysine 14 on histone 3, or AcH3K14), resulting in a tighter packing of chromatin in the nucleus. Oligodendrocytes also have many long processes with high concentrations of myelin basic protein (MBP), and other proteins that are myelin components (e.g., CNP, PLP1/2, and MAG).
Effect of Neural Activity on Oligodendrogenesis

Even though OPCs can display signs of differentiation followed by myelin production in monoculture, it appears that the complete process as it occurs in the human brain is dependent on interactions with nearby neurons. Neuronal extracellular matrix (ECM) proteins such as laminin and fibronectin that are expressed along neuron axons interact with OPC integrins. They have been shown to be crucial players in both OPC migration and the development of complex myelinating processes during oligodendrogenesis (Milner and Ffrench-Constant, 1994; Colognato et al., 2004; Laursen et al., 2011; Relucio et al., 2012). Other ECM proteins in the CNS can inhibit OPC differentiation, such as leucine-rich repeat and immunoglobulin-like domain-containing protein 1 (LINGO-1) (Mi et al., 2005). Transmembrane complexes formed with LINGO-1 between neurons and OPCs have been implicated in the decreased potential of OPCs to remyelinate adult neurons after a demyelinating lesion has occurred (Jepson et al., 2012).

In addition to the interactions derived from direct contact with OPCs, neuron electrical activity has also been shown to have significant effects on OPC development. The normal firing of action potentials by neurons has been directly linked to the successful development of myelinating oligodendrocytes in the CNS. Inhibition of action potentials by the sodium channel-blocking neurotoxin tetrodotoxin (TTX) decreases the amount of myelinogenesis in the developing mouse brain while α-scorpion toxin—a potent sodium channel activator—increases myelinogenesis (Demerens et al., 1996). Work from the Monje lab at Stanford Medical has shown that in the adult mouse brain, optogenetic stimulation of premotor cortical neurons increases oligodendrogenesis and improves motor function. HDAC inhibition using trichostatin A (TSA) inhibits this process (Gibson et al., 2014). The proposed biological mechanism for this phenomenon is based on OPC expression of voltage-operated Ca\textsuperscript{2+} channels (VOCCs).
The activation of voltage operated Ca\(^{++}\) channels (VOCCs) in the soma of an OPC can be caused by increased extracellular K\(^{+}\) resulting from the propagation of an action potential by a nearby neuron (Wake et al., 2011). These VOCCs are transiently expressed in immature oligodendrocyte progenitors, and the activation of high voltage-activated L-type VOCCs by OPC membrane depolarization has been shown to facilitate axon-glial interactions followed by OPC maturation (Cheli et al., 2015).

In summary, a higher frequency of action potential propagation by nearby axons increases the likelihood of OPC differentiation. This evidence helps to explain why dedicated physical therapy can improve motor symptoms in adult MS patients (Doring et al., 2011). Requiring patients to perform voluntary motor tasks that activate CNS neurons whose axons are demyelinated represents an endogenous mechanism of increasing the frequency of action potential propagation down those axons. This will increase the probability that those axons will be remyelinated by adult OPCs.

**Artificial Axon Surrogates**

Despite oligodendrogenesis being heavily influenced by neural activity, research must still be done *in vitro* with OPC monocultures to isolate their activity in response to chemical cues. In the drug discovery pathway, thousands of compounds from drug libraries are screened *in vitro* to test their effects on the differentiation of isolated OPCs. The environment of the OPC in these tests, though, could not be more different than their natural environment in the CNS. This discrepancy is why research has been aimed at generating artificial axon surrogates for *in vitro* drug screenings that decouple extrinsic chemical cues normally provided *in vivo* while still
Figure 2. Current axon surrogates used for studying myelogenesis. Many different methods have been developed for the fabrication of axon surrogates. (A) Electro-spun fibers provide suspended nanofibers around which differentiating OPCs can wrap their myelinating processes. The material of which these fibers are made is usually a polymer that is as stiff as polystyrene. (B) Micropillars made of compressed silica provide a more functional cell seeding platform that allows the efficiency of myelination to be quantified easily. The wrapping of myelin around the vertical cones can be imaged from the top view with a microscope. (C) Another method is to 3-D print axon surrogates using direct ink printing with compliant polymers. These fibers more closely mimic the elastic modulus of neurons, and mechanical strain can be applied to them to mimic swelling of brain tissue or directional axon growth. The compliancy of the fibers, though, prevents suspension and thereby prevents OPCs from being able to completely wrap the axon surrogates. [Graphic adapted from Lee et al. (2012), Mei et al. (2014), and Espinosa-Hoyos et al. (in review).]

Isolated OPCs are usually cultured on either polystyrene (Young’s elastic modulus, ~3 GPa) or glass (50-90 GPa), both of which are at least $10^6$ times stiffer than brain tissue (100 – 1,000 Pa). Research in the realm of material-cell interface has shown that OPCs demonstrate better adhesion, survival rates, and proliferation when seeded on gels with comparable stiffness to brain tissue compared to cells on glass or polystyrene (Jagielska et al., 2012). OPCs have also demonstrated an ability to myelinate axon surrogates based on their physical shape and diameter (Howe, 2006; Lee et al., 2012; Mei et al., 2014). Not only do these axon surrogates offer a platform for observing myelination in moniculture, the “micropillars” designed by Mei et al. (2014) offer a cross-sectional view of myelin wrapping that allows for high-throughput screening of myelogenesis efficiency in differentiating isolated OPCs (Fig 2B). These micropillars address many problems in oligodendrogenesis research, including the lack of high-throughput capabilities for quantitative detection of myelination. Current myelin detection techniques
include fluorescent imaging for myelin protein expression and detection of gene expression levels at various stages which requires isolation of mRNA from OPCs. Both techniques can be time-consuming and often require that the cells in culture be sacrificed. The potential of axon surrogates to act as instruments for detecting myelination is an area that is still largely available to investigation.

So far, the field of artificial axon development is focused mainly on developing and improving the physical representation of axons by these fibers. The hope is that in the future, such axon surrogates will be multimodal—meaning that they will be more than simple architectural structures. They will additionally have tunable properties that mimic the biochemical and electrochemical dynamics of real neurons. Multimodality is still yet to be accomplished, and it is difficult to choose which axonal functions should be incorporated into an axon surrogate when there is still the maintenance of the simplicity and controllability of the OPC monoculture to be considered. This thesis will explore the possibility of electrical conductivity as a new feature to be tested in artificial axon use.

Electrospinning of polymers is a technique that has been used for the development of axon surrogate nanofibers. Electrospinning is capable of being utilized to form fiber mats of biocompatible conductive polymers (Fig 2A) (Ghasemi-Mobarakeh et al., 2009; Lee et al., 2012). To date, the technique has not been used for seeding OPCs on electrically conductive fibers, but nerve stem cells cultured on such fiber mats have shown enhanced growth when a DC electrical potential is applied across the fiber mat (Ghasemi-Mobarakeh et al., 2009). I aim to replicate the electrospinning protocols from the literature and adjust them so that OPCs can be cultured on conductive polymer nanofibers.
It might be possible that an electrical potential applied to a conductive polymer fiber mat could be sufficient to enhance both oligodendrogenesis and myelogenesis of OPCs via VOCC signaling, but it is unlikely that the mechanism will be as direct as if one were applying a voltage across the cell membrane. Rather, the voltage across the fiber substrate will generate an electric field, the presence of which is relevant as we aim to mimic in vivo tissue conditions for in vitro cell cultures. Overlapping electric fields are abundant in the active brain due to continuously firing neural networks. These electric fields are so powerful and uniform that they can be measured through the skull with electroencephalograms (Kaufman and Watson, 1949; Kennard, 1956). In other cell types including fibroblasts, Schwann cells, and PC12 neural precursor cells, electrical stimulation via the cells’ substrate in vitro has been shown to cause morphological changes in the cell membranes as well as enhanced integrin signaling (Cho et al., 1994; Kotwal and Schmidt, 2001; Balint et al., 2014) (Fig 3). Redistribution of OPC membrane proteins, such

![Figure 3. Effects of electric fields on cell morphology.](image)

In studies performed on mammalian cells in vitro, electric stimulation via a conductive polymer substrate has shown to cause (A) electrophoretic redistribution of membrane proteins and (B) uptake of cations by the polymer. Both could affect cell membrane potentials. (C) In addition, cells have shown increased adsorption of ECM proteins (such as fibronectin) by the substrate when electrical bias is applied which can lead to increased integrin signaling, and therefore changes in gene expression.
as ion channels, and ion uptake by the polymer mat could lead to local changes in cell membrane potential that would cumulatively signal for OPC differentiation.

Electrical stimulation of cells via an applied voltage to conductive fiber mats provides methods for both a selective input (the applied voltage) and a dependent readout (electrical properties of the fiber mat). This thesis will aim to investigate the effects of electrical stimulation via a conductive nanofiber substrate as well as whether the production of myelin around conductive fibers will directly correlate with any changes in the electrical properties of the fiber mat. Cell differentiation and myelination can be confirmed with RT-qPCR of electrically stimulated and unstimulated cells. The electrical properties across the fiber mats can be measured using very sensitive potentiometers. The hypothesis of this work is that electrical stimulation of OPCs will enhance cell differentiation, and the morphological changes undergone by cells will lead to increased cell-material interaction. This increased interaction with a partially biodegradable conductive polymer substrate will alter the fiber mat’s physical and electrical properties over time. If any correlations between expression of differentiation markers and resistance changes in the fiber mat prove significant, conductive axon surrogates could potentially serve as another instrument for indirectly detecting levels of myelination. Moreover, this myelination detection method could be adjusted for quick and high-throughput readouts, providing an efficient new platform for the drug discovery of remyelination therapies.
Materials and Methods

Ethics Statement

This study was carried out in accordance with the guidelines of the National Institutes of Health for animal care and use (Guide for the Care and Use of Laboratory Animals) and the protocol was approved by the Institutional Animal Care and Use Committee at the Massachusetts Institute of Technology (MIT Committee on Animal Care).

Electrospinning of Polyaniline-based fibers for Cell Culture

The fiber mats fabricated for this study were composed of polyaniline emeraldine base (Sigma Aldrich, MW 65,000), polycaprolactone (Sigma Aldrich, MW 80,000), and gelatin (GBiosciences). Polyaniline (PANI) emeraldine base is a conductive polymer which appears dark blue or black in color. To increase the solubility of PANI for electrospinning, it was protonated with an equal mass of camphorsulfonic acid (CSA) to convert it to PANI emeraldine salt (Fig 4). This protonation process, also called “doping,” converts PANI to a more soluble polyconjugated polyradical cation salt that appears green in color (Hopkins et al., 1996; Ghasemi-Mobarakeh et al., 2009). Doping of PANI was carried out with CSA for 3 hours while

![Figure 4. Structure of Polyaniline.](image)

Polyaniline (PANI) is an organic polymer that can be transformed between different chemical states which all have different conductive properties. Polyaniline emeraldine base (PANI EB) is a common and inexpensive form. It can be converted to polyaniline emeraldine salt (PANI ES) by protonation with a strong acid. PANI ES has improved solubility compared to PANI EB, and it is also the most conductive form of polyaniline.
the mixture was stirred in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma Aldrich). After doping, a mixture of polycaprolactone and gelatin (PG) in a 70:30 mass ratio was added to the solution and stirred at room temperature for 24 hours. Because of the low solubility of PANI, fabrication of fibers became more difficult at PANI concentrations above 15 wt%; however, too low of a PANI concentration noticeably decreased the conductance of the final fiber mat. Therefore, all fiber mats described are composed of a PANI/PG ratio of 10:90 by mass. Before electrospinning, the PANI/PG solution was dissolved in HFIP to a final concentration of 6 wt%.

After stirring for 24 hours, the PANI/PG solution was drawn up through a 10 mL syringe and prepared for electrospinning (Fig 5A). Attached to the syringe was a length of tubing leading to a blunt needle with a diameter of 0.4 mm. The needle tip was placed 12 cm above an aluminum collecting plate, and the PANI/PG solution was depressed from the syringe at a flow rate of 1 mL/hr. A voltage of 26.1 kV was applied between the needle tip and the collecting plate. The charge build-up in the solution within the needle caused electrostatic repulsion between molecules, which then forced the PANI/PG mixture to break through the surface tension into a long, thin fiber. On top of the aluminum collecting plate were glass coverslips which each had two platinum wire electrodes (Sigma Aldrich, 0.10 mm diameter) attached to the surface with PELCO® conductive carbon glue (Ted Pella, Inc.). The PANI/PG fibers were collected on the glass coverslips so that the electrodes were sufficiently covered, and then the samples were carefully removed from the collecting plate and dried at room temperature for 24 hours.

Each fiber mat was adhered to the bottom of a 6-well plate by applying silicon vacuum grease to the bottom of the glass cover slip (Fig 5B). For stimulation, the platinum electrodes were soldered to silver-plated copper wire so that all fiber mats to undergo stimulation were
connected in one parallel circuit. Each fiber mat was washed with sterile phosphate buffered saline (PBS) (Gibco, pH 7.4) three times, each wash lasting 15 minutes. Next, the fibers were surface-functionalized with a 50 µg/mL (equivalent to 50 µg/cm) solution of fibronectin from bovine plasma (Sigma Aldrich) for 1 hour at room temperature and subsequently washed with PBS.

Isolation and Culture of Oligodendrocyte Progenitor Cells

Oligodendrocytes progenitor cells for this study were derived from P1-P2 Sprague-Dawley rat cortices (BioreclamationIVT). Cortices were kept on ice in Hibernate A media (Gibco) while all traces of meninges and vasculature were removed. The cortices were then divided into smaller parts, the Hibernate A media was removed, and the tissue was digested for
20 minutes at 37° C. The digestion media was composed of Dulbecco’s Modified Eagle Medium (high glucose) (Gibco), PenStrep antibiotics (Gibco), L-cysteine (Sigma Aldrich), DNase I (325 µg/mL) (Ambion), and Papain (Worthington), all of which was administered through a 0.20 µm sterile filter.

After digestion of the tissue, the sample was further broken down by centrifugation followed by manual pipetting in a solution of Dulbecco’s Modified Eagle Medium (DMEM) with PenStrep (P/S) and 10% fetal bovine serum (FBS) (Atlanta Biologicals). The mixed glial cultures were split so that there was material from 2 cortices per T75 culture flask (Falcon) in 10 mL of DMEM P/S 10% FBS. Before adding the glial cultures, each flask had been surface-functionalized with 5 µg/cm of poly-d-lysine (PDL, MW 70,000) (Sigma Aldrich). Cultures were kept in an incubator at 37° C and 5.0% CO₂.

After 10 days in culture with media changes every 3 days, the adherent mixed glial cultures were placed on a Mini Shaker (VWR) for 3 days of shaking at 300 rpm. After shaking, the supernatant was removed from each flask and replaced with fresh DMEM P/S 10% FBS. Every 10 mL of supernatant was added to an untreated 10 cm polystyrene dish (Falcon), and then was left in the incubator for 30 minutes. The supernatant contained microglia as well as OPCs, and after 30 minutes of allowing for differential adhesion, microglia were adhered to the polystyrene while OPCs remained in suspension [method described by Chen et al. (2007)]. The supernatant of each dish was collected, centrifuged for 5 minutes, and re-suspended in DMEM P/S with SATO’s modification [5 µg/mL insulin, 50 µg/mL holo-transferrin, 5 ng/mL sodium selenite, 16.1 µg/mL putrescine, 62 ng/mL progesterone, 0.1 mg/mL bovine serum albumin, 0.4 µg/mL Tri-iodothyroxine, 0.4 µg/mL L-Thyroxine]. OPCs were counted using a hemocytometer.
and plated onto polystyrene at a density of 265 cells/mm². This entire isolation process from shaking would be repeated for a single flask of mixed glia 6-8 times.

To maintain OPCs in their progenitor state, 10 ng/mL of platelet-derived growth factor A (PDGF-A) and 10 ng/mL of fibroblast growth factor (FGF) were added daily to OPC culture. After 2-3 days of expansion on polystyrene, OPCs were washed with PBS and then detached with Accutase® for 8 minutes at 37°C. The Accutase® was diluted with DMEM P/S 10% FBS, and cells were further detached from the plate surface by gently squirting them with a pipet. The suspended cells were then centrifuged for 5 minutes, and the pellet was re-suspended in freezing media [65% DMEM P/S, 25% FBS, and 10% dimethyl sulfoxide (DMSO) added to sample over ice]. After counting the cells, OPCs were placed in a -80°C freezer for 24 hours and then transferred into liquid nitrogen until later use.

**Seeding and Stimulating Oligodendrocyte Progenitor Cells on Fiber Mats**

Before seeding OPCs onto the PANI/PG fiber mats, electrical measurements of the mats in PBS were first taken using a PalmSens3 with PSTrace software (version 4.6). Voltage sweep measurements were taken from a range of -1.0 to +1.0 V after 60 seconds of equilibration. $E_{\text{step}}$ was set to 0.001 V, and the scan rate was set to 0.5 V/s. For multistep amperometry measurements, the instrument equilibrated for 60 seconds, collected a baseline current measurement for 30 seconds, and then applied 1 mV at a pulse width of 15 seconds. This pulse was repeated four times with 40 seconds in between pulses. The current across the mat was measured and plotted every 100 milliseconds.

OPCs were thawed from liquid nitrogen, diluted with 9 mL DMEM P/S 10% FBS, and centrifuged for 10 minutes. The pellet was suspended in DMEM with SATO’s modification as
well as with PDGF-A and FGF as previously described. Cells were seeded either on glass or PANI/PG fibers at a density of 265 cells/mm² and cultured at 37° C, 5% CO₂ for 24 hours. After 24 hours, the media of all cells was changed to induce differentiation (DMEM with SATO’s modification plus 0.5% FBS, sans growth factors). Additionally, at this time some of the OPCs on PANI/PG fiber mats were stimulated by applying 1 mV across each fiber mat for 1 hour using a 260 Nanovolt Source (Keithley Instruments, Inc.). Cells continued to grow in culture for 24 hours after inducing differentiation.

**Preparation of Cell Cultures for Imaging**

For OPCs cultured for imaging analysis, cells were fixed twenty-four hours after inducing differentiation. The cells were first washed with PBS and then fixed based on the type of imaging. For cells to be stained for confocal fluorescent imaging, samples were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS. Cells were left in the fixative for 20 minutes at room temperature and then washed three times with PBS. The OPCs were permeabilized with 0.1% Triton for 3 minutes, and then left in a blocking solution for 30 minutes (1% bovine serum albumin in PBS). Next, the cells were bathed in a 20 µg/mL solution of rat anti-MBP monoclonal antibodies (AbD Serotec) for 1 hour at room temperature, followed by three washes with PBS and 1 hour in 20 µg/mL of AlexaFluor 594 rabbit anti-rat secondary antibodies (Invitrogen). To stain the nuclei of cells, OPCs were washed with PBS and then bathed in a 10 µg/mL solution of Hoechst 33342 (Life Technologies), followed by 3 additional PBS wash steps. Images were obtained with a confocal fluorescent microscope.

OPCs which were to be imaged by scanning electron microscopy (SEM) were fixed with a 1% glutaraldehyde, 4% paraformaldehyde solution in PBS for 72 hours at 4° C. Cells were
washed 3 times with PBS and then dehydrated in steps: 50% ethanol (Koptec, 200 proof anhydrous) for 5 minutes, 70% ethanol for 10 minutes, 80% ethanol for 10 minutes, 95% ethanol twice for 5 minutes each, and then 100% freshly opened ethanol three times for 5 minutes each. The sample was dried by liquid CO$_2$ exchange in a critical point dryer (Autosamdr-815 Critical point dryer) provided by the W.M Keck Imaging Facility of the Whitehead Institute (Cambridge, MA). The dry sample was sputter-coated with 10 nm of gold, and images were collected with a scanning electron microscope (Merlin, Zeiss) at an accelerating voltage of 15 kV.

**RNA Collection and RT-qPCR**

To analyze differential gene expression of OPCs cultured on PANI/PG fibers with and without electrical stimulation compared to OPCs cultured on glass, mRNA transcripts were collected from samples 24 hours after differentiation induction (RNEasy Micro Kit, Qiagen). The concentration of mRNA was measured using a NanoDrop spectrophotometer (Thermo Scientific). Total mRNA from samples was converted to single-stranded cDNA with reverse transcriptase and a Qiagen random primer mix (QuantiTect Reverse Transcription Kit, Qiagen). The reaction was carried out on a 3′Prime Thermal Cycler (Techne) at 42°C for 15 minutes immediately followed by 3 minutes at 95°C to inactivate the reverse transcriptase. The cDNA samples were stored at -20°C until being used for quantitative polymerase chain reaction (qPCR).

Each qPCR reaction was conducted in triplicate in a 96-well plate. Samples from OPCs cultured on glass, PANI/PG fibers without stimulation, and PANI/PG fibers with stimulation were screened for relative expression of differentiation markers such as *Hdac11*, *Cnp*, and *Mbp* compared to a housekeeping gene: glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). The
The assumption in this measurement is that expression of Gapdh, an instrumental gene in the glycolysis pathway of all cell types, is not significantly changed upon application of an experimental variable such as electrical stimulation (Chen et al., 2009). Each qPCR reaction was 20 µL in volume, containing 10 µL of QuantiNova SYBR Green PCR Master Mix (2x, Qiagen), 1 µL of sample cDNA, 6 µL of DNase-free water, and 3 µL of forward and reverse primers (final concentration 0.75 µM). Primers were designed using ProbeFinder software version 2.50 (Roche) and ordered through Integrated DNA Technologies (Table 1). Because Mbp in rats has several different splice variants, the primers designed for qPCR were specified to target transcript variant 4, a variant which is more stably expressed both in development and adulthood than other variants found in rat brains (Padhi and Pelletier, 2012).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>GC Content</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mbp.tv4</td>
<td>F: 5’ GCA GAA GCC AGG ATT TGG 3’</td>
<td>56%</td>
<td>59</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>R: 5’ TCC CTT GTG AGC CGA TTT AT 3’</td>
<td>45%</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Hdac11</td>
<td>F: 5’ CGG CCA TTC TAC CCT GAA G 3’</td>
<td>58%</td>
<td>60</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>R: 5’ TGG GTG CTT TCC TGC CTA 3’</td>
<td>56%</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Cnp</td>
<td>F: 5’ AAA TTC TGT GAC TAC GGG AAG G 3’</td>
<td>45%</td>
<td>59</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>R: 5’ CCG TAA GAT CTC CTC ACC ACA 3’</td>
<td>52%</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Gapdh</td>
<td>F: 5’ ACT CTA CCC ACG GCA AGT TC 3’</td>
<td>55%</td>
<td>59</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>R: 5’ ACT CAG CAC CAG CAT CAC C 3’</td>
<td>58%</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

Primers for qPCR were designed using ProbeFinder software from Roche diagnostics. Optimal primers produced an amplicon between 50 and 160 bp in length and had an annealing temperature (T<sub>m</sub>) of 60° C.

The 96-well plate containing each reaction mixture was analyzed for fluorescence intensity in real time on a thermal cycler using the LightCycler® 480 Real-Time PCR System (Roche). The PCR reaction was initiated by heat activation of the DNA polymerase at 95° C for
2 minutes. Amplification of the genes of interest was carried out over 40 cycles of 2-step cycling: denaturation of double-stranded DNA for 3 seconds at 95° C followed by a combined annealing and extension step for 10 seconds at 60° C. At the end of 40 cycles, a melt curve analysis was performed to aid in confirming that amplified products were identical across conditions. PCR products were also run on a 1% agarose gel in TAE buffer to confirm that amplicon sizes matched the expected values. The C\textsubscript{t} values for each sample in the well were provided by the LightCycler® system. The C\textsubscript{t} value is the cycle at which fluorescence in the sample well reaches a supra-threshold level that is determined by the cycler. A lower C\textsubscript{t} value is associated with a higher concentration of the template being amplified.

**Statistical Analysis of Data**

Reported errors are standard errors of the mean (SEM). Statistical significance analysis was conducted by Student’s t-test or by one-way ANOVA followed by Tukey’s HSD test.
Results

Properties of PANI/PG Fiber Mats

During electrospinning, PANI/PG fiber mats appear green in color (Fig 6A). Observation under a light microscope followed by measurements in ImageJ software showed that the average fiber diameter is 735 ± 79 nm (Fig 6B). After electrospinning, fibers were washed with PBS and surface-functionalized with fibronectin. During the wash step, fiber mats underwent a visible color change from green to blue (Fig 6C). This change is associated with a deprotonation of the emeraldine salt form of PANI—which had previously been doped with CSA—so that it switches conformation to become the emeraldine base form (Fig 4).

Electrical properties of the fibers were first analyzed by simple voltage sweep measurements. PANI/PG fiber mats demonstrated nonohmic voltage-current relationships (Fig 7A), and every mat measured had a positive y-intercept, indicating a nonzero current when no

Figure 6. Electrospun PANI/PG fiber mats. (A) Fiber mats were composed of 10 wt% polyaniline (PANI), 64% polycaprolactone and 26% gelatin (PG). Shown here are mats which have been electrospun onto 12 mm and 18 mm glass coverslips. (B) Light micrograph of electrospun PANI/PG fibers. (C) Bottom: PANI/PG fiber mat shortly after electrospinning. Mat is green because of the low pH of the spinning mixture (PANI emeraldine salt doped with CSA). Top: PANI/PG fiber mat from the same experiment after 48 hours in biological conditions (pH 7, 5% CO₂, 37°C). The mat appears dark blue in color because PANI has returned to emeraldine base form at neutral pH conditions.
voltage was applied (Fig 7B). The mean y-intercept across all mats was 3.94 ± 1.49 µA, and the mean x-intercept was found to be -645.2 ± 26.1 mV (n = 11). Next, current across fiber mats was monitored during multistep amperometry (see section Materials & Methods). Measurements were taken while fiber mats were submerged in PBS with a 1 mV potential being applied across the mat for 15 seconds. Current response curves for each sample consistently followed the expected pattern for a resistor/capacitor circuit: sharp current increase at the onset of applied

Figure 7. PANI/PG fibers demonstrate unique electrical properties. The currents across electrospun PANI/PG fiber mats were measured during a voltage sweep from -1.0 to 1.0 V. (A) Sample response of a single PANI/PG fiber mat. (B) Overlay plot of all voltage sweep curves from PANI/PG mats. Each curve represents a different sample, all of which were prepared in an identical manner. (C) The current across the PANI/PG fiber mats was monitored as a 1 mV potential was applied for 15 seconds (red bar) and then repeated once every 55 seconds. To calculate resistance, the peak height of the response curve was used as a surrogate for current in amps at \( V = 0.001 \). Tau (\( \tau \)) was measured as the time (s) for the current to return 63\% of the way back to baseline from the minimum current. (D) Resistance was calculated and tau was measured for each peak of one fiber mat before and after 48 hours of cell culture (n = 4). Resistance significantly increased after culture (\( p < 0.01 \)), and tau showed a nonsignificant decrease (\( p = 0.19 \)). This trend was identical in both mats which were measured before and after 48 hours of cell culture.
voltage and a leveling-off of current over time. This was immediately followed by a sharp reversal of current at the offset of electrical potential and a slow return to baseline current (n = 4 fiber mats) (Fig 7C). Similar current responses were observed in a solid sample of PANI/PG and were not seen in measurements taken from PBS alone (data not shown). The initial change in current observed when 1 mV potential was applied to the mat was considered to be the change in current at zero capacitance. This current was used to calculate an estimate of fiber mat resistance using Ohm’s law (V = IR). Additionally, tau (τ) was measured in seconds as the length of time between the minimum current observed when the applied potential was removed to the point at which current returned 63% of the way back to baseline current. Both properties, estimated resistance and tau, were averaged across 4 pulse cycles for each mat before and after 48 hours of cell culture (Fig 7D). Only two fiber mat samples with OPCs seeded on them survived to post-culture measurements. For both samples, resistance of the mat significantly increased after 48 hours of cell culture \([t_1(3) = 11.29, p_1 = 0.002 \); \(t_2(3) = 3.66, p_2 = 0.035\)]. Tau did not change significantly for either mat, but demonstrated a decreasing trend after cell culture \([t_1(3) = -1.667, p_1 = 0.194 \); \(t_2(3) = -0.424, p_2 = 0.700\)]. Capacitance was calculated using the two parameters (\(\tau = RC\)). The first mat, whose pre- and post-culture properties are shown in Fig 7D, had a significant decrease in capacitance from 709 ± 89 nF to 432 ± 36 nF \([t_1(3) = -3.32, p_1 = 0.045\)]. The second mat showed a similar decrease in capacitance which was not found to be significant \([t_2(3) = -1.33, p_2 = 0.275\)]. During the experiment, negative control fiber mat samples had been prepared and exposed to the exact same culture conditions without OPCs seeded on them, but the samples did not survive 48 hours in biological conditions (see section Appendix). Therefore, statistical analysis of cell-material interaction effects on changes in fiber properties could not be accurately performed in this study.
OPC Morphology on Fiber Mats

The most common method of monitoring cell survival and behavior during an experiment is to observe cells under a light microscope. Due to distortions and obstructions of light caused by the PANI/PG fiber mat substrata, OPC cultured on the fibers could not be observed in this way. Control OPCs which were cultured on glass served as a surrogate for monitoring the condition of the cells which had been isolated from the same mixed glial cultures. Images of OPCs cultured on PANI/PG fibers had to be obtained through other methods.

OPCs which had been cultured for 48 hours on fibronectin-coated PANI/PG fiber substrata were fixed and immunostained for MBP, and their nuclei were stained with Hoechst dye (Fig 8). Images obtained on a fluorescent confocal microscope showed that OPCs on fibers maintained strong survival rates, indicating good cytocompatibility of PANI/PG. Cells had been seeded at a density of approximately 265 cells / mm$^2$, and after 48 hours in culture, cells were seen at a density of 210 ± 20 cells / mm$^2$. Immunostaining showed that 58.1% of cells on fibers exhibited MBP expression which was localized to the cell body.

A fiber mat saved just after electrospinning and one fiber mat which had been used in OPC culture were additionally imaged with secondary electron SEM (Fig 9). These results show

![Figure 8. Immunocytochemistry of OPCs on PANI/PG fiber mats. After 48 hours in culture on PANI/PG fiber mats, OPCs were immunostained for myelin basic protein (MBP). Cells were also stained with a DNA intercalator Hoechst 33342 for nuclei localization (DAPI). Overlay of the two channels shows that 58.1% of cells on fiber mats were MBP+ after 48 hours on fiber mats (n = 191 nuclei total).](image)
that PANI/PG fibers before cell culture are in fact randomly aligned, but under high magnification it is easy to see that many fibers are orthogonal to one another (Fig 9A). OPCs could be seen on PANI/PG fiber mats, and they displayed a simple morphology (Fig 9B-C). No processes could be distinguished from the substrate by eye, which is to be expected at such an early time point in differentiation. It was clear that the fibers of the mat had undergone major physical changes by the time images were collected. The fibers appear webbed at the surface, and they also seem to be coated in evenly-distributed, small, unidentifiable globules. It is possible that these objects are fibronectin agglomerates. They could also be artifacts which developed during the critical point drying process in sample preparation. OPCs are easily distinguishable by their size of 4-5 µm in diameter.

**Gene Expression of OPCs on Fibers with and without Electrical Stimulation**

Differential gene expression of OPCs cultured on glass, PANI/PG fibers, and PANI/PG fibers with electrical stimulation was measured by RT-qPCR (Fig 10). Genes of interest included Hdac11, Cnp, and Mbp, which were normalized to the housekeeping gene Gapdh. Raw C_t values from the qPCR run were converted into ΔC_t values (C_t Target – C_t Gapdh) by averaging across
triplicates for each gene in each condition. The ΔΔCt values (ΔCt Fiber condition - ΔCt Glass) were calculated in an all-to-all comparison method for each gene. Statistical analysis was performed on these ΔΔCt values against the null hypothesis of ΔΔCt = 0, and reported in Figure 10 is the base-2 anti-log of the means ± SEM. This anti-log value provides the relative fold change in gene expression of OPCs cultured on PANI/PG fibers compared to OPCs on glass.

Expression of Hdac11, which is a strong indicator of differentiation in OPCs, was significantly upregulated in cells which had received electrical stimulation (p < 0.0001) [F(2) = 22.1, p < 0.0001]. The same was true of Cnp, which is a gene expressed early in the formation of myelin (p < 0.0001) [F(2) = 15.0, p < 0.0001]. Expression of Mbp, a late-stage expression marker of myelin formation, was upregulated in both OPCs on PANI/PG fibers without electrical (p = 0.025) and with stimulation (p < 0.0001) [F(2) = 21.8, p < 0.0001].

Figure 10. Differential gene expression of OPCs cultured on PANI/PG fibers with and without electrical stimulation. OPC mRNA transcripts were collected and analyzed using RT-qPCR. Genes of interest included Hdac11, an epigenetic factor associated with increased differentiation, Cnp, and Mbp, an early and late marker of myelin production respectively. OPCs cultured on fibers demonstrated a modest upregulation of Mbp compared to cells on glass (p = 0.025) (n = 13). OPCs which were cultured on fibers and stimulated for 1 hour with 1 mV of DC bias across the mat 24 hours before RNA collection showed significant upregulation of Hdac11 (n = 12), Cnp (n = 18), and Mbp (n = 15) (p < 0.0001 for all).
Discussion

The goal of this work was to determine whether OPC differentiation is strongly influenced \textit{in vitro} by the application of an electric field. This was tested by seeding cells on conductive polymer PANI/PG fiber mats and applying a potential difference across the mat for one hour during cell differentiation. Stimulated cells were compared to unstimulated OPCs, and cells which received electrical stimulation via their substrate were found to show increased expression of differentiation and myelination markers. These results suggest that electrical conductivity is a feature which should be incorporated into axon surrogates for \textit{in vitro} monoculture studies of myelination. Additionally, the unique electrical properties of the PANI/PG fiber mats as well as their propensity to change over time could potentially be exploited by researchers to detect changes in cell-material interaction by measuring only the substrate’s material properties.

Conductive Nanofiber Substrata Increase OPC Differentiation

Based on the expression profiles of OPCs after receiving electrical stimulation on PANI/PG fibers compared to those without stimulation, electric fields applied \textit{in vitro} cause a significant increase in differentiation of OPCs (Fig 10). This supports the hypothesis that current \textit{in vitro} platforms do not provide the proper environment for observing oligodendrogenesis as it happens \textit{in vivo}. This is especially obvious when large fold changes in expression were detectable 24 hours after a stimulation event which had only been applied for 1 hour. The biological mechanism for this upregulation is still unknown, but it encourages further experiments which might employ different kinds of electrical stimulation, both dynamic and static.
The genes which were upregulated in OPCs that had been stimulated on PANI/PG fibers (Hdac11, Cnp, Mbp) are strong indicators of increased differentiation. Expression changes in future experiments should be confirmed at the protein translation level by repetition of the experiment followed by a longer differentiation period (5-10 days) and fluorescent immunocytochemistry coupled with Western blot analysis. Confocal fluorescent images of OPCs immunostained for MBP on PANI/PG fibers in this study were imaged 24 hours after changing to differentiating media (Fig 8). This time point was too early for OPCs to have developed extended processes that are characteristic of a more mature morphology. This time point was chosen, though, to confirm that the results of RT-qPCR were from OPCs which had similar morphologies and density to the control OPCs that were cultured on glass. This measure was necessary because of the problem that PANI/PG fiber mats pose as substrates when it comes to light microscopy. Light microscopes are often used to monitor samples over the course of an experiment, but PANI/PG fibers are not transparent, and therefore light microscopy is not an effective approach for observing the morphology of live cells. As shown in Figure 9, the best way to view cells on these fiber mats is ex post facto SEM. If these fibers are to be used in the future, new methods need to be developed for monitoring OPC development without sacrificing the cells. This can be done with live fluorescent staining, but monitoring cells could be more streamlined if the method were one which capitalized on the electrical properties of the fibers.

It has been sufficiently demonstrated that neural activity in vivo and in coculture can stimulate myelinogenesis, and that some oligodendrocytes are even capable of propagating action potentials (Karadottir et al., 2008; Kukley et al., 2010; Wake et al., 2011; Gibson et al., 2014). Researchers who aim to mimic these effects in OPC monoculture do so by altering the ion concentration of the culture media with increased potassium, which is similar to what happens in
the environment of an OPC after a nearby neuron fires an action potential. This technique has been shown to sufficiently activate voltage-operated calcium channels (VOCCs) and signal for increased OPC differentiation (Cheli et al., 2015). Few groups, though, have measured the effects of purely electrostatic conduction (as opposed to saltatory conduction of neurons) on OPC development. This includes groups who have observed increased OPC differentiation on conductive materials, such as graphene, without applying a potential bias (Shah et al., 2014). It even includes groups who had OPCs in mixed neural precursor cell cultures in electric fields on electrically conductive scaffolds. Li et al. (2013) did just that, but only focused on morphological changes in neural stem cells without monitoring OPC development. The significant change in the OPCs’ expression profile during differentiation on conductive PANI/PG nanofibers in this study rejects any remaining theory that oligodendrocyte development is not affected by an electroactive environment. Not only do the results suggest that neurobiologists should aim to further elucidate the electro-sensitivity of OPCs, it also provides evidence that engineers developing artificial axon surrogates for in vitro myelination should incorporate electrically conductive materials into their platform.

**Conductive PANI/PG Fiber Mats Demonstrate Unique Electrical Properties**

The decision to use polyaniline as the conductive polymer for this study was based on the well-developed methods by others in using PANI in electrospinning especially for neural tissue engineering (Li et al., 2006; Ghasemi-Mobarakeh et al., 2009). The nonohmic response of PANI-based fibers to a range of applied voltages (Fig 7A) was to be expected based on the different charge carriers that this conductive polymer has: charge being carried down the PANI molecule by resonance structures at low voltages, and polarons moving charge faster at higher voltages.
(Hopkins et al., 1996). What was unexpected about these fiber mats was the wide variation of voltage sweep curves between fiber mats that had been fabricated in an identical manner (Fig 7B). This may have been caused by the physical instability of fibers during electrical measurements (see section Appendix), or it may suggest a much more complex electrical system that has yet to be investigated. This “complex system” may include p-n junction-like currents or multiple time constants within the PANI/PG fibers. Fortunately, multistep amperometry provided consistent response patterns from all mats (Fig 7C).

Treating these amperometry curves from PANI/PG fibers like resistor-capacitor circuit response curves allows us to dissect out meaningful information. Of the mats measured, resistance of the mats significantly decreased after 48 hours in biological conditions (Fig 7D). This is likely due to a slow deprotonation of PANI at a neutral pH for long durations (Fig 6C), but this cannot be confirmed without more measurements from fibers kept in identical conditions without OPCs cultured on them. Still, with an increased resistance, the decreasing trend of tau and of mat capacitance is not easily explained by chemical changes in PANI. It is easy to think of how these fibers might act as capacitors. First, each fiber is not a continuous molecule of PANI—not like a metal wire, which is a continuous charge carrying material. PANI is incorporated into the mats as long chains, but there are undoubtedly spaces in between molecules of PANI where charge could build up without being allowed to flow continuously. Additionally, two adjacent PANI/PG fibers could have charge build up between them like two plates of a capacitor. Should fibers be myelinated by differentiated oligodendrocytes, the distance between adjacent fibers would increase and capacitance would be expected to decrease. This is an exciting concept that demands more investigation as it provides a feasible theory for how
electrical properties of conductive polymer fiber mats could be influenced by cell-material interactions.

**Recommendations for Future Work**

In order to strengthen the supporting evidence that *in vitro* electrical stimulation of OPCs via their substrata promotes differentiation, future work must aim to uncover the mechanism for OPC electro-sensitivity. Electric fields *in vitro* could potentially activate VOCCs in OPCs, which can be investigated by performing fluorescent calcium imaging of cells at the onset of stimulation (Cheli et al., 2015). Another possibility is that the electrical stimulation is promoting higher adsorption of ECM proteins such as fibronectin by the fiber mat (Kotwal and Schmidt, 2001). Protein adsorption after application of different stimulation patterns can be compared using an ELISA assay. Finally, RT-qPCR experiments which measure expression levels of OPCs at different time points after receiving stimulation could aid in describing the expression dynamics of cells after receiving stimulation. This time-based expression profile can also be a useful tool in determining how electrical stimulation is being transmitted to the nucleus.

Additionally, I recommend that future work investigate the interaction of fiber architecture and conductive substrata on the response of OPCs *in vitro*. This investigation would help in determining whether the effect of electrical stimulation on oligodendrogeneration is dominated by the electric field or if the nanofiber architecture augments the effect. An experiment of this type would simply have two types of substrata (flat PANI/PG and PANI/PG nanofibers) and two types of conditions (with and without electrical stimulation). The biggest motivator for considering these effects is the observation that OPCs cultured on PANI/PG fiber mats without electrical stimulation express significantly higher levels of *Mbp* compared to cells
cultured on glass (Fig 10). The question remains whether this upregulation is caused by the presence of geometric fibers or by the effect of PANI/PG material properties—such as material stiffness—on OPCs.

All future work which is built on this project would benefit from stable, long-term investigations. Most in vitro studies that allow for sufficient OPC differentiation are conducted for 3-10 days after induction of differentiation (Jagielska et al., 2012; Shah et al., 2014; Najm et al., 2015; Jagielska et al., 2017). This period is long enough for OPCs to develop complex morphologies and MBP+ processes, and it is an optimal time for data to be collected through imaging studies. To accomplish this, conductive fiber mats need to be stable and consistent during imaging. As SEM images show, PANI/PG fiber mats undergo obvious physical changes from electrospinning all the way through to post-OPC-culture analysis (Fig 9). Without proper controls, it is difficult to say whether these changes occurred during cell culture or during sample preparation (fixation and critical point drying). This problem can be addressed by either maximizing the fabrication process to produce large numbers of fiber mats so that optimal control samples are available, or the other solution is to develop a conductive fiber mat that is more physically stable. The latter poses another problem, though, in that if the substrate is too stable, its properties will be less likely to be affected by cell-material interaction.

The long-term goal of employing conductive fiber substrata is to correlate electrical properties of the mat with stages of OPC behavior. It is likely that the solution lies in using a more chemically stable conductive polymer—one whose conductance is less affected by biological conditions and pH—into a biodegradable mixture which can be electrically manipulated by physical interaction with cells. It would also be prudent of future investigators to include temperature measurements in these experiments to control for heat that is generated due
to the large resistivity of conductive polymers. Finally, the substrata must be incorporated into a high-throughput platform that secures the substrata to ensure consistent culture and analysis conditions. The findings presented here, even without these desired modifications which should be considered in future work, are still motivation enough to declare that electrical conductivity is a worthwhile feature to incorporate into axon surrogates for *in vitro* studies of myelination. Furthermore, the exploitation of electrically conductive fiber substrata could aid in guiding differentiation of cells which are more difficult to observe *in vitro* such as mouse or human OPCs. Continuing studies of the electro-sensitivity of OPCs could even lead to meaningful *in vivo* experiments which seek to examine how minor alterations in brain electric fields through techniques such as transcranial magnetic stimulation might affect how OPCs can differentiate for the purpose of repairing demyelinated lesions in the adult brain. The results of this thesis may ask more questions than are answered, but the questions are exciting and full of possibilities. Hopefully their answers will have an impact on the platforms available to future scientists who are seeking to understand, manipulate, and perfect the mysterious process of myelination.
Appendix

Improving the Platform for Cell Culture on Conductive Fiber Substrata

The most limiting factor on the comprehensiveness of this study was the instability of fiber mats during cell culture experiments. Fiber mats were electrospun directly onto a glass substrate which had platinum electrodes adhered to the surface with conductive carbon paste. When the mats were placed in cell culture wells with biological media, the fiber mats tended to delaminate from the glass surface. When fiber mats delaminate, they float easily in the media as a thin membrane. This led to multiple problems, including inconsistencies during fluorescent imaging for many samples as well as an inability to measure the electrical properties post-culture. Without stable fiber surfaces which are consistently adhered to the electrodes, high-throughput studies are not yet a possibility.

In parallel to the studies described in this thesis, work was done to develop a specialized multi-well plate which would incorporate and secure PANI/PG fiber mats uniformly (Fig 11). The original design criteria were to have a plate which would have two separate parts, a top and a bottom, which could secure the fiber mats in between the two layers. The plate itself would also provide insulation for the electrodes and connecting wires so that both electrical stimulations and recordings would be minimally affected by contact with the media. Several plate prototypes were developed over the course of this study, but almost all the prototypes failed in that they either (1) damaged the fiber mats beyond use or (2) were unable to have sufficient binding of the top and bottom layers of the plate, which is necessary for water-tight culture wells. Here I will describe the prototypes produced, how they failed, and my recommendations for future prototype developments.
Methods and Findings

Three main materials were used in the production of plate prototypes: acrylic, glass, and polydimethylsiloxane (PDMS) (Sylgard® 184, Dow Corning). These are materials which have repeatedly been used in this lab to produce custom cell culture plates (Jagielska et al., 2017). Acrylic was cut on a laser cutter to produce either individual plate parts or PDMS molds. PDMS, which is an elastomeric polymer, was developed by combining a base and cross-linking curing agent in either a 20:1 or 10:1 mass ratio and then curing overnight at 45°C. Glass-PDMS and PDMS-PDMS covalent bonds were formed by treating the surfaces with air plasma for 30 minutes (Atto plasma system, Diener Electronic). This process results in plasma oxidation reactions which bring silanol groups (SiOH) to the surface of the material (Tan et al., 2010). Bringing two plasma-treated surfaces into contact (usually with the addition of uniform pressure and heat up to 80°C) will produce a silanization reaction (formation of Si-O-Si covalent bonds).

Figure 11. Concept schematic of the ideal multi-well plate for conductive fiber mats. The concept of the customized multi-well plate was to have a bilayer plate with a top and a bottom which could be covalently bonded. (Right) This cross-sectional view shows that in between the two plate layers would be the PANI/PG conductive fiber mats. This would secure the mats and prevent delamination during experiments. Additionally, platinum electrodes (shown in black) would be completely isolated from the cell media by the plate. (Left) Developing this concept into a multi-well plate would provide high-throughput capabilities for both stimulating the mats and measuring their electrical properties.
between the two materials (Kim et al., 2011). This is the key factor which makes PDMS and/or glass optimal materials for developing a bilayer cell culture plate.

Initial plate prototypes were designed to have a PDMS top and bottom. The top would be cast from a mold which had two steps in the well: a large outer step with diameter 11.5 mm and a smaller inner step with a 6.8 mm diameter (Fig 12A). This was designed so that the inner well would have the same dimensions of the wells in a classic 96-well plate, but would have a reserve so that higher volumes of media could be added. To provide points through which the electrode wires could exit the plate, copper wire was used in the mold and could be easily removed after the PDMS had cured. When the wires were removed before the cast PDMS was taken out of the mold (Prototype #1-2), there was no binding event between the top and the bottom layers after

Figure 12. Production of multi-well plate for conductive polymer fiber mats. Shown here are different parts and prototypes of the multi-well plate platform. (A) Acrylic mold for prototypes #1-3 and #6. The electrode insertion sites are shaped with small pieces of copper wire which could be removed from the mold before or after removing the cast PDMS. (B) Plate prototype #3. The cast PDMS was removed without removal of the mold wires, and all but three wells were ripped. Intact wells are indicated by blue arrows. (C) Customized acrylic top used for prototypes #5 and #6. (D) Plate prototype #6. Three wells had PANI/PG fiber mats with platinum electrodes (far right), six wells had fiber mats without electrodes (middle), and four wells had glass bottoms (left).
plasma treatment. The cause of this is believed to be the generation of debris that occurred when the wires were pulled from the mold and the PDMS. An accumulation of PDMS debris and dust during removal could have coated the plate layer’s surface sufficiently to inhibit strong covalent bonding with another layer. When the PDMS plate top was removed from the mold without removal of the copper wires, bonding between the top and bottom PDMS layer was successful (Prototype #3). Unfortunately, this resulted in the copper wires ripping most of the wells in the plate during removal (Fig 12B).

Additional plates were made which had an acrylic top layer (Fig 12C). The bottom of the acrylic part was coated with PDMS, and the top had laser-cut holes which would act as the insertion points for the fiber mat electrodes. Prototypes #4 and #5 were produced in this manner, with a PDMS-coated glass bottom and solid PDMS bottom respectively. Those plates had some success, but will still require a few improvements for effective future use.

The last two prototypes, Prototypes #6 and #7, were unique. Prototype #6 aimed to use the same top layer mold as the first few prototypes, but the goal was to incorporate the PANI/PG fiber mats during the curing process. The mats were gently placed in the mold with the electrodes running through the insertion points, and layers of PDMS were added to the mold slowly. The first layer was partially cured before the next volume of PDMS was added. Some iterations of this technique worked (Fig 12D), but in most cases, uncured PDMS would adhere to the fiber mat surface, forming a thin film over the fibers after curing. Removal of this film resulted in the complete destruction of the fiber mats. Prototype #7 was an attempt to completely abandon previous concept designs and simply bind two simple layers of PDMS around the PANI/PG fiber mats with electrodes. The two PDMS layers were completely cured; the top had been cured in a 12-well plate mold (12 mm diameter wells). Electrode insertion points were
made in the top layer using a 2.5 mm biopsy punch. This plate was unsuccessful because the volume of the electrodes between the two layers prevented sufficient contact for water-tight bonding to occur after plasma treatment. A complete summary of all the prototypes can be found in Table 2.

Table 2. Prototypes of bilayer, multi-well plates for fiber mat incorporation

<table>
<thead>
<tr>
<th>Prototype</th>
<th>Top Layer</th>
<th>Bottom Layer</th>
<th>PDMS Ratio</th>
<th>Problem</th>
<th>Additional Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>PDMS from mold with copper wire</td>
<td>Glass</td>
<td>20:1</td>
<td>Insufficient binding of layers</td>
<td>Wires removed before PDMS</td>
</tr>
<tr>
<td>#2</td>
<td>PDMS from mold with copper wire</td>
<td>PDMS</td>
<td>20:1</td>
<td>Insufficient binding of layers</td>
<td>Wires removed before PDMS</td>
</tr>
<tr>
<td>#3</td>
<td>PDMS from mold with copper wire</td>
<td>PDMS</td>
<td>20:1</td>
<td>21 out of 24 wells were ripped by wire</td>
<td>Wires not removed</td>
</tr>
<tr>
<td>#4</td>
<td>Acrylic with PDMS-coated bottom</td>
<td>Glass with PDMS-coated top</td>
<td>20:1</td>
<td>Insufficient binding (poor contact between layers)</td>
<td></td>
</tr>
<tr>
<td>#5</td>
<td>Acrylic with PDMS-coated bottom</td>
<td>PDMS</td>
<td>10:1</td>
<td>No electrodes in the prototyped</td>
<td>Prototype used for imaging OPCs</td>
</tr>
<tr>
<td>#6</td>
<td>PDMS, partially cured layer by layer with mats already incorporated</td>
<td>PDMS</td>
<td>10:1</td>
<td>Most iterations had a thin layer of PDMS cured over the fiber mats</td>
<td>One prototype was successful</td>
</tr>
<tr>
<td>#7</td>
<td>PDMS with 12 well spaces, 12mm diameters</td>
<td>PDMS</td>
<td>20:1</td>
<td>Insufficient binding (poor contact between layers)</td>
<td>Electrodes impeded layer contact</td>
</tr>
</tbody>
</table>

Listed here are all the prototypes that were developed in an attempt to generate a bilayer, multi-well plate into which PANI/PG fiber mats could easily be incorporated for high-throughput experiments. The top and bottom layers were always exposed to 30 minutes of air plasma before bonding was attempted. The PDMS ratio indicates the mass ratio of PDMS base to cross-linking curing agent. A lower base-to-curing agent ratio results in a stiffer polymer.

**Recommendations for Future Work**

The prototypes for a multi-well cell culture plate specifically designed for the incorporation of conductive polymer mats were designed and developed based on the cost-effective tools available in the lab. If this work is continued in the same fashion, I believe that Prototype #5 showed the most promise and should be further investigated. The flexible PDMS bottom ensured a higher amount of contact which could be made between the two layers as they were bonded after plasma treatment. Additionally, having an acrylic top part with a thin PDMS coating on its bottom allowed for the facile removal and reuse of the acrylic because it does not covalently bind to PDMS. Unfortunately, the fiber mats as they are currently fabricated still
provide too great of an obstruction for all the iterations of this plate to successfully bind into a single unit, especially when Pt wire electrodes are present. This plate could be improved by redesigning the fiber mat pieces. Instead of electrospinning onto wire electrodes, electrospinning onto thin platinum foil electrodes adhered to a paper-thin substrate would reduce the volume of the mats, making them easier to incorporate. Additionally, seats could be molded into the bottom PDMS layer into which the fiber mats could cleanly snap.

If provided a broader set of tools and resources, I would have modeled my multi-well culture plates after the children’s toy Snap Circuits® (Elenco). Snap Circuits are basically components of circuits (resistors, connectors, switches, etc.) that can be rearranged by snapping them together in a different design for a different function. In this design, the conductive polymer fiber mats would be fabricated uniformly onto small chip pieces, each with an identical set of stimulating electrodes that remain isolated from the culture portion of each well. The fiber mat chips could snap into place in a multi-well plate, and then two additional pieces could be snapped on top of the plate for either stimulation or for measuring electrical properties of the fiber mats. The stimulating accessory could be easily arranged (just like a Snap Circuit playground) to only stimulate chosen wells. The stimulator would be connected to an adjustable power source. The measurement accessory would snap uniformly into all the wells and could conduct high-throughput analysis of all the fiber mats based on the techniques shown in Figure 7C (see section Results, page 23). The measurements would be multiplexed based on the design of the high-throughput corrosion sensor designed by Matt Whitfield called the CorrHT (Whitfield et al., 2014). The CorrHT system was designed to take rapid resistance measurements of steel wires in different biological conditions in a 96-well plate. Originally this project aimed to capitalize on that existing instrument, and all the plate prototypes were designed with dimensions
that would allow them to easily be incorporated into the multiplexed testing block. Unfortunately, the CorrHT is specialized for measuring materials that have very low resistances, and conductive polymers have very high resistances. The multimeter sensor in this set-up would have to be replaced to effectively capture the properties of PANI/PG fiber mats with high resolution.

Whichever direction future iterations of these plates may turn, it is important to bear in mind the reasoning behind the original design criteria. First, these plates must be optimal for cell culture. Materials used must be compatible with biological conditions. Second, uniformity across the plate must be maintained. Due to the sensitivity of cells in culture as well as the physical and chemical volatility of PANI/PG fiber mats, uniform culture, stimulation, and measurement conditions are necessary to replicate meaningful experimental results. Finally, a multi-well plate specialized for incorporating electrically conductive substrata for cell culture must be fit for high-throughput experiments. The broad goal of this thesis is to investigate the utility of conductive nanofibers in promoting oligodendrocyte progenitor cell (OPC) differentiation while providing the possibility that changes in electrical properties of the fibers may act as early indicators of cell behavior. For these nanofibers to be employable by those studying OPC differentiation and myelination—especially pharmaceutical companies who screen thousands of compounds from drug libraries on cells—the platform should be high-throughput in the sense that it is optimized for a high volume of experiments with the fastest, most-accurate readout possible.
References


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