Published in final edited form as:

Acta Biomater. 2011 July ; 7(7): 2892–2901. doi:10.1016/j.actbio.2011.04.009.

Electro-Conductive Polymeric Nanowire Templates Facilitates *In Vitro* C17.2 Neural Stem Cell Line Adhesion, Proliferation and Differentiation

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Abstract

Stem cells still remain one of the most exciting and lucrative options for treatment of variety of nervous system disorders and diseases. Although there are neural stem cells present in adults, the ability of both the peripheral and central nervous system for self-repair is limited at best. As such, there is a great need for a tissue engineering approach to solve nervous system disorders and diseases. In this study, we have developed electrically conductive surfaces with controlled arrays of high aspect ratio nanowires for the growth and maintenance of neural stem cells. The nanowire surfaces were fabricated from polycaprolactone using a novel nanotemplating technique, and were coated with an electrically conductive polymer, polypyrrole. The polypyrrole coated nanowire surfaces were characterized using scanning electron microscopy and X-ray photoelectron spectroscopy. Additionally, the surface resistance of polypyrrole coated nanowire surfaces was measured. C17.2 neural stem cells were used to evaluate the efficacy of the polypyrrole coated nanowire surfaces to promote cell adhesion, proliferation, and differentiation. The results presented here indicate significantly higher cellular adhesion and proliferation on polypyrrole coated nanowire surfaces as compared to control surfaces. The differentiation potential of polypyrrole nanowire surfaces was also evaluated by immunostaining key neuronal markers that are expressed when NSCs differentiate into their respective neural lineages.

Keywords

Nanowire surfaces; polycaprolactone; polypyrrole; C17.2 neural stem cells; neural tissue engineering

Introduction

Stem cell based tissue-engineering therapies incorporating unique scaffold architectures can have significant benefits to patients currently living with neurological diseases and disorders that involve a functional disturbance and/or a pathological change in the spinal cord. There are several causes of these types of disorders. For example, diseases such as polio, measles or herpes may cause myelitis which is inflammation of the spinal cord[1]. Vascular

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myelopathy is another type of spinal cord disorder and often arises from spinal cord vascular malformations[2]. When spinal cord disorders are caused by trauma, it is referred to as spinal cord injury (SCI)[3]. SCI represent the most troubling population of individuals with spinal cord disorders. Causes of SCI include motor vehicle accidents (44%), acts of violence (24%), falls (22%), sports (two-thirds of these are from diving accidents) (8%), with the remainder classified as “other” (2%)[4]. It is estimated that the annual incidence of SCI, not including those who die at the scene of the accident, is approximately 40 cases per million population in the United States or approximately 12,000 new cases each year[5]. In 2009, the number of people who were living with a SCI has been estimated to be between 231,000 to 311,000[5]. Further, 52% of individuals with SCI are paraplegic, i.e. their motor or sensory function of the lower extremities is impaired and they only have use of their upper extremities. The other 48% are quadriplegic, i.e. their motor or sensory function of the whole body is impaired and they have completely lost use of all their limbs and torso. In contrast to other spinal cord diseases and disorders, most people who get SCI are male (82%) and the injury comes at a young age, the median age at the time of injury is 31.7 years. Unfortunately, there is no clinically proven way to reverse damage to the spinal cord. Current SCI treatment focuses on preventing further injury and empowering people with a SCI to return to an active and productive life as opposed to regaining lost function. However, researchers are continually working on innovative treatments such as direct stem cell injections[6] and stem cell therapy coupled with tissue engineering[7, 8] that may promote nerve cell regeneration or improve the function of the nerves that remain after SCI.

Nanotechnology offers interesting avenues to explore stem cell based tissue engineering therapies to promote nerve cell regeneration or improve the function of the nerves that remain after SCI. Tissue-engineered scaffolds with a unique nanotopography have shown favorable responses to neuronal cells both *in vitro* and *in vivo*. Several *in vitro* studies have reported enhanced functionality of neuronal cells on polymer nanofiber scaffolds[9-11], nanoporous scaffolds[12] and nanowire scaffolds[8]. In addition, there is significant evidence that neural stem cells (NSC) promote extensive axonal growth when implanted with nanofiber scaffolds into the site of a spinal cord injury[13] and can also reduce the functional deficits in a sciatic nerve injury model *in vivo*[7]. Thus, it can easily be concluded that nanotopography on a scaffold surface plays a positive role in neuronal cell-surface interactions. However, most of the scaffolds do not have physiologically relevant surface properties. Mammalian cells are known to preferentially adhere and differentiate on electrically charged surfaces[14]. Therefore, while nanotopography has been shown to enhance cellular response, it seems necessary to further functionalize the scaffolds and create an electrically charged surface for growth and maintenance of NSCs.

The electrically conductive polymer polypyrrole (PPy) has garnered substantial interest for applications in neural tissue engineering due its biocompatibility[15, 16], ease of synthesis[17], and electrical properties[18]. It has been commonly used in biosensors and polymer batteries for these reasons [19]. Studies have shown that PPy has the ability to promote growth of endothelial cells[20], primary neurons[21], and even mesenchymal stem cells[22]. The need for an electrically conductive polymer for neural tissue engineering applications arises from the fact that uncharged surfaces are less than optimal for promoting normal cellular phenotypic behaviors[14]. Furthermore, the ability to deliver an electrical current to the cells via the scaffold surface may have several advantages. Current research has emerged showing that physiologically relevant electrical stimulation can enhance nerve regeneration[23] and help replenish NSCs in an injury site[24]. It is therefore advantageous to design scaffolds that not only have unique surface nanotopography but also have the ability to provide physiological levels of electrical stimulation for the NSCs to modulate and enhance their cellular response.

In this work, we have developed a solvent-free nanotemplating technique for fabricating polycaprolactone (PCL) nanowire surfaces as templates for growth and differentiation of NSCs. These nanowire surfaces were then coated with PPy to provide an electrically conductive surface for cell-surface interactions. PCL was used to fabricate nanowire surfaces due to its biocompatibility and controlled biodegradability. PPy coated PCL nanowire surfaces were characterized using X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy (SEM). Further, the stability of PPy coatings on PCL nanowire surfaces in physiological conditions was evaluated using SEM and XPS. C17.2 murine neural stem cells were used as model cells to study the viability, adhesion, proliferation, and differentiation on PPy coated PCL nanowire surfaces. This cell line was derived from the external germinal layer of a neonatal mouse cerebellum[25]. However, the C17.2 cell line is a good NSC model because after isolation they still retain their ability to differentiate into both neurons and glial cells[26]. The cell line has also been shown to express significant levels of several neurotrophic factors including nerve growth factor, brain-derived neurotrophic factor, and glial cell line derived neurotrophic factor[13].

Experimental Methods

Fabrication of PCL Nanowire Surfaces

PCL nanowire (NW) surfaces were fabricated according to a protocol previously developed in our lab and illustrated in Figure 1[27]. Briefly, nanowire surfaces were fabricated via template synthesis from PCL using commercially available nanoporous aluminum oxide membranes with 20nm pore size (ANOPORE™, Whatman). Polymer discs (10mm diameter and approximately 2mm thick) were placed on the surface of the membrane (Figure 1A), and the nanowire were extruded through the membranes in an oven at 115°C for 3 mins (Figure 1B and C). The aluminum oxide membranes were dissolved in 1M NaOH for 75 mins to remove the membrane thus releasing the extruded nanowires (Figure 1D). Nanowire surfaces were then soaked and rinsed in DI water, dried, and stored in a desiccator until further use.

PPy Coating on PCL Nanowire Surfaces

The as-fabricated NW surfaces were coated with PPy using a polymerization technique described elsewhere[17, 21]. Briefly, the nanowire surfaces were sonicated in a solution of 14mM pyrrole and 14mM sodium para-toluene sulfonate for 30 secs to saturate the surfaces with pyrrole. This was followed by gentle shaking and incubating the nanowire surfaces for 1 hour at 4°C. Next, 38mM ferric chloride was added followed by gentle shaking and incubating for 24 hrs at 4°C. After removing the excess reaction solution, PPy coated PCL nanowires surfaces (PPy-NW) were sonicated for 1 min in DI water followed by drying in vacuum oven.

Characterization of PCL Nanowire Surfaces

NW surfaces, both coated and uncoated, were characterized using SEM, XPS and surface resistivity measurements. The stability of the coating in physiological environment was also characterized using SEM and XPS.

The surface nanoarchitecture before and after PPy coating was evaluated using SEM imaging. The surfaces were sputter coated with 10nm of gold and imaged using a JEOL JSM 6500F at voltages ranging from 10-15kV.

The polymerization of PPy from pyrrole on PCL nanowire surfaces involves the incorporation of chlorine and sodium para-toluene sulfonate, which can be detected using XPS. XPS survey spectra were collected from 0 to 1100eV with pass energy of 187.85 eV,

and high-resolution spectra were collected for C1s peak with pass energy of 10eV. Data for percent elemental composition, elemental ratios and peak fit analysis were calculated using Multipack and XPSPeak 4.1 (Freeware) software.

PPy film thickness on NW surfaces was determined from the attenuation of XPS signals from the substrates. The thickness was obtained by using the standard uniform overlayer model, which is given by equation 1[28-32].

$$I = I_0 \left(\frac{t}{E_L \sin \theta} - 1 \right) \quad (1)$$

where I_0 : intensity of carbon peaks before surface modification
 I_t : intensity of carbon peaks from after modification with PPy
 t : thickness of the film
 E_L : electron attenuation length for carbon peak
 θ : angle at which X-ray hits the surface

In order to obtain film thickness from this equation, the electron attenuation lengths (E_L) for carbon peak needs to be calculated. E_L was calculated using equation 2[29, 32].

$$E_L = \frac{49}{E^2 \rho} + 0.11 \frac{\sqrt{E}}{\rho} \quad (2)$$

where E : electron energy (X-ray core energy – core binding energy for carbon) = (1486 – 285) = 1201eV
 ρ : density of PPy (1.1 gm/cm³)

The surface resistivity of PPy-NW surfaces was measured using a method described elsewhere[17]. In brief, two copper contacts, 0.4 cm wide, were placed 0.5 cm apart on the surfaces and the resistance was measured using a digital multimeter (Omega). The resistivity was calculated using equation 3.

$$\rho_s = \frac{W}{L} R_s \quad (3)$$

where ρ_s : resistivity
 W : width of the copper contacts
 L : distance between the contacts on the surfaces
 R_s : resistance measured using the digital multimeter

The stability of PPy-NW surfaces was determined under physiological conditions. The surfaces were incubated in phosphate buffer solution (PBS) at 37°C for 7 days. After 7 days, the surfaces were washed with DI water and dried followed by characterization using SEM and XPS as described previously.

Neural Stem Cell Culture

C17.2 murine neural stem cells, developed by the group of Dr. Evan Snyder, were used for the studies presented here. This cell line is known to differentiate into all neural

lineages[25]. The medium used for growing the C17.2 cells was Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented by 10% fetal bovine serum (Sigma), 5% horse serum (Sigma), 1% 2mM L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma). The medium was changed every 2-3 days, and the subculture was done at a ratio of 1:10. The cells were maintained in an incubator at 37°C and 5% CO₂ for the entire duration of culture. NW and PPy-NW surfaces were sterilized in 24-well plates by incubating them with 70% ethanol for 30 minutes followed by exposure to UV light for 30 mins in a biosafety cabinet. Prior to cell seeding, all the substrates were washed three times with sterile PBS. C17.2 cells were seeded at a density of 10,000 cells/well. Passage 3 cells from 70% confluent culture dishes were used for all of the studies. Each experiment was reconfirmed on three different substrates with at least three different cell populations ($n_{\min} = 9$). All the quantitative results were analyzed using analysis of variance (ANOVA). Statistical significance was considered at $p < 0.05$.

Adhesion and Proliferation of C17.2 Cells on PCL Nanowire Surfaces

Cell adhesion and proliferation was investigated using calcein-AM live cell stain after 1, 2, and 7 days of culture. The surfaces were removed from culture media; rinsed with PBS and incubated in calcein-AM (2 μ M in PBS) solution for 45 min at 37°C. They were then rinsed in PBS and viewed using FITC MF101 green filter with a Zeiss Axioplan 2 fluorescence microscope. The cell coverage on different surfaces was calculated using the fluorescence images and the Image J software.

Morphology of C17.2 Cells on PCL Nanowire Surfaces

The morphology of adhered C17.2 cells was investigated after 1, 2, and 7 days of culture using SEM imaging. The cells on different surfaces were fixed in a solution of 3% glutaraldehyde, 0.1 M sodium cacodylate, and 0.1 M sucrose for 45 min. The surfaces were then soaked in a buffer solution containing 0.1 M sodium cacodylate and 0.1 M sucrose. This was followed by dehydrating the cells by soaking the surfaces in increasing concentrations of ethanol (35%, 50%, 70%, 95%, and 100%) for 10 minutes each. The cells were further dehydrated by soaking the surfaces in hexamethyldisilazane (HMDS) for 10 min. The surfaces were then dried under vacuum and stored in a desiccator until examined with SEM. They were sputter coated with 10nm of gold and imaged using a JEOL JSM 6500F at voltages ranging from 10-15kV.

Differentiation of C17.2 Cells on PCL Nanowire Surfaces

In order to evaluate differentiation of C17.2 cells into different neural phenotypes, the cells were immunostained for specific marker proteins (Table 1). The cells were fixed with 3.7% paraformaldehyde for 15 mins at 37°C followed by permeabilization with 1% Triton-X in PBS for 5 mins at room temperature. After fixing and permeabilizing, the cells were washed three times with PBS and incubated in 100 μ g/ml of bovine serum albumin (BSA) and 40 μ L/mL trypan blue for 20 mins to block any non-specific interactions. Cells were then incubated in the primary antibodies (concentration 1 μ g/ml) for 1 hr at room temp. The cells were washed three times with PBS followed by incubation in 5 μ g/ml of the appropriate secondary antibody. The cells were then visualized using appropriate filters with Zeiss Axioplan 2 fluorescent microscope.

Statistics

Each experiment was reconfirmed on three different substrates with at least three different cell populations ($n_{\min} = 9$). All the quantitative results were analyzed using an analysis of variance (ANOVA). Statistical significance was considered at $p < 0.05$.

Results and Discussion

Tissue regeneration aimed at spinal cord disorders is vital due to the current lack of treatment options for ailments such as SCI. It is indisputable that a material which is able to foster neural stem cells and coax them to differentiate would be beneficial to finding a cure for many nervous system disorders and diseases. As such, there has been great interest in the field of tissue engineering for solutions aimed at such disorders. Neural cells on scaffolds with unique nanotopography have shown the most promising results for tissue engineering approaches to cure spinal cord diseases and injuries[9, 17, 33-37]. Thus, in this work, we have developed polymeric scaffolds with nanowire surfaces that were bio-functionalized with an electro-conductive polymer capable of providing physiological levels of electrical stimulation to NSCs. The results presented here show that these nanowire surfaces enhance NSC adhesion, proliferation and differentiation for up to 7 days of culture.

NW surfaces were fabricated using the nanotemplating technique described in the methods section (Figure 1). PCL was chosen as the scaffold material due to its biocompatibility and controlled biodegradability. It has been used for variety of tissue engineering applications ranging from cardiovascular repair[38-41], bone tissue regeneration[27, 42-44] and neural tissue engineering[8, 44, 45]. Our unique nanotemplating technique used for fabrication of NW surfaces is especially advantageous because it does not use solvents and can produce scaffolds with consistent surface-bound high aspect nanowires. Figure 2 (NW) shows representative SEM images of as-fabricated NW surfaces. The images show that the NW surfaces have a uniform architecture with occasional interruption in the uniformity by the presence of random microchannels formed due to surface charge effects. Further, high magnification SEM images reveal that the individual nanowires of the surface have a consistent diameter of around 100-150 nm (Figure 2, NW). The reason for the discrepancy between the membrane pore size and nanowire diameter can be accounted due to the thermodynamic expansion of the polymer upon cooling. During the extrusion step of the nanotemplating process, the molten PCL fills the nanopores of the membranes (Figure 1, B and C). As the polymer cools, it exerts pressure on the nanopore walls. Upon dissolving the membrane, the cooled polymer slightly expands resulting in nanowires that larger in diameter than the nanopores. However, by using different pore size membranes, surfaces with different size nanowires diameters can be fabricated using the nanotemplating technique. The nanowire surfaces significant vary for more commonly used electrospun nanofiber scaffolds for neural tissue engineering applications[9, 35]. The nanowires were fabricated using a bottom-up process as compared to top-down process used for fabrication of nanofiber scaffolds. Further, the surface architecture for nanowire surfaces is significantly different than nanofiber scaffolds. The nanowires are vertically aligned from the surface with high aspect ratio compared to nanofibers that are laying down more like a fabric. However, both nanowire surfaces and nanofiber scaffolds are characterized with uniform diameter in the nanoscale with cylindrical morphology.

The NW surfaces were coated with PPy using a polymerization technique as described in the methods section[17, 21]. The need for an electrically conductive polymer for neural tissue engineering applications arises from the fact that uncharged surfaces are less than optimal for neural phenotypic behavior. Additionally, the ability to deliver an electrical current to the cells via the scaffold surface may have several advantages in future tissue engineering applications. Figure 2 (PPy-NW) shows the representative SEM images of PPy-NW surfaces. The images show that the PPy-NW surfaces have a similar architecture as that of NW surfaces at lower magnifications. The nanowires on PPy coated surfaces maintain their orientation, and more importantly the individual nanowires maintain a consistent diameter. Further, high magnification SEM images reveal that the individual nanowires of PPy-NW surfaces have a slightly larger diameter as compared to those on NW surfaces. The high

magnification SEM images also indicate an altered and rougher surface architecture on individual nanowires, which is consistent with literature on PPy coatings on different polymer surfaces (Figure 2, PPy-NW)[17].

NW and PPy-NW surfaces were characterized using XPS to compare the chemical composition of each surface and in turn, determine whether successful polymerization took place on the NW surfaces or not. The surface elemental composition for NW and PPy-NW surfaces was determined using XPS survey scans. Table 2 shows the elemental composition of NW and PPy-NW surfaces. High-resolution C1s and N1s scans were also taken to determine the precise changes in the amounts of carbon and nitrogen on the surface (Figure 3). The results indicate higher amounts of carbon on the surface after modification with PPy. Further, the PPy molecule consists of two amine groups that were detected both in survey as well as high-resolution N1s scans on PPy-NW surfaces. No nitrogen was detected on NW surfaces (Table 2, Figure 3). The survey scans also detected small amounts of sulfur and chlorine on PPy-NW surfaces. This further confirms the polymerization of PPy from pyrrole on nanowire surface since the reaction involves incorporation of chlorine and sodium paratoluene sulfonate. Thus, the XPS results indicate successful modification of NW surfaces with PPy. Further, a standard overlayer model was used to determine the PPy film thickness on nanowire surfaces[29-32]. As expected, the polymerization process deposited a thin layer of PPy on nanowire surfaces. The results from standard overlayer model indicate that the thickness of PPy on nanowire surfaces is approximately 4.162 nm.

The electrical properties of the PPy-NW were analyzed by measuring the surface resistivity (R_s). The PPy-NW surfaces had an average $R_s = 3.68 \pm 1.44$ k Ω /square. For comparison, NW surfaces registered greater than 40 M Ω /square. The PPy coating results in a dramatic decrease in surface resistivity, on the order of 13,000 $\times$ less than NW surfaces. This decrease in resistivity will allow electrical current to conduct through the surface easily where it otherwise would be unable. Such a decrease in electrical resistivity and increase in conductivity may have several advantages for growth and maintenance of NSCs. Studies have shown enhanced cell adhesion on surfaces with lower resistivity *in vitro*[14, 17]. Furthermore, studies have shown enhanced functionality of neurons when exposed to electrical fields *in vivo*[24, 46]. Thus, PPy-NW surfaces fabricated in this study have a two-fold advantage, a unique nanopography and decreased surface electrical resistivity, which can attribute to enhanced NSC response.

The stability of PPy-NW surfaces was monitored for up to 7 days in PBS at 37°C under physiological conditions. High magnification SEM imaging was to determine whether there were changes to the rougher surface architecture of the individual nanowires. The results indicate similar surface morphology of PPy-NW surfaces after 7 days as that of as-coated NW surfaces implying that these coatings are robust and did not rapidly degrade under physiological conditions (Figure 4 and Figure 2 PPy-NW). Further XPS was used to determine the changes in surface composition as well as thickness of PPy coating on NW surfaces. The surface elemental composition for PPy-NW surfaces after 7 days in physiological conditions was determined using XPS survey scans. Table 3 shows the elemental composition of PPy-NW surfaces. The results indicate negligible decrease in carbon, nitrogen, sulfur and chlorine concentrations after 7 days in physiological conditions as compared to as-coated PPy-NW surfaces. Further, the thickness of PPy coating was calculated using standard overlayer model. After 7 days in physiological conditions, the thickness of PPy coating was approximately 3.987 nm as compared to 4.162 nm for as-coated PPy-NW surfaces indicating about 4% decrease in thickness. The surface resistivity was also measured to be approximately $R_s = 3.45 \pm 1.44$ k Ω /square after 7 days in physiological conditions as compared to $R_s = 3.68 \pm 1.44$ k Ω /square for as-coated PPy-NW

surfaces. These results indicate that PPy coating on stable and will be able to withstand physiological environment with minimal degradation.

In order to evaluate the ability of electro-conductive PPy coated nanowire surfaces to maintain growth and differentiated states of neural cells, C17.2 murine NSCs were cultured on NW and PPy-NW surfaces. Our previous work has demonstrated significantly enhanced functionality of PC12 cells on NW surfaces compared to smooth surfaces in terms of adhesion, proliferation, neural network formation and expression of key neuronal markers [8]. Although PC12 cells are typically used to analyze neural cell response to tissue engineering scaffolds[17, 47-49], the C17.2 NSC cell line was used for this study due to the ability of these cells to differentiate into all lineages of nervous system including neurons and glial cells[13]. This cell line was derived after v-myc transformation of neural progenitor cells isolated from neonatal mouse cerebellar cortex. The developmental potential of C17.2 cells is similar to that of endogenous neural progenitor/stem cells in that they are multipotent and capable of differentiating into all neural cell types. Based on the results presented here with C17.2 cell line, we believe that the primary cells would also behave similarly. However, the degree of multipotency may not be similar to that of C17.2 cell line and will depend on culture microenvironment.

NSC adhesion and proliferation was evaluated after 1, 2, and 7 days of culture by staining the adhered cells with calcein-AM and imaging the surfaces using fluorescence microscopy. Calcein-AM is commonly referred to as a “live” cell stain because it is readily transported into the cell and fluoresces upon interaction with esterases. Because dead cells lack the esterases to cause calcein-AM to fluoresce, only live cells appear green. The results indicate that PPy-NW surfaces supported significantly higher cell adhesion as compared NW surfaces after 1 day of culture (Figure 5, Day 1). The cells also seem to have a spreading morphology on PPy-NW surfaces indicating enhanced migration (as indicated by dotted circle). After 2 days of culture, the cells on PPy-NW surfaces have proliferated and appear to be in contact with nearby cells (as indicated by dotted circle) (Figure 5, Day 2). In contrast, while the cells are proliferating on NW surfaces, there appears to be little to no contact aggregation. After 7 days of culture, there are significantly higher numbers of viable cells on PPy-NW surfaces as compared to NW surfaces (Figure 5, Day 7). The cells appear to be forming a “neural network” on PPy-NW surfaces. The fluorescence microscopy images were also analyzed for cell coverage using open-source Image J software. The results of the cell coverage calculations confirm the visual inspection and quantitatively indicate an increase of more than 2× in cell coverage on PPy-NW surfaces as compared to NW surfaces after 7 days of culture (Figure 5, cell coverage). Thus, the results indicate that surface nanotopography enhances cell adhesion and proliferation. However, it is apparent from our results that providing nanotopography with electro-conductive surface significantly enhances cell adhesion and proliferation, thus providing an optimal template for maintaining differentiated states of NSCs.

NSC morphology on NW and PPy-NW surfaces was investigated after 1, 2 and 7 days of culture using SEM imaging. As expected from the fluorescence microscopy images, the cells on PPy-NW surfaces exhibit a more spread out morphology when compared to those on NW surfaces after 1 day of culture (Figure 6, Day 1). After 2 days of culture, the cells on PPy-NW surfaces show a significantly higher degree of spreading and appear to be in more contact with other cells when compared to the cells on NW surfaces (Figure 6, Day 2). High-resolution SEM images for cells on PPy-NW surfaces show that the cellular extensions are relatively shorter and are interacting with the nanowire architecture (Figure 6, Day 2, PPy-NW, image on the right is high magnification for the dotted circle). After 7 days of culture, the cells have formed what appears to be a complex “neural network” on PPy-NW surfaces as compared to the cells on NW surfaces (Figure 6, Day 7). Further, high-resolution

SEM images for cells on PPy-NW surfaces show that the cells have longer extensions that are interacting with the nanowire architecture (Figure 6, Day 2, PPy-NW, image on the right is high magnification for the dotted circle). Such interaction with material nanoarchitecture is an integrin mediated action and is extremely important for maintenance of differentiated phenotypes of NSCs on material surfaces[50]. Further studies are being conducted to understand the mechanisms of cellular interaction with nanowire architecture.

In order to evaluate the efficacy of PPy-NW surfaces for neural tissue engineering applications, it is important to evaluate the ability of these surfaces to maintain the differentiated states of NSCs. Thus, immunofluorescence imaging was used to ensure expression of key neuronal markers in the differentiated states of NSCs on PPy-NW surfaces after 7 days of culture (Table 1). Immunofluorescence imaging was not performed on NW surfaces due to lack of extensive “neural network” formation. The C17.2 murine NSCs are known to differentiate in all neural lineages including neurons, astrocytes and oligodendrocytes. First, the adhered cells were immunostained for Nestin. Nestin is a type VI intermediate filament protein that is expressed mostly in stem cells from the central nervous systems but not in mature central nervous system cells[51]. Its expression is commonly used as a marker for undifferentiated NSCs. Its transient expression is a critical step in the neural differentiation pathway, meaning that its expression indicates that the cells will eventually differentiate into one of the neural lineages. The results indicate that fewer cells expressed nestin suggesting that majority of the cells on PPy-NW surfaces have differentiated into neural lineages, and these undifferentiated NSCs are on the path of neuronal differentiation (Figure 7, Nestin). Further, the cells were immunostained for neurofilament-H (NF-H) and microtubule-associated protein 2 (MAP2), both of which are expressed by neurons[52]. Neurons are electrically excitable cells that processes and transmit information by electrical and chemical signaling. The cytoskeleton of neurons consists of three types of cytosolic fibers; actin microfilaments, intermediate filaments and microtubules. Neurofilaments are the major intermediate filaments found in neurons. They are critical for radial axon growth and determine axon caliber, whereas microtubules are involved in axon elongation. MAP2 is thought to be involved in microtubule assembly, which is a critical step in neuron generation [53]. The results indicate that NSCs are expressing both NF-H and MAP2 on PPy-NW surfaces suggesting that the cells have differentiated into neurons (Figure 7, NF-H and MAP2). The cells were also immunostained for glial fibrillary acidic protein (GFAP), which is primarily expressed by astrocytes. GFAP is thought to help in maintaining astrocyte mechanical strength, as well as the shape of cells[54]. The results indicate that the cells on PPy-NW surfaces are expressing GFAP suggesting that some populations of NSCs have differentiated into astrocytes (Figure 7, GFAP). Astrocytes are important cells, especially in a nervous system disorder application. Astrocytes play a role in the scarring process of the brain and spinal cord following traumatic injuries such as spinal cord injuries[55]. Additionally, astrocytes also provide biochemical support to endothelial cells that form the blood-brain barrier, they provide nutrients to the nervous tissue, and they maintain extracellular ionic balance[56]. Finally, the cells were immunostained for adenomatous polyposis coli (APC), which is primarily expressed by oligodendrocytes. The results indicate that the cells on PPy-NW surfaces are expressing APC, have differentiated, and have morphology unique to oligodendrocytes. The main function of oligodendrocytes is the insulation of axons (the long projection of nerve cells) in the central nervous system[57]. Like all other glial cells, they provide a supporting role for neurons. The expression of APC by oligodendrocytes is known to direct cholinergic synapse assembly between the neurons[58]. Thus, the results presented here substantiate the efficacy of PPy-NW surfaces in maintaining the differentiated states of NSCs into major neural phenotypes.

Conclusion

In this work, we have developed a simple nanotemplating technique to fabricate PCL nanowire surfaces. These surfaces were further coated with PPy to provide a physiologically relevant template for growth and maintenance of NSCs. The results presented here confirm that PPy coating reduces the electrical resistivity of the PCL nanowires to a functional level where electrical stimulation may be applied to control the growth of neurons. Furthermore, the results indicate that decreasing the electrical resistivity of the surface increases the functionality of C17.2 neural stem cells in terms of their adhesion, proliferation, and differentiation. Thus, by controlling the surface nanotopography and providing a physiologically relevant surface charge, neuronal cell functionality can be altered for potential applications in neural tissue engineering. Further studies are now directed towards understanding the mechanisms of NSC functionality on PPy-NW surfaces using proteomics techniques.

Acknowledgments

The authors would like to acknowledge Dr. Evan Y. Snyder, MD, PhD from the Burnham Institute of Medical Research for kindly providing the C17.2 cells. This publication was made possible by Grant Number 1R21AR057341 from NIH-NIAMS.

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Figure 1.

Schematic of PCL nanowire fabrication: **A.** A PCL pellet is placed on top of the alumina nanoporous membrane; **B,C.** The polymer is extruded through the nanoporous membrane in a vacuum oven; **D.** The alumina nanoporous membrane is dissolved in NaOH to release the nanowires; **E.** PCL nanowire surfaces.

Figure 2. Representative SEM images of NW and PPy-NW; the high magnification SEM images show an altered and rougher surface architecture on individual nanowires that were coated with PPy.

Figure 3. High-resolution C1s and N1s scan for NW and PPy-NW surfaces indicating precise changes in surface carbon and nitrogen concentrations after coating the surfaces with PPy.

Figure 4.

Similar surface morphology of nanowires after 7 days in physiological environment as that of as-coated surfaces (Figure 2, PPy-NW) indicating that the coatings are robust and will not rapidly degrade.

Figure 5.

Representative fluorescence microscopy images of NSCs stained with calcein-AM on NW and PPy-NW surfaces after 1, 2, and 7 days of culture; the dotted circle represents cell spreading and contact, and "neuronal network" formation. The figure at the bottom shows cell coverage on NW and PPy-NW surfaces calculated using ImageJ software.

Figure 6. Representative SEM images NSCs on NW and PPy-NW surfaces after 1, 2 and 7 days of culture. Significantly higher “neural network” formation is observed on PPy-NW surfaces. The high magnification images (for the dotted circle) confirm that the cells interacting the nanowire architecture.

Figure 7. Representative immunofluorescence images showing expression of Nestin, NF-H, GFAP, MAP2, and APC on PPy-NW surfaces confirming that the NSCs have differentiated into all neural lineages.

Table 1

Description of key neuronal markers, primary antibodies and secondary antibodies, and blocking agents used for immunofluorescence identification for NSCs.

Differentiation Marker	Description	Primary Antibody	Secondary Antibody	Blocking Serum
Nestin	Type VI intermediate filament protein, expressed mostly in nerve cells where they are implicated in the radial growth of the axon; expressed by undifferentiated cells	Goat Anti-Nestin Polyclonal	Donkey Anti-Goat FITC	Normal Donkey Serum
Neurofilament-H (NF-H)	Approximately 10nm intermediate filaments found specifically in neurons	Mouse Anti-NF-H Monoclonal	Bovine Anti-Mouse FITC	Normal Bovine Serum
Glial fibrillary acidic protein (GFAP)	Intermediate filament protein specific to astrocytes	Goat Anti-GFAP Polyclonal	Donkey Anti-Goat FITC	Normal Donkey Serum
Microtubule-associated protein 2 (MAP2)	Thought to be involved in microtubule assembly, which is an essential step in neurogenesis; expressed by neurons	Rabbit Anti-MAP2 Polyclonal	Bovine Anti-Rabbit TR	Normal Bovine Serum
Adenomatous polyposis coli (APC)	Protein that plays a critical role in several cellular processes; expressed by oligodendrocytes	Rabbit Anti-APC Polyclonal	Bovine Anti-Rabbit TR	Normal Bovine Serum

Table 2

Surface elemental composition (%) measured using XPS survey scans for NW and PPy-NW surfaces

	C	O	N	S	Cl
NW	73.5	26.5	0	0	0
PPy-NW	74.7	15.6	8.4	1.1	0.2

Table 3

Surface elemental composition (%) measured using XPS survey scans as-coated PPy-NW surfaces and PPy-NW surfaces under physiological environment for 7 days.

	C	O	N	S	Cl
Day 0	74.7	15.6	8.4	1.1	0.2
Day 7	73.5	17.5	7.9	0.9	0.2