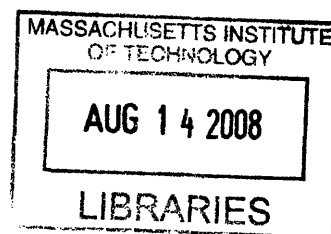


NOVEL POLYMER CONSTRUCTS FOR CONTROLLED
RELEASE AND PRESENTATION OF TOPOGRAPHIC CUES IN
SUPPORT OF NEURONAL CELLS

by
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Novel polymer constructs for controlled release and presentation of
topographic cues in support of neuronal cells.

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Abstract:

In order to improve nerve healing, a new treatment that uses conductive polymer scaffolds to bridge gaps between damaged nerve ends and deliver drugs controllably was explored. In order to optimize neuron growth across scaffolding a neuronal scaffold designed with an electrically conductive polymer, polypyrrole (PPy), will be used as a substrate to enhance nerve cell interaction in vitro. The polymer will be analyzed for the capacity to influence cellular phenotype, including via controlled biomolecular delivery and surface topography. This thesis showed that all these concepts are possible and begins to optimize these characteristics. Successful optimization of these scaffold characteristics will provide a novel method for treating injury in the central nervous system.

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Introduction:

Optimization of polymer scaffolding shows promise for use in the repair of damaged nerves. Spinal cord injury affects many people and treatment has proven to be complex due to multiple inhibitory factors in the central nervous system. In contrast, peripheral nerve injuries show some promise of independent growth. After larger injuries, they are usually treated with autologous nerve grafts. However, the grafting procedure includes several disadvantages including loss of function at the donor nerve graft site, mismatch of damaged nerve and graft dimensions, and the need for several surgical procedures. As an alternative treatment, tubular guidance channels are being examined [11]. Optimizing tubular scaffolds can provide a new viable treatment for peripheral nerve injury by guiding nerve cell regeneration while delivering therapeutic drugs.

This thesis will focus on combining various features in a novel tubular PPy scaffold. Possible advantages of polymers for treating spinal cord injury include guidance therapies, electrical stimulation, biomolecular therapies, and cellular therapies. Research has shown that nanoscale features on substrates and electrical stimuli have influenced neuronal cell growth [18]. Additionally, the capability to deliver therapeutic neurotrophic factors through a polymer based delivery mechanism or stem cell therapy would be beneficial. This thesis examines and optimizes drug delivery, electrical stimulation, and scaffold design in parallel, and investigates practical methods of combining and implementing these features.

1. Background:

1.1 Polypyrrole Scaffold

Polypyrrole (PPy), a conjugated polymer having favorable electrical properties such as conductivity, was can be used to construct scaffolds to treat spinal cord injury. For our applications, PPy was constructed by incorporating various anionic dopants including salts for structural stability and drugs for anti-inflammatory and neuroprotective treatment. Electrically conductive polymers such as PPy can be reversibly altered by electrochemical oxidation and reduction. PPy constructs in direct contact with tissue are promising due to electrical, mechanical, and biocompatibility properties of the polymer [1]. Furthermore, new methods for constructing three dimensional scaffolds have been proved facile using PPy [5, 6].

1.2 Electrically-controlled Release

The capability of electrical stimulation using conductive polymer scaffolds in vivo has the potential to provide several therapeutic treatments for injured neuronal cells [2, 3]. By changing the externally applied electrochemical potential, the surface properties of the substrate are altered which can ultimately improve growth and function of the cells. For instance, electrically stimulation of neuronal cells influences gene expression. Studies have shown that neuronal cells subject to electrical stimuli on PPy show increased neurite growth [12] and neurotrophic factor expression [9, 10]. Electroporation is a potential therapy for treating injured nerve cells. [7, 15], and the capacity of PPy to release GFP plasmid was examined..

1.3 Biomolecular Therapy

Anti-inflammatory drugs have the potential to increase cellular growth and cell regeneration. Studies show that pyrrole can be polymerized with incorporation of anionic drugs and has the potential to release them through electrochemical reduction [14]. After traumatic injury to the nervous system, excitotoxicity causes secondary injury and further damage. Injured cells may cause postsynaptic excitotoxicity by releasing glutamate which binds to post synaptic glutamate receptors. This effect can be reproduced experimentally via N-methyl-D-aspartate (NMDA) which binds to those same glutamate receptors. This thesis analyzed the mitigating excitotoxic effects of minocycline, an anti-inflammatory and neuroprotective drug, after exposure to NMDA. Minocycline was delivered via drug encapsulating PLGA microspheres, and through minocycline-doped PPy films via electrically-controlled release. Release kinetics and therapeutic effects of these methods of delivery were analyzed as well. Therapeutic effects on cells were quantified by identifying cell viability post treatment after exposure to NMDA toxicity.

1.4 Guidance Therapy

Cultured cells rely on the addition of medium supplements for proper growth and function. However, cell substrate interactions have also proven to regulate cellular growth and function [1,17]. Many mammalian cells must anchor onto a cell surface in order to proliferate, and controlling cell surface properties can control shape and function of the cells as well. Culture substrata properties such as charge density, wettability, and morphology influences cell attachment, metabolism, and function. This thesis worked to further support PPy biocompatibility.

Substrate topography influences cell behavior by causing attachment, migration, differentiation, and proliferation. Various forms of contact guidance can be presented to cells such as grooves, ridges, stops, pores, wells, and nodes in both the micro- and nanoscale. New research has shown that nanoscale topography has influenced neuronal cell growth by encouraging polarization and migration [17, 18]. Methods for fabricating PPy thin films with surface topography were analyzed.

1.5 Biocompatibility

The PPy scaffolds will need to be able to support cell growth once implanted. This thesis analyzed neuronal cell biocompatibility on PPy, seeding capability, to ultimately see the effect of implantations in vivo.

2. Methods:

2.1 Polypyrrole Scaffold

2.11 Depositing PPy on films and tubes

PPy films and tubes were polymerized using established protocols [5]. Glass slides coated with Indium Tin Oxide (ITO) were used to deposit PPy films, and platinum wires (100 and 250 micron diameter) and copper wires (1.6mm) were used to construct tubular scaffolds. PPy films (fig 2.111) were polymerized in a 0.2 M pyrrole and 0.2M sodium dodecylbenzene sulfonate (NaDBS, Sigma Aldrich 289957, fig 2.112) solution (3 min, 8mA).

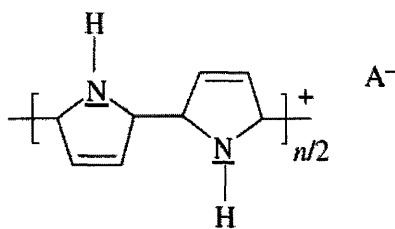


Fig 2.111 Polypyrrole [19]

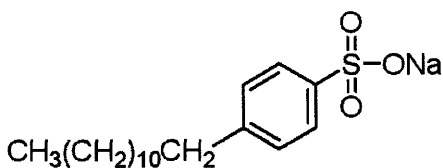


Fig 2.112 NaDBS [19]

2.2 Electrically-controlled Release

2.21 Minocycline Detection

A 20 nM sample of minocycline in water was scanned at various UV wavelengths in order to determine the optimal signal for detection.

2.22 Minocycline Release in PPy

Minocycline was used as the dopant anion (7.5 mM, Sigma M9511). Films were made with 0.2 M pyrrole and 7.5 mM Minocycline and 0.2M pyrrole, 7.5 mM Minocycline and 0.2 M NaDBS. Films were fabricated and minocycline was released in ECIS 8-well chambers (Applied Biophysics) for 24 hrs. PPy tubes (16 mm long on 250 μm platinum wire) were fabricated using a 0.1M pyrrole in acetonitrile and 0.5M Tri-n-

butyltin fluoride (TBTF) adhesion layer and another minocycline release layer (0.1 M pyrrole, 7.5 nM Minocycline, 0.25 M NaDBS) using the same solution used for the films. The adhesion layer was plated initially (8 mA, 10 min) and the minocycline layer was plated afterwards (8 mA, 70 min). Minocycline was then released from these tubes under various stimulation parameters (DC -2V, AC 166.66 Hz, AC 16.66 Hz, and no stimulation control).

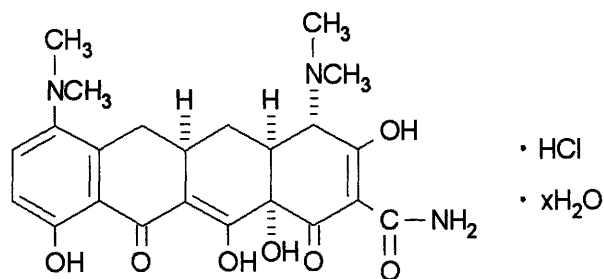


Fig 2.221 Minocycline [19]

2.23 Electroporation and Transfection

ECIS 8-well chambers from Applied Biophysics (fig 2.221) were purchased and plated (1 mA, 2 min) with PPy and DNA (0.2 M pyrrole, 0.1M NaDBS, and 0.2 M GFP plasmid, PE-GFP). Afterwards, DNA was released using various electrical stimulations (Table 3.211). The amount of DNA released was detected using the Picogreen Assay. HeLa cells were seeded on the film during stimulation and were observed for transfection afterwards using fluorescent imaging.

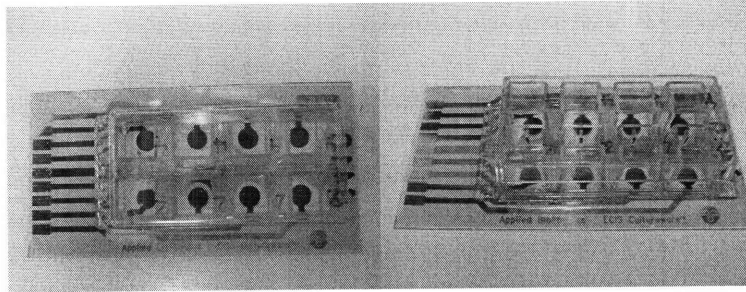


Fig 2.231 ECIS 8 well chambers

2.3 Biomolecular Therapy

2.31 Minocycline Release in Microspheres

PLGA microspheres encapsulating minocycline were made by a traditional double-emulsion w-o-w (water-oil-water) method with minocycline dissolved to saturation in Tris-EDTA (TE) buffer. Blank microspheres were made using TE-lactose instead of minocycline. Afterwards, microsphere encapsulation efficiency and release kinetics were analyzed. Encapsulation statistics were obtained after dissolving microspheres and detecting the amount of minocycline released using spectrometry (Test samples were 600 μ l 1M NaOH, 600 μ l 1M NaOH and 2.40 mg minocycline, 600 μ l 1M NaOH and 6.00 mg minocycline microspheres, and 600 μ l 1M NaOH and 4.75 mg of blank microspheres). Measurements for release kinetics were obtained by dissolving 5 mg of minocycline and blank microspheres in 1.25 mL of PBS for 6 weeks.

2.32 Toxicity Assay

In an in vitro model of glutamate excitotoxicity, E18 primary rat spinal cord neurons were cultured for 24 hours, and then exposed to 300 μ M NMDA. Experimental

groups were treated with minocycline-encapsulated PLGA microspheres, or blank PLGA microspheres, and neuronal survival was quantified via the MTT colorimetric assay.

2.4 Guidance Therapy

2.41 Fabrication of Surface Topography

Polydimethylsiloxane (PDMS) chambers with microscale and nanoscale features were constructed and were used as guides to polymerize PPy films. An ITO slide and a PDMS mold with gold plated roof were used. The gold plated roof was the ground and it contained nanoscale features. Pyrrole was polymerized inside the channel using the same polymerizing parameters used for making PPy films. The duration of the plating step was extended until proper features are seen.

PDMS molds were used to etch patterns in ITO as well, and consequently were used to polymerize pyrrole into certain patterns. Microchannel molds were fabricated using SU8 lithography techniques (fig 2.411) and used to create channels in PDMS. The PDMS was then attached to ITO slides and an acid solution (20% HCl and 2% HNO₃) was flown through (microfluidic connections from Upchurch Scientific) to etch the ITO for 20 seconds and then neutralized with K₂NO₃ (2 % by mass). The effect of the etching was observed by electropolymerizing pyrrole onto the slides.

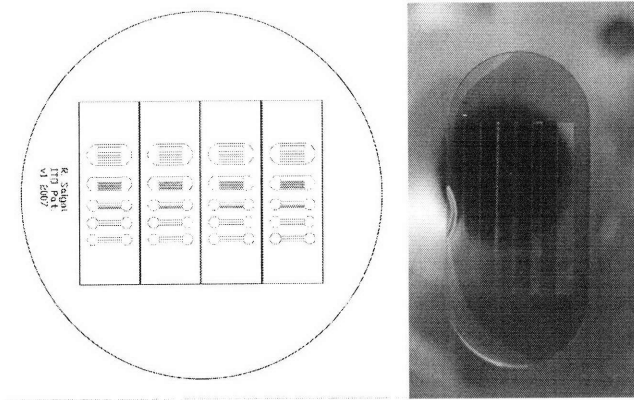


Fig 2.411 Channel mask and wafer

2.5 Biocompatibility

2.51 Biocompatibility Assays

PPy tubes were fabricated on 14AWG copper wire (McMaster 7125K412). A 30mm copper wire was submerged in pyrrole solution and oxidized for 1hr at 10mA at room temperature. Afterwards the tubes were reduced for 2min at -10V and the resulting 30mm PPy tubes cut into 7.5mm pieces. Twelve tubes were made with usual smooth texture. Twelve silicone tubes (Tygon 3350, 1/16" inner diameter, 1/8" outer diameter) were used as controls. Cells were then seeded (15.6 ul, 1.3 mill cells/ml, neural basal media) into tubes. Cells were allowed to proliferate in the tubes and MTT (Sigma) or CellTiter (Promega) assay was consequently conducted on these samples.

2.52 Cell Cultures

Primary Rat Neuronal Cells (Neuromics) were obtained and cultured in Neurobasal medium as per standard protocols.

3. Results

3.1 Polypyrrole Scaffold

3.11 Depositing PPy on films and tubes

The resulting PPy films and tubes are shown in the fig 3.111. Fig 3.111a shows PPy tubes of different sizes. The larger tubes have a 1.6 mm inner diameter and the smallest tubes have 100 μm diameter. The larger tubes were released from their copper wires and the smaller tubes are still attached to their platinum wire. The pyrrole polymer is brittle, smooth, slightly transparent, and black.

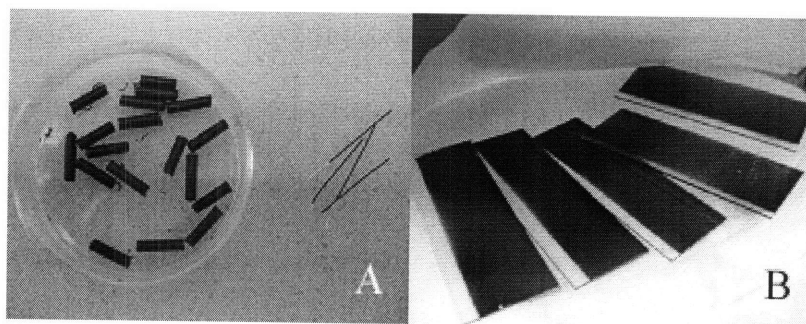


Fig 3.111 PPy tubes (A) and films (B)

3.2 Electrically-controlled Release

3.21 Minocycline Detection

After the minocycline solution was scanned through a range of UV wavelengths, the resulting signal intensities are graphed in fig 3.221.

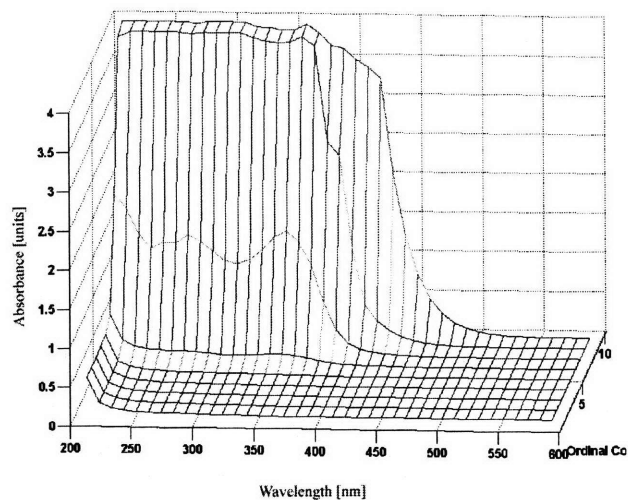


Fig 3.211 Minocycline has a characteristic absorbance peak at 350nm, allowing detection by UV/vis spectrophotometry.

3.22 Minocycline Release in PPy

The amount of minocycline released from PPy films is plotted in fig 3.221. PPy films were plated with pyrrole and minocycline or pyrrole, minocycline, and sodium dodecylbenzene sulfonic acid (NaDBS). Plating and releasing parameters were optimized from this initial release experiment and used when releasing minocycline from tubular scaffolds (fig 3.222).

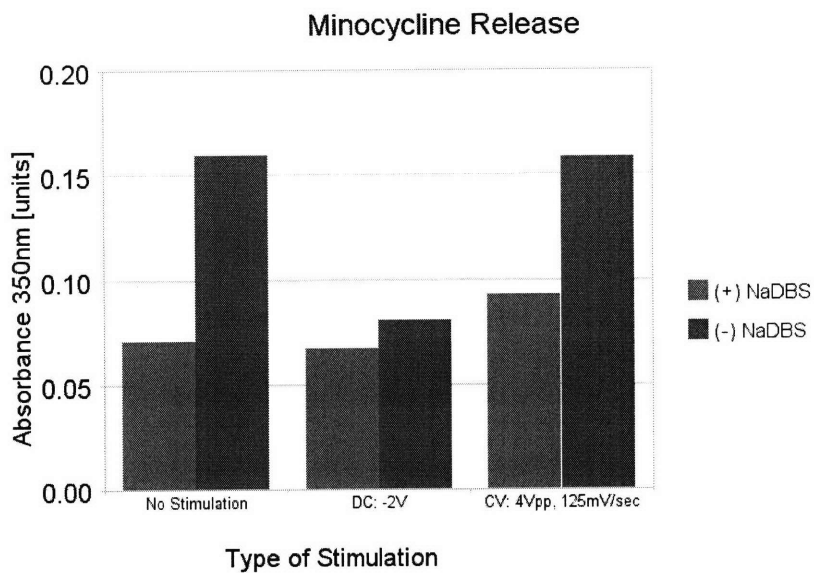


Fig 3.221 Minocycline release from PPy films

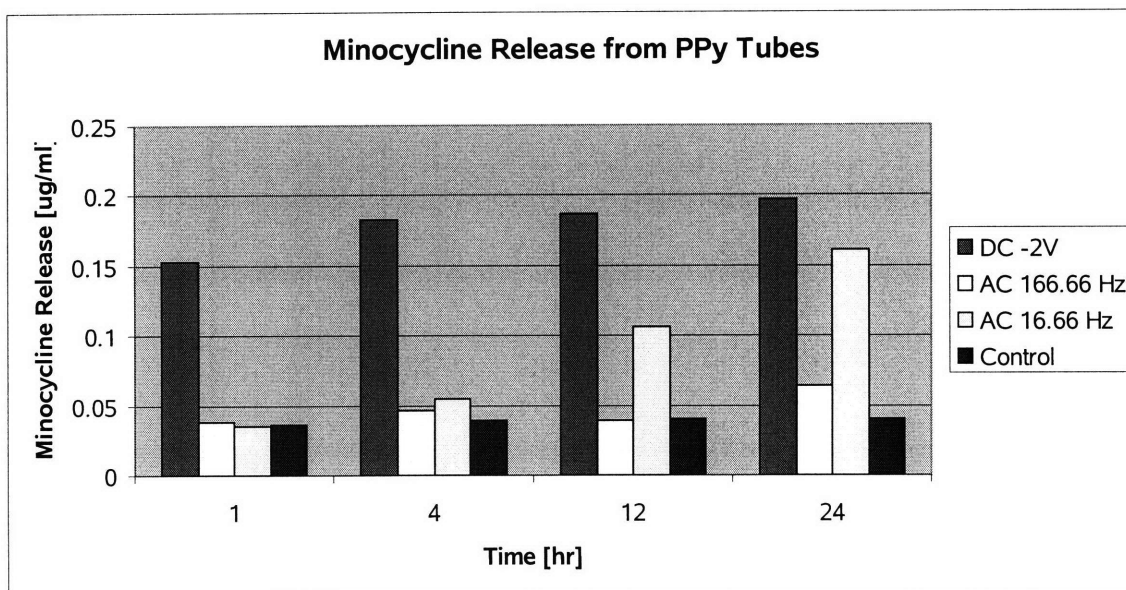


Fig 3.222 Minocycline Release

3.23 Electroporation and Transfection

Figure 3.231 shows how much DNA was released after electrical stimulation of PPy films containing DNA. The calculated release for the no DNA control (type E in

figure below) was -1.2664 ng/ml so it was omitted from the graph. Cells transfection was assayed by FACS for transfection afterwards and a minute number of cells showed GFP expression.

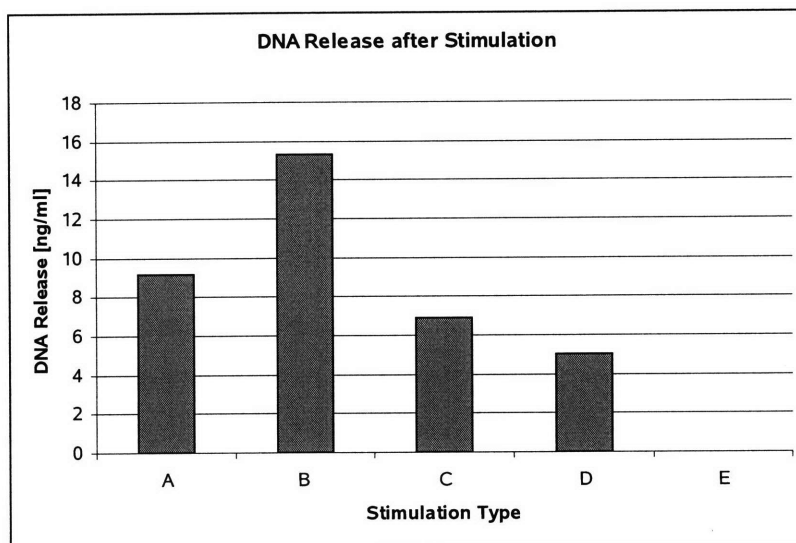


Fig 3.231 DNA release from PPy films. Stimulation types are referred to in Table 3.231.

	Stimulation
A	-2V x 1 hr
B	3x -250V Pulse
C	CV 4V _{peak-to-peak} , 16.667 mHz x 1hr
D	-2V x 1hr, then 3x -250V pulse
E	-2V x 1hr, then 3x -250V pulse (NO DNA plated in well)

Table 3.231

3.3 Biomolecular Therapy

3.31 Minocycline Release in Microspheres

In addition to release from PPy, minocycline released from microspheres was analyzed as well. The microsphere statistics are given in table 3.321 and the loading characteristics are given in table 3.322. The microsphere release kinetics is displayed in fig 3.321.

Mean	Std. Dev.	Mode
4.1 μm	1.4 μm	5 μm

Table 3.311 Microsphere size statistics.

	10mg/mL drug	50mg/mL drug
Loading efficiency	22.7%	11.1%
Yield	16.8%	7.0%
$\mu\text{g drug / mg}$ microsphere	2.84	6.93

Table 3.312 Loading characteristics

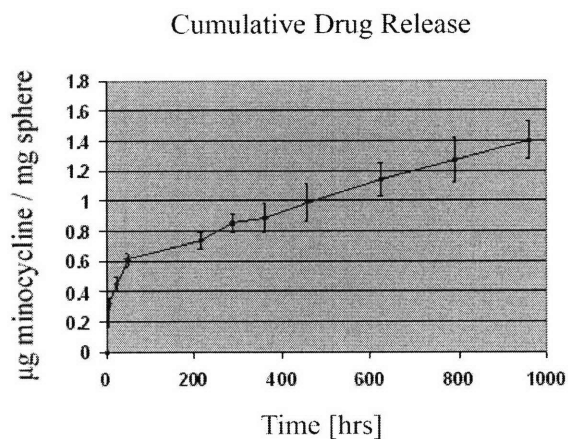


Fig 3.311 Cumulative drug release

3.32 Toxicity Assay

The results of minocycline microsphere treatment are provided in fig 3.331. There was a larger amount of cell viability with minocycline treatment using microspheres.

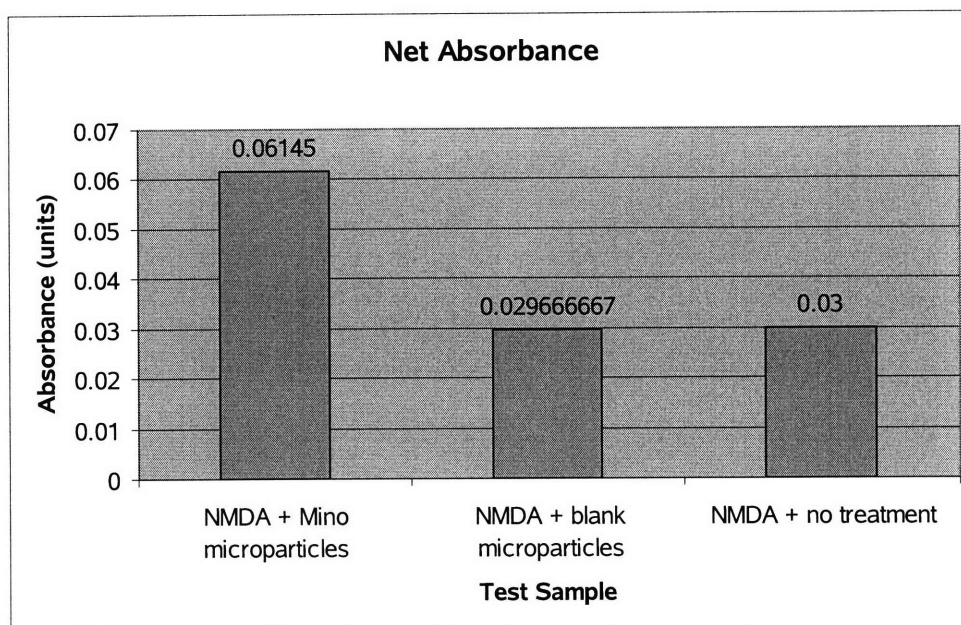


Fig 3.321 Neuronal cell survival (MTT)

3.4 Guidance Therapy

3.41 Fabrication of Microscale Topography

There was evidence of polymerization inside the mold and there was evidence of some microscale features on the pyrrole film. Additionally, acid proved to be able to etch ITO into certain patterns (fig 3.411).

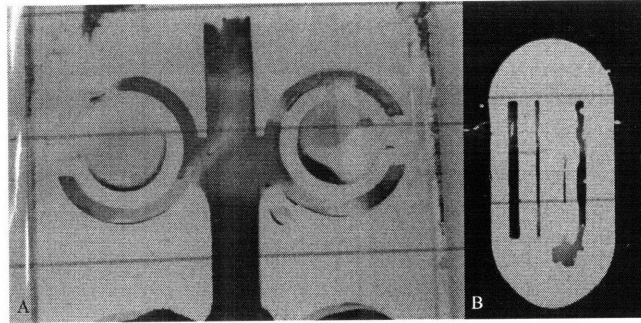


Fig 3.411 ITO patterned with acid solutions and polymerized with PPy

3.5 Biocompatibility

3.51 Biocompatibility Assays

Primary neuronal cell viability was measured after 7 days and plotted below in fig 3.511. Higher absorbance in the CellTiter assay (Promega) corresponds to a greater number of viable cells.

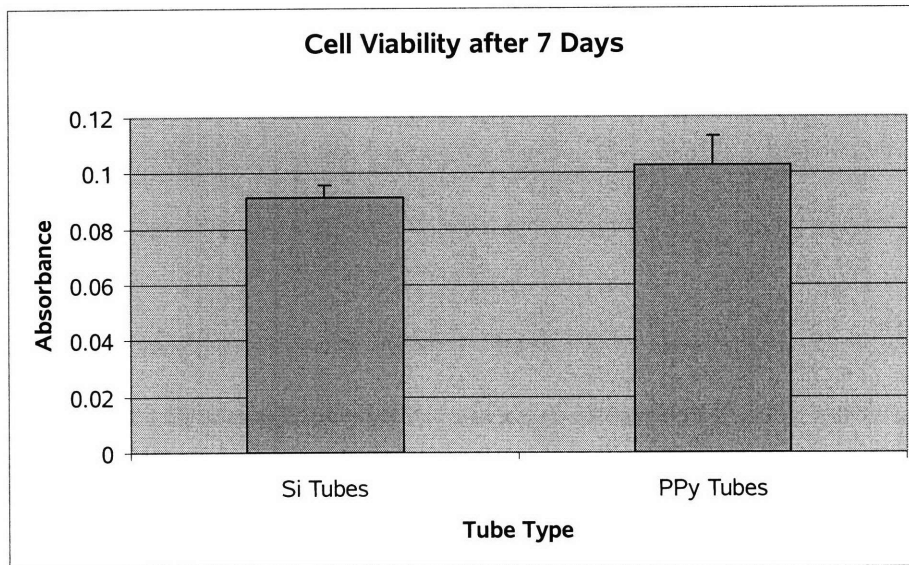


Fig 3.511 Primary Neuronal Cell Viability

4. Discussion:

4.1 Polypyrrole Scaffold

4.11 Depositing PPy on films and tubes

The pyrrole polymer has important characteristics that make it a useful biomaterial. Several of those qualities are being tested and analyzed in this thesis. Fig 3.111 shows two possible applications of the PPy polymer. The electropolymerization of pyrrole into PPy is straightforward and allows for fabrication of scaffolds of arbitrary geometry. Separating PPy structures from their electrode templates can be achieved by a brief period of electrochemical reduction, allowing for mechanical separation of the scaffold [11]. Applying a reducing potential may also cause the polymer to release its dopant anion (fig 2.111), so optimization of scaffold release is necessary to maintain mechanical integrity. This characteristic provides the potential to release therapeutic anionic molecules by applying a reducing potential.

4.2 Electrically-controlled release

4.21 Minocycline Detection

Ultraviolet (UV) spectrometry at 350nm was concluded to be an optimal wavelength for measuring minocycline absorbance. This wavelength is ideal due to the absorbance of UV light from the conjugated double bonds in minocycline. There was some concern of signal interference by pyrrole which has conjugated double bonds as well. This dilemma was addressed by including controls for this scenario in the experiments. Additionally, the degradation of the film can provide some interference in absorption

measurements due to the large, pieces interfering with the light path. This information proved helpful in designing an assay for minocycline release.

4.22 Minocycline Release in PPy

Fig 3.221 provides evidence of drug delivery using PPy. The graph shows that minocycline release can be better controlled by incorporating NaDBS, an anionic dopant salt, alongside the drug when plating the film. The PPy film plated with only minocycline was not as mechanically stable as the film plated with drug and salt, which can be seen by the large amounts of minocycline released without stimulation. This might be due to the polymer readily degrading. Each oxidized pyrrole molecule in the polymer backbone requires a dopant anion to maintain charge neutrality (Fig 2.111). Minocycline is a larger molecule (MW 457.477) with more charged functional groups but not intrinsically anionic (fig. 2.221). Due to its larger size and non-anionic nature, it might be harder to stably incorporate in the polymer. Additionally, its multiple charged functional groups might add to the polymer instability. Inclusion of a smaller, negative dopant anion salt (NaDBS, MW 348.48) allowed for formation of a more stable polymer.

The results from the first experiment were used to analyze release of minocycline from PPy tubes. Fig 3.222 shows a steady release of minocycline using an alternating current (AC). The higher frequency resulted in less release. During higher frequencies, the polymer is exposed to the negative potential for a shorter duration per period resulting in less release. The 16.66 mHz stimulation release resulted in a steadier release profile than direct current (DC). The AC group was subjected to a 4 Volts peak-to-peak triangular wave. During the release phase, the polymer was thus exposed to a maximum of -2 V as

well. A DC potential appears to cause a more dramatic burst effect, leading to inability to achieve steady-state long-term release. This may be a result of structural damage inflicted onto the polymer thin film due to the DC potential. This can possibly introduce experimental artifact and overestimate of drug release, if released pyrrole monomers increase absorbance at 350nm. However, the experiment shows the potential of using a PPy scaffolds as a means of delivering minocycline using AC. It provides the potential to administer drugs locally which should minimize adverse effects of systemic administration.

4.21 Electroporation and Transfection

Minocycline detection using UV spectrometry may be hindered due to reduction and release of pyrrole monomers into the sample. The PPy film that was exposed to 1hr of -2V x 1hr, then 3x -250V pulses did not release as much or more than samples at either the DC potential or pulses alone. This may be due to inadequate loading of DNA in the PPy film. The experiment should be repeated with higher sample size and more DNA loading. A small number of cells were found to express GFP, indicating proof of concept. More experiments need to be conducted to optimize transfection parameters.

The results show that PPy has the potential to incorporate and release DNA, which can be used for various therapeutic applications. More DNA was released with quick pulses than with a steady direct voltage. It will be difficult to use such large pulses in the human body, however, the study did show that measurable amounts of DNA was released with application of lower voltages for longer periods. Large pulses of voltage may cause problems with the structural integrity of the scaffold since there were some visible signs of film degradation with the pulses. Cyclic voltammetry elicited observable release of DNA

even though it was less than the DC or pulsatile groups. This could be due to DNA being reincorporated in the polymer during phases of positive potential. The PPy film did show less signs of degradation under cyclic stimulation.

4.3 Biomolecular Therapy

4.31 Minocycline Release in Microspheres

Microspheres have demonstrated the ability to controllably release minocycline. This vehicle of drug delivery provides the potential to deliver drugs locally as well. The release profile (fig 3.311) shows that the minocycline microspheres released constant amounts of drug for roughly six weeks. The loading characteristics were not ideal (fig 3.312), due to the small amount of minocycline encapsulated in the microspheres. The protocol for drug encapsulated microspheres takes advantage of drug solubility properties. Due to minocycline's charged functional groups, the molecule is not completely hydrophobic, so some drugs diffuse into the aqueous phase during microsphere encapsulation. Therefore, much of the drug is lost before it is encapsulated inside the PLGA polymer. Faster transfer during these steps might prove helpful in improving the loading efficiency.

4.32 Toxicity Assay

Minocycline encapsulated microspheres were able to aid survival of primary neuronal cells in vitro (fig 3.321). Improved survival was already present at 24 hrs and with a final minocycline dosage of 200 nM. These results provide the potential for using minocycline microspheres as a therapeutic approach for treating nerve injury.

4.4 Guidance Therapy

4.41 Fabrication of Microscale Topography

Results show that pyrrole can be polymerized inside PDMS molds and potentially be able to form microscale patterns. The protocol needs to be optimized, but the concept has some possible applications in fabricating smaller features on pyrrole, possibly down to the nanoscale. Prospects for improvement include a more robust and sealed fluidic network and more robust connection pads for the polymerization steps. The possibility of etching ITO in order to polymerize pyrrole into patterns is possible using microfluidics and acid etchants (fig 3.411). Inadequate sealing resulted in the broken patterns. Additionally, it was difficult to attach and the positive and negative electrodes on the ITO during the electropolymerization step, which helped create the broken patterns as well. Fig 3.411b show polymerization of pyrrole channels of various length. The smallest channel width that was etched effectively is 100 μm . The smaller channels were not large enough to completely seal off the acid. Pyrrole polymerizes isotropically [6] so mask dimensions and electropolymerizing durations need to be designed accordingly.

4.4 Biocompatibility

4.51 Biocompatibility Assays

In order to provide cellular therapy, pyrrole scaffolds need to be hospitable to cellular proliferation. Results of the MTT assays show that PPy tubes are at least as biocompatible as silicone tubes, with a similar cell survival one week after seeding on each substrate. PPy allows for the possibility of electrically stimulating cells while in culture.

Additionally it supports the idea of having cells grow onto the scaffold during healing in vivo.

5. Conclusions

This thesis analyzed novel polymer constructs based on polypyrrole or PLGA for treatment of injuries in the peripheral and central nervous system. Potential uses of these polymers that have been analyzed include possibilities for electrically-controlled release of clinically relevant biomolecules, cellular therapy, and guidance therapy. The results show that PPy can be fabricated into potential scaffolds and its conductive properties allow for electrical stimulation of cells. The potential for controlled, local drug release has also been shown both in PPy polymers and in PLGA microspheres. PPy has the capacity to be patterned and methods for implementing nanopatterns on the scaffolds are being studied.

The research conducted in this thesis will be continued and these characteristics will be further analyzed, optimized, and ultimately compiled into a single scaffold. Future work will include characterizing ideal stimulation parameters for drug release, identifying ideal electrical stimulation patterns on cells and determining the resulting change in morphology and neurotrophic factor secretion. Biocompatibility will be further analyzed on PPy tubes for potential cell therapy as well as on PPy containing microscale and nanoscale features. Survival assays will continue to be done on biomolecular therapy applications as well as cellular therapy applications. Once cell survival is adequate, scaffolds will be constructed for future in vivo experiments. Additionally, after determining an ideal protocol for patterning nanoscale topography onto the scaffold, in

vitro experiments can be conducted to assess synergistic effects of mechanical, electrical and biomolecular cues on neuronal growth.

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