

Grant Project

ChE 575

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Specific Aims

The spinal cord is the body's most important organ for relaying nerve signals to and from the brain and the body. However, when an individual's spinal cord becomes injured due to trauma, their quality of life is greatly diminished. In the United States today, there are an estimated quarter of a million individuals living with a spinal cord injury (SCI). With an additional 12,000 cases being added every year. Tragically, there is no approved FDA treatment strategy to help restore function to these individuals.

SCIs are classified as either primary or secondary events. Primary injuries occur when the spinal cord is displaced by bone fragments or disk material. In this case, nerve signaling rarely ceases upon injury but in severe cases axons are beyond repair. Secondary injuries occur when biochemical processes kill neural cells and strip axons of their myelin sheaths, inducing an inflammatory immune response. In the CNS, natural repair mechanisms are inhibited by proteins and matrix from glial cells, which embody the myelin sheath of axons. This actively prevents the repair of axons, via growth cone inhibition by oligodendrocytes and axon extension inhibition by astrocytes.

A promising treatment to SCI use tissue engineered scaffolds that are biocompatible, biodegradable and have strong mechanical properties *in vivo*. These scaffolds can secrete neurotrophic factors and contain neural progenitor cells to promote axon regeneration, but further research is required to develop this into a comprehensive treatment. This proposal will present the framework required to build a scaffold harnessing multiple methods from different directions to solve the problem. Here, we seek to systematically construct a collagen-poly[N-(2-hydroxypropyl) methacrylamide] (PHPMA) hydrogel scaffold with fibrin neurotrophic factor eluting nerve guidance channels to promote axon regeneration and to direct growth from the proximal to the distal nerve stump for primary SCI injuries.

AIM 1: Determine the impact of chondroitinase ABC, and mouse monoclonal antibody 11c7 on the formation of glial scars, chondroitin sulfate proteoglycans, and axonal regeneration.

Hypothesis: Chondroitinase ABC and 11c7 will break down extracellular matrix that down regulates axonal nerve growth and inhibit its down regulating effect, and will promote larger axonal nerve growth.

Method: Use an *in vitro* model similar to a spinal cord nerve and create surgical lesion. Treat with chondroitinase ABC and 11c7 and measure the results with time lapsed microscopy.

AIM 2: Prevent ependymal cell differentiation to scar forming astrocytes.

Hypothesis: Introduction of neurogenin-2 to a spinal lesion site will both promote axonal regeneration and prevent glial scar formation by directing the differentiation of ependymal cells.

Method: Two groups of mice will receive induced spinal cord lesions. One group will be administered treatment through neurogenin-2 injection; the other group will be a control receiving no treatment. Both groups will be modified using tamoxifen-dependent Cre recombinase to allow yellow fluorescent protein to bind to the Connexin 30 gene, and green fluorescent protein to the Olig2 gene. The expressions of these genes are markers for the proliferation of scar forming astrocytes and myelinating oligodendrocytes respectively. A comparison of the gene expression between the two groups of mice using electron microscopy will reveal the fate of ependymal cells post-lesion.

AIM 3: Construct multiple fibrin nerve guidance channels (NGCs) within a collagen scaffold, embedded with nerve and neurotrophic growth factors, which is to be injected with poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA) hydrogel for increased axonal cell proliferation.

Hypothesis: Collagen-PHPMA hydrogels will provide an adhesive surface and an adequate microenvironment for axonal regeneration. NGCs and growth factors will help to facilitate and to improve the functional recovery, tissue preservation, and neuronal regeneration in SCI.

Method: Collagen scaffolds will encase electrospun fibrin NGCs and PHPMA that is injected into the interstitial space. Collagen is highly biocompatible and biodegradable, and will aid in mimicking the extracellular matrix (ECM). Similarly, PHPMA has been shown to promote tissue ingrowth, angiogenesis, and axonal regeneration, as well as limit glial scar formation *in vivo*. The following proteins are spin coated within the NGCs to build a powerful regeneration construct: nerve growth factor, neurotrophin-3, neurotrophin-4. Anterograde axonal tracing is used to determine the combination that maximizes neuron regeneration.

Significance

SCI can vary in both severity and cause. Most SCI are a result of falls (28.5%) and vehicular accidents (36.5%), putting anyone at risk for SCI. These injuries are often difficult to treat due to their neurological roots in motion and sensation, and are a large unmet medical need. Due to the rather arbitrary occurrence and varying levels of severity of SCI, no well-developed FDA-approved regenerative treatments have been created for these patients [1].

The costs to these injuries are enormous. For the first year after the injury, cost can range from \$340,000 to \$1,000,000 depending on severity. Figure 1 illustrates how location of injury and loss-of-function are related. The average annual cost for people living with SCI can be over \$70,000, which puts the lifetime costs from some over \$4,500,000 [1].

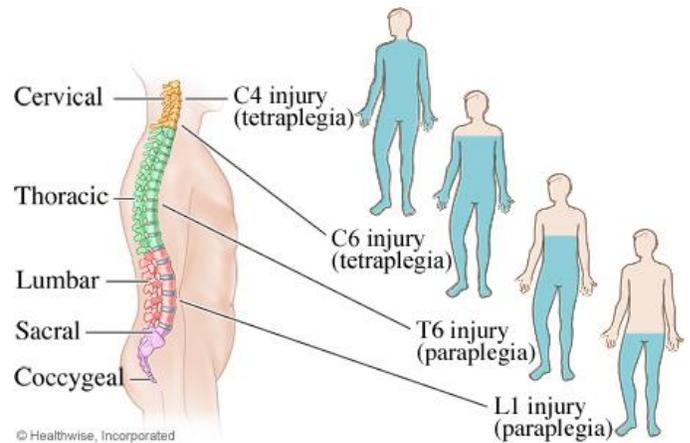


Figure 1: Loss of function is governed by the location of injury within the spinal cord [2].

Figure 2 shows the life expectancy for varying levels of SCI compared to the average American lifespan. Normal life expectancy is considerably higher than those sustaining any type of spinal cord injury when compared to all patients that survive the primary cause of injury [1]. Patients that sustain severe injuries at older ages can see a 91% decrease in longevity compared to those without SCI. Almost all stages of SCI will require a patient to need help in managing basic bodily functions and day-to-day tasks. This can lead to increased stress for families, caretakers and health insurance companies. Although advances in technology have increased overall survival rates, there has been no increase in long-term survival in the past 30 years for those sustaining SCIs [3]. We plan to design a biodegradable combinational collagen-PHPMA hydrogel to help increase longevity in those patients currently suffering with SCI.

		Life expectancy (years) for post-injury by severity of injury and age at injury										
		For persons who survive the first 24 hours					For persons surviving at least 1 year post-injury					
Age at Injury	No SCI	AIS D - Motor Functional at Any Level	Para	Low Tetra (C5-C8)	High Tetra (C1-C4)	Ventilator Dependent- Any Level	AIS D - Motor Functional at Any Level	Para	Low Tetra (C5-C8)	High Tetra (C1-C4)	Ventilator Dependent- Any Level	
20	59.0	52.0	44.6	39.6	35.3	18.6	52.4	45.1	40.4	36.6	24.9	
40	40.0	33.7	27.2	23.1	19.7	8.4	34.0	27.7	23.7	20.7	12.3	
60	22.7	17.5	12.7	9.9	7.8	2.0	17.7	13.0	10.3	8.4	3.7	

Figure 2 Overall life expectancy for people living with a SCI based on varying levels of injury and age of occurrence (2013).

PHPMA-collagen hydrogels with neurotrophin-2 and 3 growth factors are our proposed way to target and regenerate nerve growth at the site of injury. By using neurotrophin-2 in our hydrogel scaffold, we believe we will see a reduced expression of glial scar cells, allowing neurons to regrow. By returning function to the body, we will be able to increase patient quality of life and longevity. The treatment will be a viable alternative to current treatment by reducing the lifetime cost of treatment of SCI.

We will focus on designing a hydrogel scaffold with guidance channels that induce neurological growth in the correct direction. A series of animal model experiments will be conducted as a basic proof of concept trial of neuronal cell growth in our combinational hydrogel. The experiments will be used further to observe the results of our method to suppress glial scar growth and guide neuron regrowth within our hydrogel.

Innovation

Current methods to treat SCI on the basis of scaffold implantation and cell-based regeneration are clinically ineffective to date. However, this platform serves to broaden the notion of combinatorial tissue engineering approaches as a robust way to treat SCI. Research is largely ongoing in designing therapeutic scaffolds for

SCI treatments, yet many approaches lack a multi-layered design that is capable of mimicking the entire complexity of the SCI lesion for regenerative purposes. The proposed hydrogel scaffold will be a pioneer in its ability to direct neuronal tissue growth in the lesion cavity and rebuild synapses for functional recovery. This novel approach is superior to other current design strategies due to two main advantages that it presents.

1. The collagen-PHPMA hydrogel scaffold will enable tissue ingrowth, angiogenesis, and axonal regeneration, as well as limit glial scar formation, which provides a strong basis for regeneration.

Woerly et al. have shown that PHPMA (Neurogel™) hydrogels can promote tissue repair and reduce necrosis in SCI [4]. Collagen is a key addition to the PHPMA model because it will aid in recapitulating the ECM and mimicking the neural tissue that we seek to regenerate. Our collagen-PHPMA hydrogel scaffold will be the base of regeneration, and will functionalize the SCI lesion by providing a biocompatible, biodegradable bridge in the transected spinal cord.

2. The hydrogel scaffold will release neurotrophic factors from nerve guidance channels over time, which allows for targeted neuronal and functional recovery.

Kim et al. have developed microsphere-releasing chitosan guidance channels (Figure 3) to deliver therapeutic molecules to enhance regeneration through a novel spin-coating technique [5]. They achieved favorable sustained release, but no meaningful recovery of function. Johnson et al. observed increased neural fiber density within the SCI lesion through the controlled release of neurotrophin-3 [6]. Their findings support the hypothesis that certain proteins can promote neural fiber sprouting, but their design has several limitations. Our model will release the protein which enhances the highest level of neural fiber sprouting from engineered nerve guidance channels.

The proposed research holds immense potential to shift the basis of current research to encompass combinatorial tissue engineering approaches to help rebuild after SCI. Our work is the crucial foundational work that is needed to lead to the implementation of advanced techniques in the development of a technology that can be used to treat patients with SCI in the clinic in the near future.

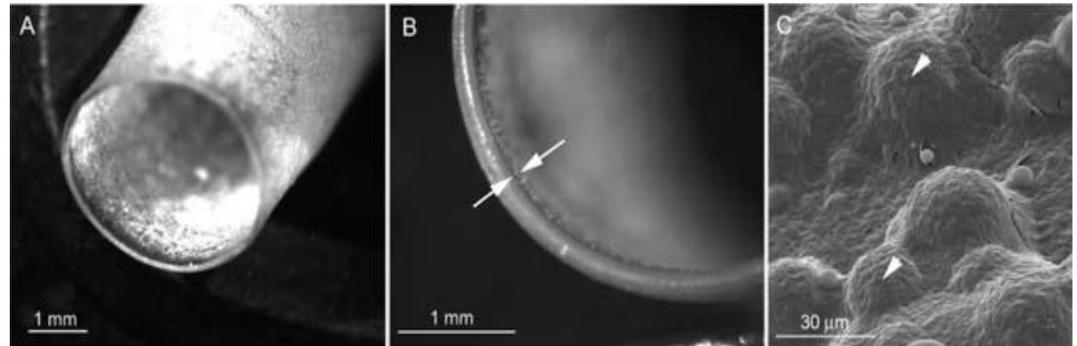


Figure 3: (A, B) Light micrographs of the 200 μm thick microsphere-releasing chitosan guidance channels fabricated by Kim et al. White arrows show 20 μm thick secondary chitosan coating (C) Microspheres, indicated by white arrows, are visualized by SEM [5].

Approach

AIM 1: Determine the impact of chondroitinase ABC, and mouse monoclonal antibody 11c7 on the formation of glial scars, chondroitin sulfate proteoglycans, and axonal regeneration.

Hypothesis: Chondroitinase ABC and 11c7 will break down extracellular matrix that down regulates axonal nerve growth and inhibit its down regulating effect, and will promote larger axonal nerve growth.

After an injury to the central nervous system, the formation of glial scars, a dense matrix chondroitin sulfate proteoglycans (GSPGs) occurs. These scars interact with nerve cell receptors to inhibit cell growth and create a mechanical barrier that is difficult for the new axonal nerve growth to penetrate [7].

It has been shown that immediately after injury axonal nerve cell growth begins only to stop approximately six hours after injury [8]. It has been hypothesized that this is a result of the scar tissue that is forming co-currently after a spinal cord injury. Some research has demonstrated that by degrading the chondroitin sulfate matrix, greater axonal regeneration has been shown to occur [9]. Separate research into monoclonal antibody inhibitors for the Nogo-A receptor has also shown an increase in nerve cell growth [10]. The inhibition of the Nogo-A receptor leads to a change in regulation of the GTPase RhoA, which is a transcription factor that regulates actin cytoskeleton pathways [11].

This aim seeks to test the effects of chondroitinase ABC, a protease for GSPGs, and monoclonal antibody mediated inhibition on the Nogo-A receptor, using an *in vitro* model of a nerve. The *in vitro* model will be generated by modifying a method published by Kaech and Banker. Astroglial cells are harvested from the meninges of the fetal pig brain, and cultured in a media containing antibiotics to prevent contamination. After ten days, a cranial nerve also taken from fetal pig brain is positioned on top of one glial cell culture, and other glial culture placed on top of the other two cultures, laminating the nerve cell. This will ensure that the nerve is well supported [12]. After five days excess glial cells will be removed from the media leaving the final *in vitro* model that can be used for experimentation.

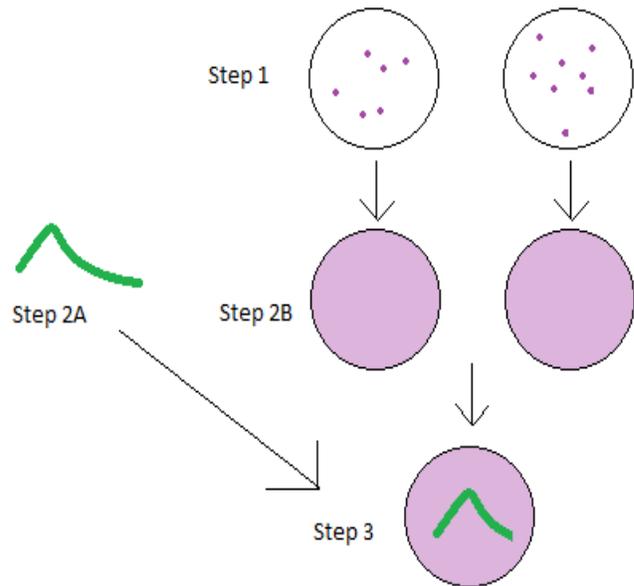


Figure 4: Represents the procedure used to generate the *in vitro* model to see the effects of GSPGs and mAb 11c7 on the regeneration of nerves. Glial cells are harvested from pig brain, and cultured. A fetal pig cranial nerve is then laminated between two glial cell cultures.

The experiment will consist of three experimental groups and a control. The *in vitro* nerve culture will have had a 5mm segment removed. The experimental group's media will be tested with Nogo-A antibody 11c7 at concentrations of $1 \mu\text{g}/\text{mL}$, $3 \mu\text{g}/\text{mL}$, and $12 \mu\text{g}/\text{mL}$, and chondroitinase ABC levels of $2 \text{mg}/\text{mL}$, $4 \text{mg}/\text{mL}$, and $8 \text{mg}/\text{mL}$ [13][10]. Nerve growth will then be recorded with time lapsed bright field microscopy. These results will be further characterized by using a modified neurite outgrowth procedure given by Hu et al [14]. The nerves will be stained with anti-neurofilament and anti-human neuronal protein. The growth will then be measured using the Image Express Automated Cellular Imaging and Analysis system. Results for this will be used to determine to total magnitude of axonal generation and how this varies at the different levels.

AIM 2: Prevent ependymal cell differentiation to scar forming astrocytes.

Hypothesis: Introduction of neurogenin-2 to a spinal lesion site will both promote axonal regeneration and prevent glial scar formation by directing the differentiation of ependymal cells.

The proliferation and migration of reactive astrocytes and the proteins involved in these processes have been observed *in vivo* in mice ages 2-5 months. Analysis of the behavior of various lineages of neural cells found within the spinal cord, both before and after an induced lesion, revealed that most scar-forming astrocytes are derived from ependymal cells found in the core of the spinal cord [15]. Genetic fate mapping has shown that these ependymal cells have the ability to proliferate to either scar-forming astrocytes or myelinating oligodendrocytes [16]. A schematic outlining the results of this genetic fate mapping is provided in Figure 5. Neurogenin-2 (Ng2) is a transcription factor found in both humans and mice that has been shown to play a role in directing the differentiation of neuronal stem cells to active motor neurons, suppressing astrocytic differentiation and promoting myelination in damaged spinal tissue [17][18]. It is postulated that scar formation may be decreased and axonal regeneration may be increased by introducing this protein to the lesion site before significant scarring has occurred.

Mice will be used to conduct animal testing, with experimental treatments administered and a control. A lesion will be induced in all mice; the control group will receive no treatment while the experimental group will be injected with Ng2 close to the injury site once daily. The fate of the ependymal cells post-lesion will be monitored using genetic fate mapping techniques. All mice will receive tamoxifen-dependent Cre recombinase (CreER) treatment in order to monitor the expression of marker genes using fluorescent tags. Astrocytes and oligodendrocytes will be identified by their expression of the Connexin 30 and Olig2 genes, respectively. After administration of tamoxifen to the drinking water of these mice, the incorporation of CreER to the targeted cells becomes permanent and heritable. Expression of the genes specified will be monitored using yellow fluorescent protein (YFP), green fluorescent protein (GFP) and electron microscopy [15]. Tamoxifen is to be

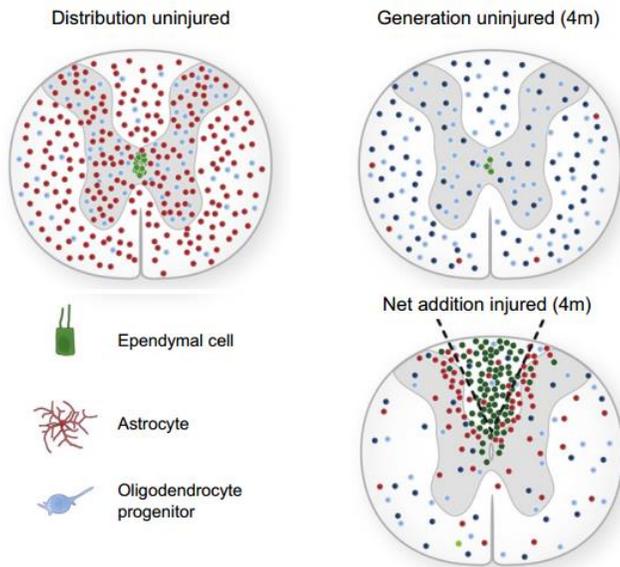


Figure 5: Three schematics of the cell distribution in a spinal cord cross section are shown. “Distribution uninjured” shows the initial distribution of cells: ependymal cells are found concentrated in the core surrounded by many astrocytes and oligodendrocytes. The following two cross sections show new cells that have proliferated over a four month period. In the uninjured spinal cord, ependymal cells remain in the core and most newly formed cells are oligodendrocytes. In the injured spinal cord, ependymal cells have migrated to the lesion site and new astrocytes have formed on the border of the injury [15].

observed with a bright field microscope equipped with a camera [16].

It is expected for the proliferation of astrocytic cells, and thus glial scar formation, to be more prevalent in the population of mice not receiving Ng2 treatment. The mice receiving Ng2 injections are likely to show dramatic increase in oligodendrocyte proliferation and a decrease in glial scar formation. Success of this experiment may lead to the infusion of a hydrogel with the Ng2 protein to be applied directly to the injury site with the hope of providing a targeted and effective administration method without the need for daily injection.

AIM 3: Construct multiple fibrin nerve guidance channels within a collagen scaffold, embedded with nerve and neurotrophic growth factors, which is to be injected with poly[N-(2-hydroxypropyl)methacryl-amide] (PHPMA) hydrogel for increased axonal cell proliferation.

Hypothesis: Collagen-poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA) hydrogels will provide an adhesive surface and an adequate microenvironment for axonal regeneration. Nerve guidance channels and neurotrophic growth factors will help to facilitate and to improve the functional recovery, tissue preservation, and neuronal regeneration in SCI.

When a nerve is severed, a support channel must exist that links the damaged nerve together in order to promote regeneration as myelin begins to deteriorate and axonal degeneration occurs at both the distal and proximal axonal stumps (Figure 6) [19]. A nerve guidance channel (NGC) serves as a conduit to bridge the two ends of the impaired axon together. It is essential for the NGC to maintain the correct orientation of the axon as the proximal stump of the nerve migrates and connects to the distal stump. Other important functions of the NGC are that it can provide nerve growth factors (NGF) to promote axon regeneration as well as decrease scarring of the nerve tissue [20].

Fibrin is used for cell transplantation because of its participation in blood clotting due to its composition of fibrinogen and thrombin. Neural cell growth on these models depends on the concentration of these two factors. Neural cells have been shown to be able to grow and to develop on fibrin based structures when the optimal levels of these two blood proteins are present [22]. Fibrin can be modified with other peptides, and, with these extensions, has demonstrated increased rates of neurite elongation and axon regeneration [23].

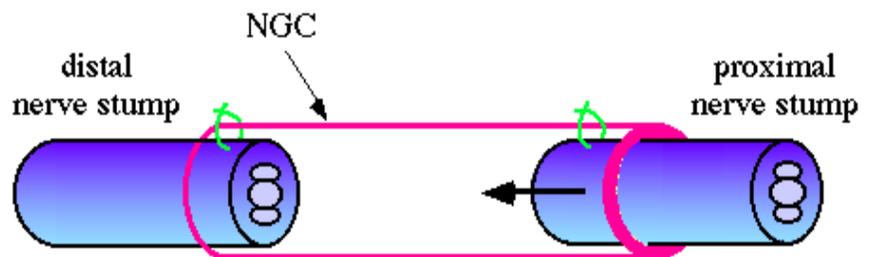


Figure 6: A diagram of two severed stumps of an axon within a neuron guide conduit. The channel maintains correct orientation while the proximal nerve stump regenerates toward the distal nerve stump. [21]

administered once daily for five days after the lesion. Cell proliferation and migration will be observed both five days and four months following the injury. The mice will be anesthetized and the spinal cord must be surgically removed to observe cell activity. The extracted spinal cord will be exposed to formaldehyde, YFP, and GFP and

The model we seek to develop will be increasingly effective through the incorporation of nerve growth factors and neurotrophic growth factors. When an organism sustains a SCI, levels of NGFs and neurotrophic growth factors increase in the area of injury to promote faster axonal recovery [24]. NGFs and neurotrophic growth factors control differentiation and viability of specific neuronal cells, but only neurotrophic growth factors 3 and 4 have the ability to prevent premature neuronal cell apoptosis [25].

Collagen will be used to generate a scaffold that will hold the Neurogel™ and nerve guidance channels together, while also promoting nerve regeneration. The NGCs will be composed of fibrin because of its efficiency in enhancing clotting.

Synthesis of the nerve guidance channels

Electrospinning will be used for the generation of the fibrinogen-thrombin nerve guidance channels NGCs, following the recommendations of Perumcherry's team [26]. Fibrinogen and thrombin will be simultaneously administered in the electrospinning process, through a common needle as shown in Figure 7. One syringe will be loaded with thrombin in a calcium chloride solution and another will contain fibrinogen in a polyvinyl alcohol solution. A 15 kV voltage, and a 0.4 mg/mL solution flow rate will be applied in both syringes, to yield a scaffold diameter within the 50-500 nm range, which has been shown to promote optimal nerve proliferation. The concentration of fibrinogen will be varied (50, 100, 150 and 200 mg/mL) and the thrombin concentration will be adjusted accordingly (2 units for every 1 mg of fibrinogen). Four NGCs will be electrospun at each concentration. Scanning Electron Microscopy will be used to characterize the scaffolds for pore size and pore diameter. They will also be tested for mechanical strength, and later for cell proliferation and exceptional nerve guidance capabilities.

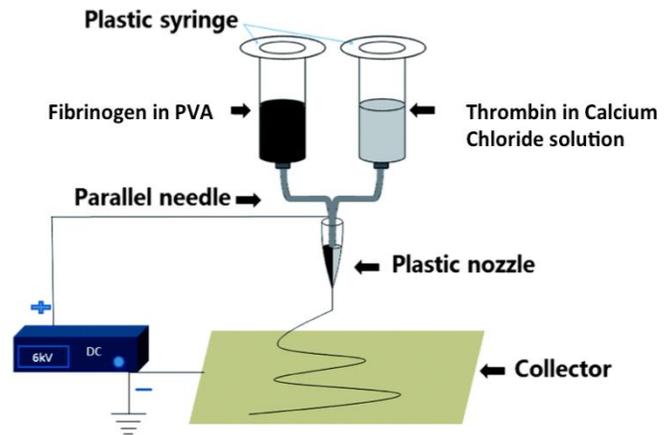


Figure 7: An illustration of electrospinning

Synthesis of the Collagen Matrix

A collagen scaffold will be electrospun to hold the NGCs and Neurogel™ in place, while also assisting the nerve regeneration process, as shown in Figure 8. Electrospinning will be used in the process to generate a high mechanical strength scaffold that will be able to withstand exposure to high pressure. A high voltage of 20 kV, will be used and the resulting fibers will be collected on a rotating rod. It is crucial for the scaffold to have a compressive modulus of 3-4kPa [27], which matches the one of the spinal cord. Collagen concentration and electrospinning parameters will be adjusted throughout the experimental period to obtain a scaffold that matches the spinal cord specifications. Testing for the compressive moduli will be conducted using an Instron, an automated materials sensor. One possible experimental challenge will be the inability to obtain the desired mechanical strength. Should that be the case, cross-linking with other biopolymers, such as hyaluronic acid will be considered for further research.



Figure 8: A schematic showing the expected scaffold, with collagen holding the channels and hydrogels [28]

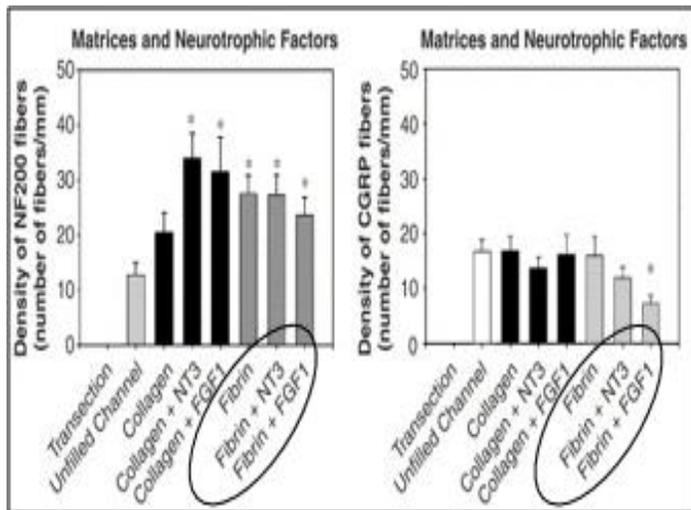


Figure 9: Comparisons of nerve fiber growth using fibrin and other various components are circled in black. Fibrin alone is shown to produce the most neuron fiber growth in contrast to the channels that

Four different solutions will be prepared prior to spin coating the fibrin nerve guide channels. The following components are to be mixed with a fibrin solution: nerve growth factors, Neurotrophin-3, Neurotrophin-4, and a solution comprised of all three constituents. To compare which concoction will induce rapid nerve regeneration, the solutions will be spin coated into the chambers to embed the various elements in the conduits [5]. Other research shows that fibrin alone seem to produce the best nerve regeneration results, which can be seen in Figure 9.

In vitro testing will consist of seeding human mesenchymal stem cells (HMSC) on the scaffold ends and injecting Neurogel™ into the scaffold cavity. We will determine the success of the model based on HMSC cell differentiation into neuronal cells after 5 days by observing them with light micrographs.

For *in vivo* testing, paraplegic adult Sprague Dawley rats will be used. The scaffold is connected to the proximal and distal nerve stumps and is sutured onto the tissues. Neurogel™ will be injected into the void between the two stumps to promote faster cell regeneration. Rats will be sacrificed after 5 days to determine the regenerative capabilities of our fibrin-hydrogel scaffolds. Anterograde axonal tracing is used for analysis of the modified regeneration construct. This observation method will allow us to judge the efficacy of our model by monitoring axonal repair of the fluorescently labelled cells over the span of 5 days [28].

For *in vivo* testing, paraplegic adult Sprague Dawley

If *in vitro* testing leads to poor neuronal cell differentiation and proliferation, it would suggest that our fibrin channels do not have the optimal fibrinogen and thrombin concentration, as the NGF and neurotrophic growth factor coating is meant to enhance the regenerative capabilities of our platform. We would assume the same issue for *in vivo* testing if the second experiment did not produce the desired results. However, if both situations provide positive results, the success of this aim would conclude that the fusion of all elements is able to create an environment that promotes improved axonal regeneration.

Summary

In summary, this proposal seeks to combine the advantages of multiple approaches into a single treatment. This grant will lay the foundation for developing a possible treatment for the hundreds of thousands of individuals living with spinal cord injuries in the United States today. Funding for this grant will lead to a better understand of how to prevent the growth of scar tissue and prevent the cells the cause those scar tissues, while also developing a scaffold to support these regenerating neurons. It is hoped that the combinations of these three factors, used in combination with each other will lead to the development of a clinical treatment that can be used to treat the thousands living with this injury.

References:

1. The National SCI Statistical Center. "Spinal Cord Injury Facts and Figures at a Glance." University of Alabama Birmingham, Feb. 2013
2. "Spinal Cord Injury; Critical Research From PVA | MMRF". *Michelson Medical Research Foundation*. N.p., 2014.
3. Middleton JW, et al. "Life Expectancy After Spinal Cord Injury: A 50-Year Study. - Pubmed - NCBI". *Ncbi.nlm.nih.gov*. N.p., 2016.
4. Woerly, Stéphane et al. "Spinal Cord Reconstruction Using Neurogel™ Implants And Functional Recovery After Chronic Injury". *J. Neurosci. Res.* 66.6 (2001): 1187.
5. Kim, H., C.H. Tator, and M.S. Shoichet. "Design Of Protein-Releasing Chitosan Channels". *Biotechnol. Prog.* 0.0 (2008): 0-0.
6. Johnson, Philip J., Stanley R. Parker, and Shelly E. Sakiyama-Elbert. "Controlled Release Of Neurotrophin-3 From Fibrin-Based Tissue Engineering Scaffolds Enhances Neural Fiber Sprouting Following Subacute Spinal Cord Injury". *Biotechnol. Bioeng.* 104.6 (2009): 1207-1214.
7. Zhang, Ning, Yan, Honghai, Wen, Xuejun, Tissue-engineering approaches for axonal guidance. *BRESR Brain Research Reviews* **2005**, 49, 48-64.
8. Schwab ME, B. D., Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol. Rev.* **1996**, 76, 319-70.
9. Zhang T, Shen Y, Lu L, Fan Z, Huo W, Effect of chondroitinase ABC on axonal myelination and glial scar after spinal cord injury in rats. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* **2013**, 27, 145-50.
10. Yazdi, I. K., Taghipour, N., Hmaidan, S., Palomba, R., Scaria, S., Munoz, A., Boone, T. B., Tasciotti, E., Antibody-mediated inhibition of Nogo-A signaling promotes neurite growth in PC-12 cells. *Journal of Tissue Engineering Journal of Tissue Engineering* **2016**, 7.
11. Deng, Q.; Cai, W.; Li, S.; Zhang, Y.; Su, B. Small Nogo-66-binding peptide promotes neurite outgrowth through RhoA inhibition after spinal cord injury. *Brain Res. Bull.* **2013**, 99, 140-144
12. Kaech, S.; Banker, G. Culturing hippocampal neurons. *Nat. Protoc.* **2006**, 1, 2406-2415.
13. Wilems, T. S.; Sakiyama-Elbert, S. E. Sustained dual drug delivery of anti-inhibitory molecules for treatment of spinal cord injury. *J. Controlled Release* **2015**, 213, 103-111.
14. Hu, F.; Liu, B.; Budel, S.; Liao, J.; Chin, J.; Fournier, A.; Strittmatter, S. Nogo-A interacts with the nogo-66 receptor through multiple sites to create an isoform-selective subnanomolar agonist. *J. Neurosci.* **2005**, 25, 5298-5304.
15. Barnabe-Heider, Fanie; Goritz, Christian; Sabelstrom, Hanna; Takebayashi, Hirohide; Pfrieger, Frank W.; Meletis, Konstantinos; Frisen, Jonas. Origin of New Glial Cells in Intact and Injured Adult Spinal Cord. *Cell Stem Cell.* **2010**. 7, 470-482.
16. Meletis, Konstantinos; Barnabe-Heider, Fanie; Carlen, Marie; Evergren, Emma; Tomilin, Nikolay; Shupliakov, Oleg; Frisen, Jonas. Spinal Cord Injury Reveals Multilineage Differentiation of Ependymal Cells. *PLoS Biology.* **2008**. 6, 1494-1507.
17. Lai, Helen C.; Johnson, Jane E. Neurogenesis or Neuronal Specification: Phosphorylation Strikes Again!. *Neuron.* **2008**. 58, 3-5.
18. Hofstetter, C. P.; Holmstrom, N. A.; Lilja, J. A.; Schweinhardt, P.; Spenger, C.; Wiesenfeld-Hallin, Z.; Kurpad, S. N.; Frisen, J.; Olson, L. Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. *Nature Neuroscience.* **2005**. 8, 346-353.
19. Jenkins, Phillip M., Melissa R. Laughter, David J. Lee, Young M. Lee, Curt R. Freed, and Daewon Park. "A Nerve Guidance Conduit with Topographical and Biochemical Cues: Potential Application Using Human Neural Stem Cells." *Nanoscale Research Letters.* **2015**. 10, 264.
20. Rochkind, Shimon, Yuval Shapira, and Zvi Nevo. "The Potential Clinical Utility of Novel Methods for Peripheral Nerve Regeneration: Where Are We Now?". *Future Neurology.* **2014**. 9(2):105-107
21. http://biomed.brown.edu/courses/bi108/bi108_2001_groups/nerve_regeneration/guidance_channels/guidance_channels_page_1.htm
22. Willerth, Stephanie M., Kelly J. Arendas, David I. Gottlieb, and Shelly E. Sakiyama-Elbert. "Optimization of Fibrin Scaffolds for Differentiation of Murine Embryonic Stem Cells into Neural Lineage Cells." *Biomaterials*. U.S. National Library of Medicine. **2006**. 27(36):5990-6003
23. Straley, Karin S., Cheryl Wong Po Foo, and Sarah C. Heilshorn. "Biomaterial Design Strategies for the Treatment of Spinal Cord Injuries." *Journal of Neurotrauma.* **2010**. 27(1): 1-19.

24. Chung, Hyo-jin, Wook-hun Chung, Jae-Hoon Lee, Dai-Jung Chung, Wo-Jong Yang, A-Jin Lee, Chi-Bong Choi, Hwa-Seok Chang, Dae-Hyun Kim, Hyun Jung Suh, Dong-Hun Lee, Soo-Han Hwang, Sun Hee Do, and Hwi-Yool Kim. "Expression of Neurotrophic Factors in Injured Spinal Cord after Transplantation of Human-umbilical Cord Blood Stem Cells in Rats." *Journal of Veterinary Science*. 2016.17(1): 97–102.
25. Scarisbrick, Isobel A., Paul J. Isackson, and Anthony J. Windebank. "Differential Expression of Brain-Derived Neurotrophic Factor, Neurotrophin-3, and Neurotrophin-4/5 in the Adult Rat Spinal Cord: Regulation by the Glutamate Receptor Agonist Kainic Acid." *The Journal of Neuroscience*. 1999. 19(18): 7757-7769
26. Perumcherry, S. R.; Chennazhi, K. P.; Nair, S. V.; Menon, D.; Afeesh, R. A Novel Method for the Fabrication of Fibrin-Based Electrospun Nanofibrous Scaffold for Tissue-Engineering Applications *. *Tissue Engineering Part C: Methods* **2011**, 17 (11), 1121–1130
27. Bakshi, A.; Fisher, O.; Dagci, T.; Himes, B. T.; Fischer, I.; Lowman, A. Mechanically engineered hydrogel scaffolds for axonal growth and angiogenesis after transplantation in spinal cord injury. *Journal of Neurosurgery: Spine* **2004**, 1 (3), 322–329
28. Tsai, E. C.; Dalton, P. D.; Shoichet, M. S.; Tator, C. H. Matrix inclusion within synthetic hydrogel guidance channels improves specific supraspinal and local axonal regeneration after complete spinal cord transection. *Biomaterials* **2006**, 27 (3), 519–533 DOI: 10.1016/j.biomaterials.2005.07.025.