A HYDROGEL TOOL-KIT FOR *IN VITRO* NEURAL REGENERATION MODELS

AN ABSTRACT

SUBMITTED ON THE SIXTEENTH DAY OF JANUARY, 2015

TO THE DEPARTMENT OF BIOMEDICAL ENGINEERING

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF SCIENCE AND ENGINEERING

OF TULANE UNIVERSITY

FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

BY

Parastoo Khoshakhlagh

Approved: Michael Moore, Ph.D.
Director

Approved: Ronald Anderson, Ph.D.

Approved: Lee Murphy, Ph.D.
ABSTRACT

Soft tissue reconstruction in the nervous system is sensitive to the mechanical and chemical cues of the growth microenvironment. Many technologies have been designed to study these stimuli and their effect on the regional extracellular matrix (ECM). Because of the hard-to-achieve nature and costliness of these technologies, biologists are usually reluctant to employ them to study cellular behaviors. In addition, the complexity of the nervous system, particularly in cases of nerve repair and reconstruction, necessitates the development of facile high-throughput investigational tools.

The objective for this proposal is to develop a series of novel tools to manipulate neuronal cell-cell and cell-ECM responses to varying nervous system microenvironment stimuli in a 3-D *in vitro* model. In Specific Aim 1 a structurally tunable hydrogel is synthesized to investigate the effect of mechanical stimuli on nerve regeneration. We developed a novel interpenetrating network of polymers (IPN) of photocrosslinkable hyaluronic acid and Puramatrix components to promote cellular growth and allow adjustment of mechanical properties by varying the degree of methacrylation. The results demonstrated that neurite outgrowth in 3-D was significantly higher in the more compliant and less interconnected environment.

In Specific Aim 2 we analyzed the effect of different light wavelengths on cultured neurons in a 3-D dual hydrogel model. We performed cellular studies including neurite growth, neurite viability and DNA fragmentation. These studies agree that utilizing visible light is more practical for cellular encapsulation in photopolymerization applications as it is less damaging to the cells in our studies than UVA light. However,
visible light still causes damages to our cells. These data also confirm that increasing the irradiation dosage through raising the exposure time will result in more cellular damage and DNA fragmentation.

We then utilized the optimized system from Specific Aim 3 to examine myelination events in co-cultures of dorsal root ganglion (DRG) with Schwann cells. Here, we demonstrated that this co-culture setting provided us with aligned, highly fasciculated neuronal growth with myelin sheath nicely wrapped along them. We also used two culture systems, and also studied the influence of collagen on neuronal growth and myelination. We also studied the influence of collagen and ascorbic acid (AA) exposure duration on neuronal growth and myelination. We demonstrated that the longer exposure to AA along with the presence of collagen in the system would lead to higher neurite growth and more myelination.
A HYDROGEL TOOL-KIT FOR In VITRO NEURAL REGENERATION MODELS

A DISSERTATION
SUBMITTED ON THE SIXTEENTH DAY OF JANUARY, 2015
TO THE DEPARTMENT OF BIOMEDICAL ENGINEERING
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
OF THE SCHOOL OF SCIENCE AND ENGINEERING
OF TULANE UNIVERSITY
FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY

BY

Parastoo Khoshakhlagh

Approved: Michael Moore, Ph.D. Director

Approved: Ronald Anderson, Ph.D.

Approved: Lee Murtagh, Ph.D.
ACKNOWLEDGEMENT

Obtaining a PhD degree over the 5-years that I spent at Tulane would not have been possible without the many people whose presence had a major impact on my life. The work presented here would not have come together without the help and insight of my advisor, Dr. Michael Moore. I am really grateful for the opportunity to work in his lab and to work with all of my wonderful colleagues including Gary Catig, Dr. Elaine Horn-Ranney, Dr. Lowry Curley, Renee Huval, Chris Rodell and Joe Majdi, who were tremendously receptive and welcoming when I first came here and gave me support throughout these years of working in the lab. I also want to thank my new lab mates Devon Bowser, Ash Sivakumar, Sion Wards, Dan Sazer and Clayton Ford, who are keeping the environment of the lab as friendly as it has always been. I also want to thank my dear friends Dr. Russell Wolf, Dr. Travis Haas, Sarah Giltz and Dr. Jesse Ranney, who made my experience at Tulane University unique and pleasant. In addition, I want to acknowledge Dr. Will Glindmeyer and Dr. Anne-Marie Job for being extremely helpful not only with their editorial assistance but also with their moral support throughout all these years. I cannot express how much impact it has had on my performance to have colleagues who are receptive, helpful and friendly. I also want to thank my committee members: Dr. Murfee, who helped me learn how to present my data and results in a scientific manner and Dr. Anderson, who has been a wonderful mentor and professor whose advice was invaluable throughout all these years. I also want to thank everyone who supported me at Tulane University including Dr. Scott Grayson, Dr. Don Gaver, Dr. Cedric Walker, Dr. Quincy Brown, Dr. Jeff Tasker, Dr. Lars Gilbertson, Dr. San Aung, Dr. Vijay John, Dr. Jibao He, Dr. Eiichiro Yamaguchi, Cindy Stewart, Lori McGinley,
John Sullivan, Megan Ohar and Anne Nguyen. I want to express how grateful I am for having a wonderful mentor, Professor Edward Karp and his wonderful family who have been guidance and support in all aspects of my life was incredibly invaluable.

I am extremely thankful for having wonderful friends throughout all these years of studying. I want to thank Sadegh Azimi for being such an incredible, thoughtful and supportive friend since I started my graduate studies and throughout my studies at Tulane University. I also want to appreciate having great friends such as Elham, Parisa, Negin, Milad, Vahid, Ali, Shaghayegh, Eram, Maryam, Mahsa and Azadeh, for making my educational journey fun and joyful. In addition, I want to thank Mohammad, my amazing friend, for being with me in all of the hardship I went through after my father passed away and for helping me keep my spirit high since then. This is also a great opportunity for me to appreciate having incredible advisors throughout my studies including Dr. Roshanak Moradi, Dr. Vahak Marghussian, Dr. Fathollah Moztarzadeh, Dr. Mahmood Rabiee, Dr. Mohammad Rabiee and Dr. Bijan Eftekhari.

I want to acknowledge my family for all of their support. I would like to thank the Setoudeh Family for being unconditionally loving, caring and supportive. I also would like to thank my cousins Shirin, Tahmineh and Reyhaneh and my in-laws, Sahar and Kaveh for being encouraging through the hard days of my life. I want to thank my sister and brother, Pooneh and Pedram, for helping me build my personality and become a better person since I was a kid. And lastly, I want to dedicate this work to my mother and father, Maryam and Eddie, for being consistently encouraging and loving and I could not become who I am without having them in my life. I could not be luckier to have their
sincere love and guidance during all these years that they taught me how to be caring and affectionate towards everybody.
TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION .......................................................................................................................... 1

CHAPTER 2: BACKGROUND ............................................................................................................................. 6

2.1 Biology of the Nervous System ................................................................................................................. 6
2.1.1 Neurons and Importance of Myelin Sheath ......................................................................................... 7
2.1.2 Influence of the Microenvironment of the Nervous System .............................................................. 11
2.2 Damage to the Nervous System .................................................................................................................. 13
2.2.1 Wound Healing Process in the Nervous System ................................................................................... 13
2.2.1.1 Current Therapies for Injured Nervous Tissue ............................................................................... 16
2.2.1.2 Growth Cone Machinery .............................................................................................................. 18
2.2.1.3 Mechanical Guidance Cues and Growth Cone Response .............................................................. 19
2.3 Benchtop Approaches to Neuron Regeneration ......................................................................................... 21
2.3.1 Hydrogel-based Models ....................................................................................................................... 22
2.3.1.1 Photomicro patterning ............................................................................................................... 24
2.3.1.2 Effect of Light on Cellular Behavior ............................................................................................ 25
2.4 Motivation ................................................................................................................................................. 28
2.5 References .................................................................................................................................................. 30

CHAPTER 3: To Synthesize and Characterize Photoreactive Interpenetrating Networks of Polymers as Tunable Scaffolds for Neurite Growth (Specific Aim 1) ....... 42

Abstract ............................................................................................................................................................. 42
1. Introduction .............................................................................................................. .43
2. Materials and Methods .......................................................................................... .45
  2.1. Synthesis of Glycidyl Methacrylate Modified Hyaluronic Acid ...................... .45
  2.2. Preparation of PM–GMHA Interpenetrating Network Gels ............................. .46
  2.3. Fabrication of Dual Hydrogel Culture Systems .................................................. .46
  2.4. Confocal and Gel Distribution ............................................................................ .48
  2.5. Swelling Measurements ...................................................................................... .49
  2.6. Rheological Characterization ............................................................................. .49
  2.7. Tribometry ........................................................................................................... .49
  2.8. Scanning Electron Microscopy ............................................................................ .50
  2.9. Primary Tissue Culture ....................................................................................... .50
  2.10. Staining Procedure ............................................................................................ .51
  2.11. Image Processing, Neurite Growth and Extension Analyses, and Schwann Cell Migration .................................................................................................................. .52
  2.12. Photomicropatterning ....................................................................................... .54
  2.13. Statistical Analysis ............................................................................................. .54
3. Results ...................................................................................................................... .54
  3.1. Hydrogel Network Formation ............................................................................. .54
  3.2. Structural and Mechanical Properties of IPN ..................................................... .58
  3.3. Incorporation into Micropatterned Hydrogel Model .......................................... .61
  3.4. Influence of Microenvironment on Neurite and Schwann cell Behavior ............ .63
4. Discussion .................................................................................................................. .67
5. References .................................................................................................................. .75
CHAPTER 4: To Evaluate the Cytotoxicity of Ultraviolet A (UVA) and Visible Light on Cultured Neurons in a 3-D Dual Hydrogel Model For Tissue Engineering

(Specific Aim 2) ...........................................................................................................................................80

Abstract ........................................................................................................................................................80

1. Introduction ..............................................................................................................................................81

2. Materials and Methods ..........................................................................................................................83

2.1. Dextran Synthesis and Characterization ..............................................................................................83

2.2. Gel Composition ....................................................................................................................................83

2.3. Photolithography Setup .........................................................................................................................83

2.4. Photomicropatterning ............................................................................................................................85

2.5. Mechanical Properties ..........................................................................................................................86

2.6. Neurite Growth ......................................................................................................................................86

2.7. DRG Dissociation ..................................................................................................................................90

2.8. Ethidium Homodimer-1 Assay ..............................................................................................................90

2.9. TUNEL Assay .........................................................................................................................................91

3. Results .........................................................................................................................................................92

3.1. Photomicropatterning Ability ...............................................................................................................92

3.2. Correlation of Irradiation Time with Mechanical Properties of MeDex Gel .........................................93

3.3. The Effect of Visible and UV Light on Neuronal Viability ......................................................................95

3.4. The Influence of Visible and UV Light on DNA Fragmentation .............................................................98

3.5. The Influence of Visible and UV Light on Neurite Growth ................................................................99

4. Discussion ..................................................................................................................................................101
CHAPTER 5: To Investigate Myelination of Neuronal Cells Incorporating with Schwann Cells in an in vitro 3-D Model (Specific Aim 3)........................................................................................................113

1. Introduction .............................................................................................................................................118

2. Materials and Methods ..............................................................................................................................119

2.1. Fabrication of Dual Hydrogel System .................................................................................................119

2.2. Dextran Synthesis and Characterization and Gel Composition ............................................................121

2.3. Primary Tissue Culture in the Dual Hydrogel System ........................................................................122

2.4. Schwann Cell Culture ............................................................................................................................122

2.5. SC Encapsulation and Incorporation in the Dual Hydrogel System ....................................................123

2.6. Media Regimen for the DRG/SC Co-culture in 3-D Hydrogel System ................................................123

2.7. Immunohistochemistry .........................................................................................................................124

2.8. Image Processing, Neurite Growth, Myelin Formation .......................................................................125

2.9. Transmission Electron Microscopy ......................................................................................................127

3. Results ......................................................................................................................................................128

3.1. 3-D Dual Hydrogel System and DRG/SC Co-culture ........................................................................128

3.2. The Influence of Collagen on Neurite Growth in 3-D Co-cultures .....................................................129

3.3. Myelin Development in 3-D Co-culture Model in Our Dual Hydrogel System ....................................130

3.4. The effect of Ascorbic Acid (AA) on Myelin formation in 3-D ..............................................................133

3.5. The Impact of Collagen on Myelin Development ...............................................................................134

Discussion ...................................................................................................................................................136

5. References ...............................................................................................................................................146
CHAPTER 5: CONCLUSION

153
LIST OF FIGURES

Chapter 1

Figure 1. Myelinated neurons in schematic illustrations .......................................................... 8
Figure 2. Depiction of compact myelin periodicity ................................................................. 10
Figure 3. Axonal growth cone interacting with biomolecules in the microenvironment ....... 21

Chapter 2

Figure 1. *In vitro* model was made by fabricating the PEG mold utilizing DMD and related photomask ........................................................................................................................................ 47
Figure 2. Network microstructures were examined using scanning electron microscopy (SEM) at 100X ........................................................................................................................................... 56
Figure 3. Visualization of PM and GMHA mixtures ...................................................................... 57
Figure 4. A) Swelling ratio and compressive moduli of IPN .......................................................... 59
Figure 5. Storage moduli (G’) and loss moduli (G’”) of the gels were measured to study the viscoelastic behavior of the IPNs and S-IPNs ........................................................................................................ 60
Figure 6. Integration of the dual hydrogel system is confirmed by mixing 1.75 x 10^7 beads/ml fluorospheres with GMHA/PM before crosslinking ................................................................................................. 62
Figure 7. Micropatterned hydrogels with IPN and S-IPN regions .................................................. 63
Figure 8. DRG neurite growth and cell migration in dual hydrogel constructs ......................... 64
Figure 9. Analysis of length of neurite extension and volume of neurite outgrowth in constructs IPN₉₀ and IPN₃₂ ................................................................................................................................. 66
Figure 10. Analysis of Schwann cell migration in IPN constructs .............................................. 67
Chapter 3

Figure 1. Depiction of the methodology for gel formation for TUNEL and Ethidium Homodimer-1 assays .......................................................... 89

Figure 2. Photomicropatterned hydrogels ......................................................... 93

Figure 3. Storage moduli (G’) and loss moduli (G”) of the IPNs ............................ 94

Figure 4. Depiction of the number of dead cells as stained by an Ethidium Homodimer-1 assay .............................................................................. 97

Figure 5. Depiction of the studies that describe the number of apoptotic cells as stained by a TUNEL assay ................................................................. 98

Figure 6. Depiction of the volume of cell growth ................................................... 100

Chapter 4

Figure 1. Depiction of the methodology for co-culturing SCs and DRGs ............... 120

Figure 2. Development of myelin protein (MBP) after 25 days ................................ 126

Figure 3. 3-D rendering of confocal images .......................................................... 127

Figure 4. Quantification of the amount of neuronal growth in each of the four culture models in 3-D .............................................................................. 129

Figure 5. The immunohistochemistry for neurofilaments (beta-III) and MBP ........... 130

Figure 6. The immunohistochemistry for neurofilaments (beta-III) and P0 .............. 131

Figure 7. The immunohistochemistry for neurofilaments (beta-III) and MAG ........... 132

Figure 8. Transmission electron microscope of neural culture cross-sections ........ 133
LIST OF TABLES

Chapter 4

Table 1. Media components for co-culture systems over a 25-day period ......................135

Table 2. Nomenclature of the experimental groups .........................................................135
CHAPTER 1: INTRODUCTION

The nervous system synchronizes voluntary and involuntary behaviors in the human body and transfers signals throughout. Furthermore, it regulates body functions in order for the body to adapt to changes, maintain homeostasis, and survive. Neurons, the main cells of the nervous system, are specialized to act as the communication network that allow organisms to interact with their external and internal environment. This system is supported by glial cells, which perform functions such as the formation of myelin, a lipoprotein that increases the speed of conduction and acts as insulation. Myelin is part of the myelin sheath, a structure that prevents electrical current leakage. In between myelin are periodic spaces known as nodes of Ranvier. Signals travel from node to node, increasing the conduction velocity. If neurons are unmyelinated, which results from disease or trauma, these signals are smaller and slower.

Neuronal pathfinding during development and repair is influenced by many attractant and repellent cues throughout the body. Growth cones are the motile sections of neurons that are located at the end of neuronal extensions. They navigate neurons in response to haptotactic and chemotactic guidance stimuli. The mechanical and chemical guidance factors play an important role in the pathway that neuron takes in order to reach to its target. For instance, the elastic modulus of the substrate alters the opposing tensile force felt by the axon. These mechanical cues change growth dynamics.

The study of neuronal pathfinding provides an understanding of nervous system regeneration and repair. Damaged nerves are able to initiate wound-healing responses following injury or degenerative disease. Neuronal cells develop growth cones and utilize
biological cues to reconnect the disrupted neural signaling pathway. However, injuries and damage that are difficult for the body to repair reduce the lives’ quality of the patients. Based on data from the National Spinal Cord Injury Statistical Center there are nearly 265,000 cases of spinal cord injuries with 12,000 new cases are identified each year in the United States (US) alone. One example is Multiple Sclerosis (MS), an immune-mediated chronic inflammatory disease that attacks myelinated neurons and destroys myelin and the associated neurons. There are nearly 350,000 patients in the US with MS, 50% of whom will need help walking within 15 years of the disease onset. [1]

The Neuropathy Association states that peripheral neuropathy, a disorder of the peripheral nerves, influences 1 out of 15 Americans each year and is a leading cause of adult disability. There are nearly 15 to 18 million patients with diabetic peripheral neuropathy, 575,000 patients with HIV-neuropathy, and 420,000 patients with chemotherapy-induced neuropathy. Twenty million Americans suffer from peripheral nerve injury caused by trauma and medical disorders. [2] For example, there were 50,000 surgical procedures on peripheral nerve repair in 1995 in the US alone. Even after extensive surgical treatment, poor nerve regeneration and lack of functional recovery can be an outcome of severe injury. These reports emphasize the need for more extensive studies in order to develop new clinical strategies for nervous system therapies. [3]

The following Specific Aims (SAs) describe a series of neuronal guidance models that investigate axonal growth and pathfinding during development, a system heavily influenced by variations in mechanical and structural cues in vivo. We utilized novel hydrogel motifs for these studies, with SA1 designed to introduce tunable matrices that allow precise manipulation of mechanical and structural cues more finely than existing
methods in order to analyze neuronal response. In SA2 we investigate the effect of
different light wavelengths on neuronal behavior in a novel spatially and temporally
tunable 3-D matrix. In SA3, we use the optimized system from SA2 to investigate
neuronal cells and Schwann cells integration. This hydrogel system will provide us with a
unique high-throughput tool to study neuronal development and behavior in varying
environments.

Specific Aim 1: To Synthesize and Characterize Photoreactive Interpenetrating
Networks of Polymers as Tunable Scaffolds for Neurite Growth

An interpenetrating polymer network (IPN), defined as two polymer networks
physically entangled but not chemically crosslinked, combines the strengths of individual
polymer networks into a single structure. For these studies we utilize photocrosslinkable
Glycidyl Methacrylate Hyaluronic Acid (GMHA) and Puramatrix. Adjustment of
mechanical and structural properties can be achieved by altering GMHA. We
characterized the developed tunable IPN systems’ structural and mechanical properties.
We compared the properties of the IPN system before and after irradiation and
incorporation of the GMHA network with Puramatrix. Consequently we introduced the
IPN system to the previously formed dual hydrogel network and quantify volume of
neurite growth and length of extension in the newly developed hydrogel while tuning the
system’s mechanical properties.

Specific Aim 2: To Evaluate the Cytotoxicity of Ultraviolet A (UVA) and Visible
Light on Cultured Neurons in a 3-D Dual Hydrogel Model For Tissue Engineering
A spatiotemporally tunable 3-D matrix was developed to analyze the effects of irradiation of different wavelength on neural cells. Here we utilized functionalized Dextran along with Riboflavin and L-Arginine as photoinitiator and co-initiator respectively. Riboflavin has similar absorption peaks in UVA and visible light therefore this unique model allowed us to study the influence of UVA and visible light on cell toxicity. We characterize the mechanical and physical properties of the newly developed gels formed upon UVA and visible light irradiations. We quantified the effect of UVA and visible light on cytotoxicity and apoptosis pathway in neurons. We also compared the neuronal viability and growth in the 3-D culture formed using UVA and visible light irradiation.

Specific Aim 3: To Investigate Myelination of Neuronal Cells Incorporating with Oligodendrocytes or Schwann Cells in an In vitro 3-D Model

The optimized 3-D in vitro model from SA2 was utilized to investigate the development of Dorsal Root Ganglia (DRG) and Schwann cells and their incorporation to develop myelination. Using the spatial tunability of the hydrogel, the in vitro model would be photopatterned to incorporate two different cell types and study proliferation and myelin formation processes as a result of the co-culture. Initially we optimized the hydrogel system in order to introduce DRGs and SCs. Then we quantified the axonal growth and extension in the 3-D structure and optimized the dual hydrogel system in order to get robust growth. Subsequently we investigated myelin formation and development in the 3-D culture as a result of incorporating two cell types in one culture model.
CHAPTER 2: BACKGROUND

2.1 Biology of the Nervous System

The nervous system is split into two components: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS, composed of the brain and the spinal cord, and the PNS, which extends throughout the body, synchronizes signals and responses to various stimuli. Signals are transported throughout the system via neurons, cells that are specialized for carrying information as electric impulses. Neurons contain axons and dendrites that extend out of the cellular body and assist in the transmission of electrical signals. Dendrites are capable of transmitting the electric impulses, or action potentials, to the next neuron in the signal pathway. As the signal travels to the end of the dendrite it reaches the synapse, a small junction between cells. Here, signals are transmitted through the use of neurotransmitters that are received by the neuron at the other end of the gap.

Neurotransmitters are received by dendrites of the next neuron and result in the neuronal cell body, known as the soma, beginning an intracellular signal cascade that leads to the generation of a signal at the axonal hillock, a specialized part of the soma that connects to the axon. If this signal cascade reaches a certain threshold, an action potential is created. This action potential propagates down an axon, another extension of the neuron that facilitates transduction. The terminal region of the axon translates this impulse into a chemical signal, which can be picked up by nearby cells including neurons and possibly result in the generation of another action potential event.
Although neurons are the basic building blocks of the nervous system, the CNS and PNS other cells are necessary for the systems to function. Supportive specialized cells in the CNS or PNS are termed glial cells. [1]–[3] Glial cells in the CNS such as astrocytes, oligodendrocytes, and microglia play major roles in system behavior. Astrocytes are capable of altering the chemical microenvironment, oligodendrocytes form myelin sheaths around axons that improve the conduction of electrical signals throughout the system, and microglia prevent neurons from the outside environment. The PNS, alternatively, has supportive cells that encapsulate neuronal bodies. Schwann cells, which serve a similar role as oligodendrocytes, facilitate action potential propagation by myelination. This assortment of cells helps form the healthy nervous system environment, and disruption of their function can lead to debilitating effects throughout the human anatomy.

The communication between neurons and glial cells influences nervous system function including signal conduction, synapse transmission, and signal processing. Signals that are sent between these cell types include neurotransmitters, ion fluxes, adhesion molecules and a variety of signaling molecules from both the synaptic and nonsynaptic portions of neurons. Through the release of these signals, glial cells are able to influence neuronal cell activity and synaptic transmission. Disruption of this system may lead to serious issues that can prevent signal transmission. [4]

2.1.1 Neurons and Importance of Myelin Sheath

Neurons are highly specialized cells responsible for communicating information in both chemical and electrical forms. In this section we discuss the structure and
importance of the myelin sheath and comment on approaches to broaden our understanding of its development, such as utilizing novel 3-D in vitro models. The myelin sheath is a specialized cell membrane with a multilamellar spiral structure that surrounds the axon. Well-myelinated nerves are completely surrounded by myelin sheaths except for nodes of Ranvier, tiny gaps that are exposed to extracellular environment. Myelin sheaths reduce nervous system capacitance, reducing current flow across the intermodal axon membrane and therefore promoting salutatory conduction.

[5]–[8] Figure 1 shows a myelinated neuron in the PNS.

![Figure 1](image)

**Figure 1.** Myelinated neurons in schematic illustrations. A) Depicts the structure of a neuron (light blue) with three Schwann cells in panel a shows the structure of a neuron with three Schwann cells that wrap around the axon (green). Nodes of Ranvier (red) are the myelin sheath gaps. Three black curves show the action potentials saltatory propagation. B) Cross-section of a myelinated axon. Four concentric layers are extended from Schwann cell to form the myelin sheath. [9]

The myelin sheath contains a pair of domains made of compact and noncompact myelin, each containing a non-overlapping set of proteins. The majority of the myelin internode is composed of compact myelin, a spiral of tightly packed membranes with a periodicity of 14 nm. [10], [11] Compact myelin is principally composed of lipids such as cholesterol and sphingolipids. [6], [8], [12] Experiments on targeted gene disruption with a focus on disrupting the lipids within myelin led to a significant decrease in axonal
conduction velocity, demonstrating the importance of maintaining myelin structure in the event of injury. Figure 2 demonstrates the structure of compact myelin, which is composed of spiraling layers of tightly packed membrane that do not contain a cytoplasm. Non-compact myelin results from the cytoplasmic regions that connect the Schwann cell cellular body to the wraps on the axon side along with the Nodes of Ranvier, Paranodes and Schmidt-Lanterman incisures (small pockets of cytoplasm left during myelination).

Different proteins make up the myelin sheaths native to the CNS and PNS. In the CNS, compact myelin has Myelin-associated Oligodendrocyte Basic Protein (MOBP) and Myelin Proteolipid Protein (PLP). Compact myelin in the PNS contains Protein Zero (P0). Myelin Basic Protein (MBP) is common between the CNS and PNS. Non-compact myelin in the PNS contains Myelin-associated glycoprotein (MAG). It is noteworthy to mention that in the PNS, one myelinated axon is produced per one Schwann cell and in the CNS a bundle of up to 50 myelinated axons is produced by one oligodendrocyte.
Figure 2. Depiction of compact myelin periodicity. A) Compact myelin as a cell process. B) Compact myelin wraps around the axon spirally. C) During myelin compaction the cytoplasm (pale green) is excluded and major dense lines (dark green) are formed as a result of cytoplasmic membranes (dark green, MDL in 2.D) fusion and extracellular leaflets form intraperiod lines (red, IPL in 2.D). The structures of three different proteins that impact myelin integrity (P0, PLP and MBP) are also described. P0 and MBP are found in the PNS myelin while PLP and MBP are found in the CNS myelin sheath. [7]

Analyzing the factors involved in myelin diseases and degeneration as well as finding clinical approaches for myelin regeneration requires precise biomimetic environments due to its complex structure and behavior. The majority of existing in vitro studies utilize neuronal/glial cell co-cultures to evaluate myelination, investigate intracellular proteins and myelin formation in standard 2-D cell culture environments. [8], [19] However, research has shown that there will be major differences, including morphology and gene expression, in neuronal behavior between 2-D and 3-D systems. This raises the need for a model that would mimic the support and 3-D architecture of the
extracellular matrix that can utilize previously studied immobilized or soluble chemical cues and drugs independently and evaluate their effects on myelination. [13], [20]

2.1.2 Influence of the Microenvironment of the Nervous System

In the previous section we described neuronal structure, myelin complexity and the importance of biomimetic 3-D cultures. Herein we briefly discuss chemotactic, haptotactic, structural and mechanical signals involved in neuronal guidance and development and the influence of mechanical behavior on cellular behavior.

Chemotactic and haptotactic cues are parameters with a major role in neurite mapping during development. Extending neurites interact with a dynamic extracellular environment that dictates the direction of growth to ensure the neurites synapse with their intended targets. The processes observed in vivo in relation to nerve growth and guidance are often exploited to develop novel therapies for neural regeneration. A wide variety of signals influence cellular functions including chemical signaling through ligand binding to cell receptors and the mechanical characteristics of the microenvironment.

Chemical signaling can occur through soluble biomolecules as well as bound or transmembrane proteins. The local environment usually releases soluble cues that can function as long-range stimuli and can be either repulsive or attractive biomolecules. However, short-range molecules are usually bound to the microenvironment and can also attract or repel growth. Many of these factors have concentration gradients in neural tissues where axons respond robustly. These signaling pathways are usually important during development and neuronal injuries.
Recently it has been shown that, in addition to chemical cues, the structure and mechanical properties of the microenvironment can also influence the biological activity through adhesion receptors. This is especially noticeable in the investigation of 2-D vs. 3-D representations of the nervous system. For instance, cells grown in 3-D collagen hydrogels demonstrated a markedly different morphology than similar cells grown on 2-D surfaces. [21], [22] Studies that compared fibroblasts on 2-D substrates versus 3-D structures established that those grown in 3-D systems more closely resembled physiological behaviors. [21], [23] It was demonstrated that the growth of stress fibers in 3-D structures led to a less compliant cellular body that was closer to those found in vivo. [24] Additionally, substrate deformability influences cellular response to growth factors, as exposure to TGFβ1 in 3-D versus 2-D resulted in increased up-regulation of smooth muscle actin. [25] It has been previously established that deformability influences cellular behavior using polyacrylamide gels with varying crosslinking density and therefore different mechanical properties. [26] In a different study, cultured fibroblasts on stiffer regions of a hydrogel system did not transition to softer regions. [27]

Researchers investigated the influence of mechanical signals in 2-D and 3-D models on neuronal responses. Lampe et al. developed elastin-like polypeptide hydrogels in order to stimulate neurite outgrowth in a 3-D environment. They evaluated the impact of length, rate and density on growth in the design of their hydrogels. The researchers tuned the system’s cell-adhesive RGD ligand density and showed that increasing the elastic modulus with a constant RGD ligand density led to decrease in neurite outgrowth. [28] Research by Balgude et al. in a primary sensory ganglia model utilizing agarose hydrogels validated that neurite growth inversely correlated with mechanical stiffness in
agarose gels. Furthermore, they note that the rate of neurite outgrowth maybe be directly related to stiffness, and there may be an equation relating the two. [29] Another study demonstrated that neurites prefer to grow in less stiff environments in 3-D gels. The study identified an optimum range of neurite outgrowth based upon the Young’s modulus, and noted that increases past this range leads to a decrease in cellular growth. It was also noted that there was a minimum growth threshold as well, where the cells did not have enough mechanical support for growth. [30] All of these works address the influence of haptotactic of the microenvironment on cellular, especially neurons, and the impact of 3-D cultures on neuronal responses which portrays the *in vivo* phenomenon more closely.

2.2 Damage to the Nervous System

2.2.1 Wound Healing Process in the Nervous System

Nerves constantly remodel throughout the lifetime of an organism, forming new connections and elongating. In this section we briefly describe wound healing processes in the PNS and CNS. Upon injury, nerve cells degenerate and the growth process is initiated. Proteins produced during regeneration are similar to those that spur axonal growth in developing embryos. [31], [32] Growth associated proteins (GAPs), tubulin, and actin are up regulated, while neurofilament proteins are down regulated. [33] Axonal regeneration occurs within hours of injury, with multiple sprouts extending from terminal nodes of Ranvier of damaged axons. These sprouts extend towards their previous connection, with the branches that do not reach an eventual target disappearing. The mechanisms of repair are different in the PNS and CNS.
In the PNS, glial cells, phagocytic cells and axonal debris all influence nerve repair after injury. Regeneration of these structures is easier if the system is crushed instead of being cut by injury, as the Schwann cells remain intact. [34] If a cut is introduced into the system, the architecture is severely disrupted and the newly formed gap may be too large to cross. Schwann cells promote regrowth and axonal extension, as it has been shown that the absence of them results in stunted axonal regrowth. It has been demonstrated that fibroblasts and the basal lamina that are present in the injury environment are not enough to spur regeneration. The arrival of macrophages to the injury site triggers an increase in the local Schwann cell population. [31] Malfunctions to the impulse conduction and signaling systems throughout the PNS can occur due to diseases, birth defects, or injuries. There should be communication between axons and glial cells in order to develop and maintain myelination and avoid myelin degeneration. In mice, myelin degeneration can occur when there is a lack of PLP or myelin cell adhesion molecule MAG. [35], [36] Neuropathy, a PNS disease, affects axons, Schwann cells and myelin. It is a result of neurons and Schwann cells not communicating correctly and is not well understood. It is known that neuropathy results in axon death and the immediate breakdown of myelin. One clinical approach may be to encourage remyelination as normal impulses can possibly be restored.

The regeneration process in the CNS, termed glial scarring, is similar in response to most injuries or trauma. Damage to the system results in a number of cellular and molecular responses caused by microglia, oligodendrocyte and astrocyte precursors. Initially, macrophages arrive from the bloodstream at the injury site. A few days later
oligodendrocyte precursors are recruited to the system. Microglia also play a role, as they rapidly divide due to the injury and perform a variety of maintenance functions such as the removal of myelin debris from the system over the course of several weeks. Ultimately, the scar tissue is astrocytic in origin and astrocytes proliferate and increase in size due to injury, filling the newly created void. There are inhibitory molecules that are also present in the damaged region, has been shown to inhibit growth both in vitro and in vivo. Removal of the various sources of the inhibitory molecules is incredibly difficult. Multiple sclerosis (MS) is a CNS disease that occurs when myelin is lost in the system and leads to wide ranging problems stemming from unnatural action potential conduction. MS is a neurodegenerative disease arising from malfunctioning neuron-glial communication in unmyelinated regions. Research that targets neuron-glial cell communication as well as demyelination due to injury and disease is essential in developing preventive treatment strategies. [4]

As a conclusion for this section, it is noteworthy to emphasize that neuronal regeneration and remyelination are difficult despite both oligodendrocytes and Schwann cells being abundant in damaged sites. For instance, there are a variety of theories for why remyelination does not occur in MS: axons are too damaged to attract myelin, the oligodendrocyte precursors that are attracted to the region are not able to migrate to the axons, or there are simply unknown factors that have not been discovered yet. Regeneration and remyelination require cell division, migrations and myelination activities, regardless of whether it is in the CNS or PNS, and synchronizing these activities is essential for future treatments and needs thorough studies of individual factors that are involved which raises the need for in vitro platforms. [3]
2.2.1.1 Current Therapies for Injured Nervous Tissue

Previously we described the wound healing processes in the PNS and CNS. Herein, we discuss some of the current and potential strategies for the nervous system regeneration. Injuries to the nervous system are very common, and the lengthy paralysis that frequently results requires clinical treatment. Surgeons attempt to repair nerves by restoring as many severed connections as possible at the injury site. However, unsuccessful treatment often occurs either due to failure to jumpstart regeneration or due to the wrong targets being innervated. This can lead to poor muscle control and deficient sensory recovery. [31], [39]

Techniques that are developed to increase the success of these treatments vary due to the wide range of injuries that may occur. Important factors such as scar formation and restoration of blood supply significantly impact these processes. An easily managed cut that can be stitched together may be sufficient to restore nerve function, as it repairs naturally over the course of months due to the natural nerve growth rate of ~1 mm/day. [40] More significant cuts require more advanced strategies. [41] Severely injured nerves are frequently removed in order to prevent scar tissue formation, and sewing cannot connect the nerve ends. In these cases, scaffolds such as autografts can be inserted into the injury site. These approaches are not fully successful, and alternative approaches are therefore necessary.

Regenerating neurons, with little ability to travel in the appropriate direction for regrowth, require guidance. Frequently they will travel down endoneurial tubes, the connective tissues that form around myelin sheaths and are left behind after nerves degenerate. Utilizing these tubes in the PNS can repair peripheral nerves when they are
guided across the injury site as nerve bundles, also known as fascicles. [42] Current strategies do not perfectly match severed axons with their previous endoneurial tube, but by guiding fascicles along artificial paths it is feasible to restore function. [43] One approach utilizes conduits that enhance and direct neuronal regrowth that are inserted into the site of injury. These conduits may be seeded with Schwann cells or growth factors to encourage regeneration. Clinical usage of conduits does not include these factors, and successful therapy is currently limited by gap size between injured nerve segments.

Engineered scaffolds provide several advantages to restore damaged systems, as they provide structural gaps for reconnection, frameworks for neurite outgrowth, and bridges for cell migration. To replace injured CNS tissue microenvironments, peptide-based hydrogels have been utilized as neurite growth scaffolds. Puramatrix, a 16-amino acid chain that can self assemble into a 3-D network when exposed to salt, is used in these instances. For example, Puramatrix has been placed in the brain at incision sites, where it successfully replaced the natural extracellular matrix and led to a growth-permissive environment. [44] Neurites grew throughout the scaffold and formed synapses with their target site. Similarly, the placement of Puramatrix in the optic tract let to axons penetrating the scaffold and forming synapses in the visual cortex, leading to restoration of some visual function. This work validated the idea that replacement of inhibitive or injured environments can led to signal restoration. [45]

Hyaluronic acid has also been shown to enhance neuronal regeneration specifically in the PNS. Hyaluronic acid, a naturally occurring linear polysaccharide that contains repetitive disaccharide units of N-acetylglucosamine and glucuronic acid is naturally found in the body. It is vital to cell-matrix interactions, principally in cell
proliferation and migration as well as the deposition and restructuring of the naturally occurring extracellular matrix. A study utilizing a hyaluronic acid implant in rat sciatic nerve showed a significant increase in conduction velocity in the PNS when the 3-D hydrogel was implanted into a nerve guide port over a 28-day period. [46]

Although there are approaches to encourage nerve regeneration, there are many unknown factors that need more investigation. Having a better understanding of the mechanisms of axonal pathfinding during development and repair can assist with better therapeutic and preventive approaches.

2.2.1.2 Growth Cone Machinery

Nerve growth during regeneration involves extending axonal navigation throughout the physiological architecture. [47] Here we describe the mechanism of neuronal pathfinding and navigation throughout the body. The growth cone, a dynamic extension of a developing neurite, leads axonal navigation. Growth cones at the end of the axon respond to stimuli that guide it toward the appropriate target to restore signal transmission. Growth cones grasp to cell adhesion molecules (CAMs) [48] or to the extracellular matrix (ECM). [49] These structures provide a pathway of permissive surfaces that growth cones travel along towards their destination. Mechanical cues such as varying elastic moduli [28] and chemical cues such as neurotransmitters [50] also impact this process, leading to a wide range of signals that steer the growth cone along its path. Further guidance is found throughout the body in the form of prohibitive surfaces that are covered in anti-adhesive surface molecules such as ephrins. [51], [52]
Research into nerve growth has led to a basic understanding of how the growth cone progresses, including the intricate cytoskeletal remodeling after translation of environmental cues. Core to this process is the dynamic properties of actin and the associated polymerization events. F-actin treadmilling and retrograde flow, or the constant motion of F-actin towards the growth cone’s center, propels the growth cone forwards. Retrograde flows result from two properties: the ability of motor protein myosin II to contact as well as F-actin polymerization in the peripheral region of the growth cone that results in a pushing motion. The movement and progression of the growth cone necessitates structures that integrins and cadherins on the filopodia may attach. These structures, found throughout the ECM and on cell surfaces, bind to the F-actin bundle. Adhesion sites are pulled on by retrograde flow of actin, leading to a tension force being applied to the F-actin bundle. Myosin II proceeds to tug on the microtubules of this bundle until there is no longer tension in the structure. [53] At this point, the adhesion complexes detach due to this lack of tension, and ongoing actin treadmilling causes the filopodia to progress forward and restart the cycle.

As the filopodia creates tensile forces on the surrounding environment during axon progression, the equal and opposite tensile force of the environment affects the growth cone's own response. The elastic modulus of the substrate alters the opposing tensile force felt by the axon. These mechanical cues alter growth dynamics. [54]

2.2.1.3 Mechanical Guidance Cues and Growth Cone Response

As discussed previously, the movement and progression of the growth crown necessitates structures that integrins and cadherins on the filopodia may attach. These
structures, found throughout the ECM and on cell surfaces, bind to the F-actin bundle. Adhesion sites are pulled on by retrograde flow of actin, leading to a tension force being applied to the F-actin bundle. Myosin II proceeds to tug on the microtubules of this bundle until there is no longer tension in the structure. At this point, the adhesion complexes detach due to this lack of tension, and ongoing actin treadmilling causes the filopodia to progress forward and restart the cycle.

As the filopodia create tensile forces on the surrounding environment during axon progression, the equal and opposite tensile force of the environment affects the growth cone's own response. The elastic modulus of the substrate alters the opposing tensile force felt by the axon. These mechanical cues alter growth dynamics.

While filopodia are not responsive to adhesion model gradient, growth cones have been shown to maneuver away regions without adhesion sites. Encountering adhesion molecules on a surface generates a tension that stimulates recruitment of filopodia that are under less tension, therefore leading to the growth cone following along the path of the filopodia that are under the most tension.

Growth cones also utilize chemical guidance cues during development. These signals may appear as membrane-bound ligands or as a gradient of chemicals secreted by cells that may either attract or repel the growth cone in order to influence its direction. Guidance cues vary both spatially and temporally, resulting in a dynamic gradient of signals that are interpreted by the growth cone. The cone itself will decide how to react to the signal based upon variables such as receptor availability, presentation of the cues, downstream regulation of metabolic protein expression, and the status of the internal environment. [55] Figure 3 shows a cultured rat hippocampal neuron. Shootin1
phosphorylated, a protein involved in the organization of an asymmetric signal for neuronal polarization, interacts with F-actins to transduce Netrin1 signaling into force for axon outgrowth.

**Figure 3.** Axonal growth cone interacting with biomolecules in the microenvironment. Actin filaments depicted in red and microtubules in blue. Demonstrates the signal-to-force pathway during axonal outgrowth. Phosphorylate Shootin1, a signaling protein, is shown in green. [56]

### 2.3 Benchtop Approaches to Neuron Regeneration

In section 2.2 we discussed regeneration mechanisms and its complexity in the nervous system. There are many unknown factors involved during development and repair that necessitate further studies. An approach to understanding and resolving these significant neural regeneration complications is through the introduction of high throughput *in vitro* platforms that mimic the nervous system environment in order to recognize the important factors that influence the CNS and PNS and propose preventive and regenerative strategies regarding the nervous system repair. [45]

*In vitro* platforms can mimic the complex anatomical structure of the nerve injury sites. Furthermore, they have tunable properties that allow investigations into the possible
parameters involved in cellular mechanisms. These settings are usually a lot cheaper than \textit{in vivo} models and they allow isolation of individual parameters to investigate.

Simplification of the nervous system for benchtop research leads to that ability to precisely control mechanical and chemical stimuli in order to elucidate the effect of various signaling pathways on neuronal behavior. Isolation of these variables leads to more accurate observations that greatly expand our understanding of both the CNS and PNS. Studies have been performed which utilize the previously mentioned Puramatrix and hyaluronic acid [57] as well as collagen, [58], poly(L-lactic-co-glyolic acid), silk and similar structures in order to replicate the nervous system ECM and create a more biomimetic platform that more closely portrays \textit{in vivo} phenomena. [59], [60]

2.3.1 Hydrogel-based Models

Nerve growth models often account for variables such as mechanical or chemical cues due to their impact on regeneration. These models are designed to recreate physiological processes in an effort to design new clinical approaches for nerve disease or damage. Accurate portrayals of the \textit{in vivo} environment require 3-D matrices that can mimic the extracellular matrix (ECM) and cell microenvironment. Recently, headway has been made in the development of highly reproducible and tunable structures that take advantage of the unique properties of hydrogels. Hydrogels, biocompatible networks of polymer chains that are hydrophilic with high water content, have been widely used for tissue engineering applications. [61], [62] These networks can be modified with biomolecules and functional groups.
Hydrogels often form upon converting liquid monomer or macromers into a gel. They may undergo a sol–gel transition upon exposure to various stimuli, such as temperature, pH, and light. Hydrogels that are light sensitive and allow guidance cue immobilization have been widely utilized for tissue engineering applications. [20], [63]–[65] The ability to trigger crosslinking by exposing the system to light allows spatiotemporal control over crosslinking density and protein binding. [62], [66] Features such as this have been utilized to recreate in vivo phenomena. It provides us with a platform where we can create gradients of entrapped or bound proteins to investigate the response of proliferating nerves. [67] As an example, previous studies have utilized photolabile nitrobenzyl-based caging moieties in hydrogels in order to covalently bind proteins in desired patterns to the matrix network. [63] In addition, alteration in crosslinking density of the hydrogel system, as a result of chemical or optical manipulation, can impact the elastic moduli of the setting. Consequently, the influence of the mechanical stiffness of the microenvironment on neurite extension and growth can be investigated. [28], [68]

We have previously developed a facile and rapid technique that uses a digital micromirror device (DMD) incorporated with a simple microscope objective to photopattern desired 3-D hydrogels. DMDs are capable of structural and molecular 3-D micropatterning. By projecting UV light in defined geometries onto photocrosslinkable substrates, the DMD enables us to create a dynamic mask that could irradiate the photosensitive gel solutions throughout the depth of the gel. [20], [63]–[65] This gives us the ability to create a hydrogel system with a 3-D microenvironment in order to generate
an ideal in vitro environment for investigating neurite growth in the presence of varying chemical and structural properties. [62]

2.3.1.1 Photomicropatterning

Microfabrication tools have opened up new research pathways for studying cell-cell and cell-protein interactions. Photopolymerization is a favorable microfabrication method for forming hydrogels due to the simplicity, convenience, adjustability of photocrosslinking techniques and precise spatiotemporal control over microfabrication. [69], [70] We have previously described the widespread applications of photocurable hydrogels in neural tissue engineering. Here we describe the drawbacks of using light for biomedical applications that necessitates further investigation.

Photopatterning provides a facile tool for microfabrication and has been widely used in biomedical applications such as islets microencapsulations, controlled platelet deposition in blood vessels and hard tissue reconstructions. [69] This method is also very simple and quick that allows converting liquid monomer or macromers into a gel in situ and under physiological conditions.

Despite all of the advantages for this methodology, there are a few drawbacks as well. Bryant, et.al showed that radicals associated with initiators could react with cell membranes, proteins and nucleic acids. They performed a comprehensive study on the effect of the photoinitiators on cytotoxicity of encapsulated fibroblasts. Another limitation of using photosensitive hydrogels is the possible harmful effect of irradiation on the cells. [71], [72] The two principal wavelength spectra utilized for this purpose are UVA (315-400 nm) and visible light (400-700 nm). It has been shown that both UVA and
visible light can induce cell toxicity, leading to cell apoptosis. However, there are no studies that compare the cytotoxic effects of these two wavelengths. [73]

2.3.1.2 Effect of Light on Cellular Behavior

As mentioned above one of the downsides of photomicropatterning in in situ cell encapsulation applications is the effect of light on cellular damage. There have been multiple studies on the effect of light of different cell types, mainly skin, retinal or oral cells. [74]–[77] These studies are mostly done on 2-D cultures.

It has been demonstrated that UV exposure induces apoptosis in keratinocytes as a protective mechanism for highly damaged cells to prevent the formation of malignant transformations. In order for UV to create a response in cells it must be first absorbed by a cellular chromophore. It has been shown that in some cases UV wavelength absorption would be equivalent to that for DNA. [75], [78] It has also shown that the DNA damage caused by UV light has both cytotoxic and genotoxic effects and a single photon hit may induce carcinogenic and lethal responses in the body. While many studies show that UV-B is the main cause for the DNA damage because of its absorption by the cellular DNA, there is evidence that UV-A will have similar influences on cell damage. Native DNA will not absorb UV-A spectra but they are capable of initiating secondary photoreactions in existing DNA photoproduccts. Another mechanism that causes DNA damage as a result of UV-A is indirect photosensitizing reactions. The biological responses to UV-A and UV-B include decreases in the survival rate and growth in different cell types and destruction in protein structure. [78] There are also repair mechanisms that accurately transmit the genetic information from one cell to its daughter, which is key for survival of
the organisms. [79] UV irradiation can also cause reactive oxygen species (ROS), which can be major mediators of oxidative cell damage. From the action of UV-A and endogenous photosensitizer such as flavins and porphyrins single oxygens can be produced that can generate oxidative damage and can oxidize the cellular DNA. [80], [81]

Although the effect of visible light on cellular damage has not been vastly researched, there are studies that show the influence of the visible light irradiation on cellular responses. Liebel et al. investigated the effect of visible light on ROS and matrix metalloproteinase (MMP) responses in skin grafts in an extensive in vitro study. They reported that visible light would induce ROS production. ROS mediated the release of proinflammatory cytokins and MMP expression. They showed that the skin cells exposed to filtered visible light could contribute in skin damage and skin aging. [76] Keilbassa et al. demonstrated that short wavelengths of visible light could generate 8-Oxoguanine DNA damage. [74] Sparsa et al. used the cytotoxicity of the blue light in order to decrease the survival of melanoma B16F10 cells. They postulated that the growth was hindered and the cells stopped dividing during the period of time that they were exposed and for four days after illumination. [82] In a different study, Li et al. determined whether visible light could directly trigger the death pathway by damaging the DNA. Their study showed that the exposure of retinal ganglion cells (RGCs) to visible light directly affects nuclear DNA damage. [83] The results was also confirmed previously that visible light can be unfavorable for the RGCs and this effect was more detrimental in cells with compromised function such as glaucoma, diabetics retinoplasty and ischemia. RGC axons are particularly rich with mitochondria to produce more energy for nerve conduction and
maintaining the neuronal function. Osborne et al. showed that mitochondria are mostly affected in visible light exposed RGCs. In addition, mitochondrial respiration and homeostasis will be deteriorated after visible light exposure. [77], [84], [85]

Although several researchers have found that DNA integrity and cellular responses are severely influenced with visible light exposure, [86]–[88] the use of it for therapy has been examined for a long time. The visible light has been used as a therapeutic agent that can potentially activate the photoreactive therapeutic agents. [89]–[91] In multiple studies, in order to direct the leading edge of nerve cells, a laser spot was located in front of the nerve’s leading edge. The nerve’s leading edge grew into the beam focus and the laser beam influenced actin polymerization driving lamellopieda extension. [92], [93] In a different study the effect of 710 nm Light-emitting Diode (LED) on protecting neurons suffering from neurodegeneration was investigated. The data showed that LED treatment might promote synaptogenesis. [94] Mathew et al. studied the impact of distant femtosecond laser pulses on growth cone fillopodia. They demonstrated that the light induces a measurable effect on fillopodia of a single axon from primary cell cultures. They postulated that the resulted attracting effect can be a characteristic of ultrashort pulses of light. [95] In a comprehensive study PC12 cells were irradiated with monochromic and mixed LED light and the neurite outgrowth was studied. The light slightly suppressed the neurite growth both upon mixed and single wavelength irradiation. The behaviors of neurites were following similar patterns in most of the cases but the blue light (470 nm) irradiation. There was no long connected neuronal outgrowth upon any of the irradiations other than blue light. This may be due to the fact that blue light is responsible for the transductional signaling for the long connection of neuronal
outgrowth. The study showed that the blue light might have the potential to be utilized for therapeutic purposes in nerve regeneration. [96]

### 2.4 Motivation

Soft tissue reconstruction is sensitive to the mechanical and chemical cues of the growth environment, and this is especially true in the nervous system. It is critical that optimal 3-D growth environments be characterized to increase the rate of axonal regeneration and neurite proliferation. The present study was designed to develop *in vitro* environments that mimic the ECM and promote neurite extension. We develop novel hydrogels and provide analyses that measure the effects of the mechanical and microstructural changes on neuronal growth and guidance. The simplicity of hydrogel models enabled us to evaluate how different variables, such as mechanical or chemical signals, individually affect neurite growth. Herein we describe a series of 3 specific aims that utilize novel hydrogels to analyze cell proliferation during regeneration events in the nervous system. We focus on the effect of the nervous system microenvironment, creating tunable (SA1) and photomicropatterened (SA2) hydrogel designs that may contribute to the understanding of the interplay between the nervous system, the regional microenvironment, and engineered biomaterial scaffolds. In SA3 we employ our system to investigate intracellular events and their impact on myelination activities among neurons after incorporation with supportive cells. This model acts as a substrate to study the effects of biomolecules such as growth factors and their individual influence on de/remyelination to study degenerative diseases. Drug screening for various medications such as chemotherapy treatments with neuropathy side effects can also be investigated.
Furthermore, this model can be easily expanded to integrate other cell types in a unique 3-D \textit{in vitro} model in order to study the intracellular biology.
2.5 References


CHAPTER 3: To Synthesize and Characterize Photoreactive Interpenetrating Networks of Polymers as Tunable Scaffolds for Neurite Growth (Specific Aim 1)

Abstract: The reconstruction of soft tissue, such as that which is found in the nervous system, is governed by the mechanical cues of the growth microenvironment. The complexity of the nervous system, particularly in cases of nerve repair and reconstruction, necessitates the development of facile high-throughput investigational tools. This study assesses the hypothesis that a mechanically tunable photoreactive interpenetrating network (IPN) of Hyaluronic Acid and Puramatrix can be manipulated in order to demonstrate that 3-D environmental stiffness influences neurite growth and proliferation. For these studies we employed photocrosslinkable Glycidyl Methacrylate Hyaluronic Acid (GMHA) and Puramatrix, a self-assembling peptide scaffold, leading to a structurally adjustable IPN system. Our in vitro model provides us with a simple, reproducible environment to generate different properties in a single specimen. Mechanically manipulated IPN systems with different degrees of methacrylation were fabricated using a dynamic mask projection photolithography apparatus and characterized. To gauge the impact of IPN stiffness on neurite outgrowth, dorsal root ganglia (DRG) explants were cultured in the hydrogels. We found that neurite outgrowth in 3-D was more likely to happen in an environment with lesser degree of methacrylation, which corresponded to structures that were more compliant and more porous. Overall, tuning the mechanical behavior of our IPN systems led to statistically significant (p < 0.05) differences in cellular growth and extension that warrants further investigations.
1. Introduction

The regional architectural morphology and mechanical properties of the nervous system microenvironment significantly influence nerve regeneration. Hydrogels with inherently tunable mechanical properties, provide a useful, biocompatible platform to investigate the structural-functional interdependence. The microstructure of a hydrogel can be tweaked through chemical manipulation to establish a micromechanical environment that most closely mimics that of the nervous system. The present study examines the effects of altering the regional composition of a novel hydrogel on neurite growth and development in an effort to improve future nervous system regeneration therapies. To this end we have examined a novel dual hydrogel system that incorporates Hyaluronic Acid (HA) and Puramatrix (PM).

HA is a glycosaminoglycan polymer composed of disaccharides D-glucuronic acid and D-N-acetylglucosamine that are linked by alternating β-1,4 and β-1,3 glycosidic bonds. [1,2] Abundant throughout the extracellular matrix (ECM), HA is an important structure within the nervous system and throughout the body. [3-5] HA has been shown to improve peripheral nerve regeneration in vitro by organizing the ECM into a more favorable structure for axon migration. [6] The present analysis investigates the chemical modification of HA utilizing polymerizable methacrylate groups to alter the regional structural and mechanical properties of a hydrogel scaffold in an in vitro model of nerve regeneration.

While HA has been utilized in biomaterial applications to create tissue-like scaffolds, there is no single ideal design that can closely mimic the body’s properties. As such, considerable research has been devoted towards its application as a biomimetic
scaffold. [7-12] Incorporating HA with other hydrogels in order to achieve a more desirable cell support and protein adhesion has been repeatedly reported for tissue engineering applications. [11,12] There are a variety of methods for utilizing the functional properties of HA in order to employ it for nerve tissue studies. One of these approaches is through the use of an interpenetrating network of polymers (IPN).

IPNs are hybrid structures composed of two or more polymers that are physically or chemically crosslinked into a network that is then re-entangled. These highly entangled networks retain the structural and mechanical properties of component polymers while strengthening and reinforcing the overall scaffold. Incorporation of PM into the IPN system designed in this article adds cell-adhesive properties to mechanically tunable HA, which is not typically cell-adhesive on its own. [11-15]

In this study, we created an in vitro model that incorporated HA with PM. PM is a self-assembling peptide scaffold structurally composed of 99% water and synthetic peptide (1% w/v) that is 16 amino acids which have alternating hydrophilic and hydrophobic side chains. [16-19] It is widely used to recreate the 3-D microenvironments that are favorable for cell growth in absence of animal-derived materials and pathogens. [16]

We utilized an integrated system with a digital micromirror device (DMD) capable of structural and molecular 3-D micropatterning to create the dual hydrogel tissue culture constructs used in our investigations. Our model consisted of a photocrosslinkable polyethylene glycol (PEG) that surrounded an IPN hydrogel of methacrylated-HA and PM. By projecting UV light in defined geometries onto the photocrosslinkable substrates, the DMD enabled us to create a dynamic mask that could irradiate PEG and the IPN
throughout the depth of the gel. [17,20-23] We took advantage of this method in order to create a dual hydrogel system with a 3-D microenvironment to create an ideal *in vitro* environment for investigating neurite growth in the presence of varying mechanical properties.

In summary, this work describes a novel hydrogel that utilizes photocrosslinkable HA and PM components to promote cellular growth and allow adjustment of mechanical properties by varying the degree of methacrylation. This analysis measures the effects of the mechanical and microstructural changes on neuronal growth and guidance.

2. Materials and Methods

2.1. Synthesis of Glycidyl Methacrylate Modified Hyaluronic Acid

All materials were obtained from Sigma–Aldrich, St. Louis, MO, unless otherwise specified. Photocrosslinkable glycidyl methacrylate-modified HA (GMHA) was synthesized as previously described by adding methacryloyl groups to HA (Molecular Weight: 1.5-1.8×10^6 Da) to obtain 32% and 90% degrees of methacrylation (%Me). [24] Briefly, 1.0 g of HA was combined with 200 mL PBS (pH 7.4), 67 mL of dimethyl sulfoxide (DMSO), 13.3 g glycidyl methacrylate (GM), and 6.7 g of triethylamine (TEA) to prepare 32% GMHA (%Me); similarly 132 mL PBS, 132 mL DMSO, 40.8 g GM, and 13.3 g TEA to prepare 90% GMHA (%Me). After 10 days of reaction at room temperature, the solutions were dialyzed against d-H_2O for 3 days and then lyophilized for 3 days. %Me was confirmed with ^1H NMR.
2.2. Preparation of PM–GMHA Interpenetrating Network Gels

For the preparation of interpenetrating network (IPN) and semi-interpenetrating network (S-IPN) hydrogels, both 32%Me and 90%Me GMHA were dissolved overnight to 4% (w/v) in PBS, pH 7.4, containing 1% (w/v) Irgacure 2959 (Ciba Specialty Chemicals, Basel, Switzerland) and 0.03% (v/v) N-vinyl-pyrrolidinone. 0.001% (v/v) laminin (Invitrogen, Carlsbad, CA) was added to 1% (v/v) PM solution (BD Biosciences, Bedford, MA). While PM alone will support axon outgrowth, laminin was added according to the manufacturer’s protocol to obtain optimal DRG neurite outgrowth in PM. To choose the best composition for each IPN system, either with 32%Me (IPN\textsubscript{32}) or 90%Me (IPN\textsubscript{90}), three different proportions of PM/GMHA were prepared: 1:2, 1:1 and 2:1. To form S-IPNs the solutions were placed in an incubator at 37\textdegree C and 5% CO\textsubscript{2} for at least 30 mins to allow PM to complete its gelation in the presence of PBS or neuronal growth media and GMHA. The S-IPNs then were changed into complete IPN systems by a 14 mins constant irradiation with UV light. Photopolymerization of GMHA chains occurred by free radical polymerization, and so gelation of IPNs was assumed to be complete after 30 mins of incubation followed by 14 mins of UV irradiation.

2.3. Fabrication of Dual Hydrogel Culture Systems

Dual hydrogel culture systems were fabricated via digital projection photolithography, as previously described and seen in Figure 1 [17]. Briefly, photoreactive hydrogel solutions contained in permeable cell culture inserts with 0.4µm pore size (Corning Inc., Corning, NY) were irradiated using an apparatus consisting of a collimated UV light source (OmniCure 1000 with 320–500 nm filter, EXFO, Quebec,
Canada), a digital micromirror device (DMD) (Discovery™ 3000, Texas Instruments, Dallas, TX) as a dynamic photomask, and a 2X Plan Fluor objective lens (Nikon Instruments, Tokyo, Japan). [17,20,25,26] A solution of 10% (w/v) PEG-diacrylate (M_n 1000; Polysciences Inc., Warrington, PA) and 0.5% (w/v) Irgacure 2959 in PBS was irradiated with 181 mW/cm^2 UV light, as measured by a radiometer (306 UV Powermeter, Optical Associates, San Jose, CA), for 55 s to make a bipolar PEG micromold (Figure 1).

![Diagram showing steps of the procedure]

**Figure 1.** *In vitro* model was made by fabricating the PEG mold utilizing DMD and related photomask (Mask 1). Each side of the mold was filled with either S-IPN₉₀ or S-IPN₃₂. For each side, a different photomask (Mask 2, 3) was used to form a complete IPN system. Dorsal root ganglion (DRG) E15 was cultured in the center of the model. Step 1: PEG mold fabricated utilizing DMD. Step 2: Mold filled with semi-IPN₉₀ and irradiated for 14 min to form IPN₉₀. Step 3: Other mold half filled with S-IPN₃₂ and irradiated to make IPN₃₂. Step 4: DRG explants cultured in the center of the model.

This process would make a gel thickness of about 480 µm. [17] PEG micromolds were filled with small volumes (~2-3 µl) of GMHA-PM, which was also crosslinked *in situ* by irradiating via the DMD photomask. Hydrogel constructs consisting of IPN₃₂ in one half and IPN₉₀ in the other were formed by successively filling the mold, irradiating
half of the void region, aspirating uncrosslinked gel, and repeating with the other solution, as described in Figure 1. Hydrogel constructs were washed in DPBS with 1% antibiotic-antimycotic additive to inhibit contamination.

To assess the integration of GMHA-PM gels within the PEG molds, $1.75 \times 10^7$ beads/mL of fluospheres (Molecular Probes, Eugene, OR) were incorporated into GMHA-PM gels prior to placing into PEG micromolds. For each experimental group (IPN$_{90}$ and IPN$_{32}$), 6 samples were crosslinked in situ via projection lithography, and 6 were left uncrosslinked. All were submerged in 3 ml of PBS within 6-well plates and agitated on a rotary shaker at 300 rpm overnight. Constructs were examined with fluorescence microscopy, and GMHA-PM gels were considered integrated if fluospheres remained in place within PEG molds. [17]

2.4. Confocal and Gel Distribution

Gel distribution in the IPN system was evaluated by adding fluorescein o-acrylate (0.01% w/v) to the pre-crosslinked solution before forming the network. This experiment was performed on IPN$_{32}$. Two groups were defined: not-mixed IPN$_{32}$ (the precrosslinked solution was not mixed after adding two components) and mixed IPN$_{32}$ (the precrosslinked solution was vortexed for 15mins following by vigorous pipetting). The control group consisted of PM mixed with fluorescein o-acrylate and flourscein-GMHA. Confocal microscopy (Leica, Wetzlar, Germany) was used to map the depth-intensity of GMHA. Z-stack images, taken at 2.4µm intervals across a gel’s 190µm depth, were compressed into a maximum projection image. In addition 100mm × 100mm area was
randomly selected in each 3-D projection to measure the fluorescence intensity throughout the depth of the gel. [14]

2.5. Swelling Measurements

Equilibrium swelling experiments were performed to evaluate the relative effect of %Me on hydrogel crosslinking density. IPNs were submerged in PBS at room temperature overnight, blotted dry, and weighed to obtain the hydrogel swollen weight (W₅) before being lyophilized overnight and re-weighed to measure the hydrogel dry weight (W₅). [11] The swelling ratio (SR), was calculated as follows:

\[
SR = \frac{W_S - W_D}{W_D} \quad (1)
\]

2.6. Rheological Characterization

The viscoelastic properties of IPNs and SIPNs were measured using a shear rheometer (TA Instruments, AR2000, New Castle, DE). Four samples of each hydrogel formulation were soaked in PBS overnight to reach their swelling equilibria and then loaded between the rheometer cone and plate (1⁰ steel cone, 4 cm diameter). Storage (G’) and loss (G’’) moduli were measured over a frequency sweep range of 0.1–100 Hz with constant 4% strain. All measurements were taken at room temperature [27].

2.7. Tribometry

*In situ* compressive moduli of IPNs were measured using a tribometer (CETR Enterprise Inc., Campbell, CA) with a cylindrical borosilicate probe of 0.625mm radius attached to a force sensor with cantilever spring constant of 53 N/m. IPN samples were formed in PEG molds as described in Section 2.3, were soaked in PBS and placed on
coverslips. The probe was brought into contact with the surface of these IPN samples and then vibrated at a vertical speed of 10 µm/s. Compressive elastic moduli (E), were calculated from force and displacement data according to equation (2) where a = radius of the cylinder, d = total displacement, and \( F_n \) = normal force:

\[
F_n = 2 \times a \times E \times d \times (2) \quad [28]
\]

2.8. Scanning Electron Microscopy

Samples were prepared for scanning electron microscopy (SEM) as follows: Hydrogels were placed on a sample holder and plunged into liquid nitrogen slushed lower than -190°C (Gatan, Alto 2500, Pleasanton, CA). Under high vacuum protection they were withdrawn into a vacuum transfer device and placed in a cryo-preparation chamber with a temperature of -130°C, while the anticontaminator was at -188°C. Once the sublimation procedure was complete, the stage temperature was changed to -130°C, and the sample was sputter coated with platinum at 10mÅ for 85 seconds. The samples were then placed on a cold stage module (-130°C) fitted on the stage of the Field Emission SEM (Hitachi S-4800, Tokyo, Japan) through an interlocked airlock. Images were taken with a voltage of 3 kV at a distance of 6 mm and 100x magnification.

2.9. Primary Tissue Culture

The dual hydrogel IPN constructs fabricated and characterized using the methods outlined above, were then cultured with Long Evans rat embryo dorsal root ganglion (DRG) tissue, in keeping with the guidelines of the Institutional Animal Care and Use Committee (protocol number: 0362R2). To start, the constructs (described in section 1.3)
utilized in these experiments were left in Neurobasal medium supplemented with B27 (2%v/v), L-glutamine (0.25%v/v), nerve growth factor (NGF) (0.02 µg/ml) and penicillin/streptomycin (1% v/v) (Invitrogen) in an incubator (37 °C, 5% CO₂) overnight.

In preparation for culture, dorsal root ganglia (DRG) were isolated from embryonic day 15 Long Evans rat embryos (Charles River, Wilmington, MA) and trimmed. In each gel construct, a single DRG explant was then placed on top of the center of the channel and gently pressed into the gel in between the two different IPNs (IPN₉₀ and IPN₃₂). Using this method, the DRG was encapsulated into the hydrogel and could stimulate 3-D growth. The IPN systems were incubated in 37 °C for 7 days, and the medium was changed 24 hours after initial culture, and then every 48 hours thereafter.

2.10. Staining Procedure

To evaluate neurite outgrowth and extension in the IPN systems, standard immunohistochemical techniques were utilized. Initially, a fixing procedure was performed with 4% paraformaldehyde for 2 hours. Next, neurites were tagged with mouse monoclonal [2G10] neuron-specific beta III tubulin primary antibody and Cy3.5 conjugated goat antimouse immunoglobulinG (H + L) secondary antibody (AbCam, Cambridge, MA). Each step was carried out in PBS with 0.1% Saponin and 2.0% BSA overnight followed by three washes in PBS with 0.1% Saponin. Schwann cells were labeled with a similar protocol using the anti-S100 antibody glial marker (AbCam, Cambridge, MA) as the primary antibody and the secondary antibody Cy2 conjugated goat antirabbit immunoglobulinG (H + L) (AbCam, Cambridge, MA). Cell nuclei were labeled using the nucleic acid stain DAPI (Molecular Probes, Eugene, OR).
2.11. Image Processing, Neurite Growth and Extension Analyses, and Schwann Cell Migration

2-D fluorescence was used to evaluate neurite extension. Images were captured using a Nikon AZ100 stereo zoom microscope and processed and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Neurite extension was defined by the average length of the 5 longest neurites in each construct that had outgrown from the center. Since this measurement might be vulnerable to outliers we also measured the volume of neurite growth.

The volume of growth into 3-D hydrogels was determined using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany). It was impossible to count the number of individual neurites due to the dense and highly fasciculated growth that extended into the gels. Therefore, in order to quantify the amount of growth in 3-D, the volume of neurite growth in the system was calculated from pixel counts. Each sample was imaged in 3-D with an optical slice greater than 75µm depth, an average of 32 slices per sample, and a resolution of 1024×1024 pixels and with a 10X objective. The resulting image stack was compressed into a maximum projection image. Due to the size of the constructs, 3 images were taken from each channel to visualize all cell growth across the entire depth of the hydrogel. The DRG body was excluded from the cell by considering the end-points of each leg to be the joint point of the curvature (where the DRG was cultured) and the beginning of the channel. Pre-processing steps including thresholding and transformation into a binary representation were applied uniformly across all images. Data analysis was performed using ImageJ and a custom algorithm in Matlab.
Neurite growth was quantified using pixel counts of the threshold images over the depth of the gel.

3-D images of neurons, Schwann cells and nuclei were captured using a Leica TSC SP8 confocal microscope with a 10X objective (Leica, Wetzlar, Germany). Since the constructs are too long to be imaged in one step, a tile scan was used in which the constructs were divided into 10 different 3-D projections. All 3-D images were stitched afterward using the LAS AF 3 (Leica Application Suite Advanced Fluorescence) microscope software. Depth coding for glial cell marker and nuclei were also quantified using the Leica TSC SP8 confocal microscope and software.

In order to study Schwann cell migration, constructs were stained for glial cell markers according to the procedure described in section 2.10 and imaged with a Nikon A1 Confocal Microscope. Samples were imaged in 3-D with an optical slice of 25μm depth, an average of 20 slices per construct and a resolution of 1024×1024 pixels and with a 4X objective, and then Z-stacks were compressed into maximum projections and binarized using a manual threshold. Schwann cell migration was quantified according to the following procedure: a custom Matlab code was used to find the average area of the cells aggregated in the ganglion on day 0. A circle with the same area was then transferred to the center of each experimental group, and the changes in the minimum distances of the 8 farthest cells (each side) at day 7 were measured from the circle’s circumference representing day 0. (n= 4 constructs)
2.12. Photomicropatterning

Initially, fluorescent hydrogels were created using fluorescein o-acrylate (0.01% w/v, MW 386 Da) that was mixed into a 1:1 GMHA/PM photocrosslinkable solution and incubated to allow the S-IPN system to set as described in section 2.2. Fluorescein o-acrylate incorporates only into regions where GMHA exists and has been irradiated. The addition of the fluorescein o-acrylate may have influenced the crosslinking density minimally; therefore the samples were not used for any quantitative analysis, but only for visual demonstration. To avoid meniscus formation, 12-well plate inserts were treated with rain-x before 120µl of S-IPN was added into the inserts. The S-IPN was micropatterned using the DMD device following the methods described in section 2.3. This solution was irradiated with UV light by the photolithography apparatus for 14 mins per construct to induce crosslinking via free radical chain reaction. A star-shaped photomask was utilized to show the photopatternability of both IPN$_{90}$ and IPN$_{32}$.

2.13. Statistical Analysis

Statistical analysis of significance was determined using a two-tailed t-test with equal variance at a p < 0.05 for all experiments. The reported mean represents different sample sizes, which are reported throughout.

3. Results

3.1. Hydrogel Network Formation

The IPN system was formed, evaluated and incorporated into our microengineered *in vitro* model. Three different ratios of GMHA/PM were selected and
evaluated for gel formation. Visual observation showed that the structure and consistency of the gel drastically changed after irradiation. Hydrogel curing was evident upon qualitative observation of gel consistency before (S-IPN) and after (IPN) irradiation for both IPN$_{90}$ and IPN$_{32}$. In addition, there was a considerable difference in integrity and uniformity between IPN$_{90}$ and IPN$_{32}$ samples, with the IPN$_{90}$ formulation yielding a more consistent structure. The composition with the highest uniformity and consistency in each group was selected for further material and biological studies.

SEM micrographs were taken of IPNs, S-IPNs, PM and GMHAs in order to investigate the formation of the IPN hydrogel microstructure in comparison with related constituents (Figure 2). Cryo-SEM was used here to ensure that the microstructure and morphology of all samples were well-preserved, and the observations discussed herein are consistent with reality. All hydrogels demonstrated a porous microstructure with interconnected fibrillar morphology. IPNs (Figure 2 E, F) showed a higher crosslinking density than GMHAs (Figure 2 C, D) of the same degree of methacrylation; likewise, GMHA-only hydrogels contained less crosslinks and a smoother microstructure (Figure 2).
Figure 2. Network microstructures were examined using scanning electron microscopy (SEM) at 100X. The GMHA fibers and the fibrous PM structure combined in the well-mixed S-IPNs (A, B) and IPNs (E, F). GMHA-only networks (C, D) were largely constructed of fibrillar porous structures. PM-only networks (G) were defined by a fibrous structure. Lower crosslinking is evident in compositions with less %Me.
The higher degree of methacrylation in GMHA led to a superior crosslinking density and more interconnected network. PM (Figure 2 G) exhibited a fibrillar, more discrete microstructure, on par with that of S-IPNs (Figure 2 A, B).

Fluorescent microscopy was employed to confirm the distribution of network components, throughout a given IPN. Figure 3 describes the fluorescent intensity plots for each hydrogel. The plots of the hydrogels indicated that GMHA and PM were

![Graph](image)

**Figure 3.** Visualization of PM and GMHA mixtures. Fluorescein o-acrylate incorporates into the GMHA network after photopolymerization. The homogeneity of PM-only (blue line), unmixed PM-GMHA (red line), mixed PM-GMHA (green line), and GMHA-only (violet line) were examined using confocal imaging. There is a slight slope for GMHA and mixed-IPN plots. A sharp decreasing trend is observable for unmixed-IPN.
uniformly distributed in the well-mixed samples. Minimal phase separation was expected, as GMHA is hydrophilic by nature and PM nanofibers contain highly hydrophilic cavities in their structure into which water can easily penetrate. PM-only and GMHA acted as the negative and positive controls, respectively. As expected GMHA-only samples were the brightest of all four groups and exhibited a high degree of uniformity, since fluorescent-GMHA was evenly distributed in the GMHA-only hydrogel. The well-mixed GMHA/PM hydrogels demonstrated a reduction in intensity relative to GMHA-only samples, though overall trend, are similar. Unmixed GMHA/PM samples, on the other hand, resulted in a decreasing trend in fluorescence that was not apparent in the GMHA-only and well-mixed GMHA/PM fluorescence intensity plots. Finally, PM-alone, which lacked any fluorescent components revealed a near zero intensity through the depth of the gel.

### 3.2. Structural and Mechanical Properties of IPN

Swelling behavior, tribometry and rheology tests were performed on IPNs in order to evaluate the relationship between methacrylation degree and hydrogel structure and mechanics. As shown in Figure 4 the swelling ratio of IPN hydrogels decreased significantly (p < 0.05) with increasing degree of methacrylation. This demonstrates that increasing the crosslinking density negatively impacts swelling behavior. Or conversely, the smaller degree of methacrylation yields a looser, more swollen system, which was also shown in our SEM results. The degree of crosslinking can simply be modulated by controlling the degree of methacrylation in the second component of the interpenetrating network.
Figure 4. A) Swelling ratio of IPNs. (n=5-7 gel samples) There is a significant increase in swelling ratio in IPN\textsubscript{32} with smaller crosslinking density. (p < 0.05) B) Compressive moduli of IPNs. (n=5-7 gel samples) Higher GMHA crosslinking density led to a significant increase in compressive moduli. (p < 0.05)

Tribometry was employed to assess the in situ compressive moduli of fully formed IPNs within the PEG molds. The results of this analysis, depicted in Figure 4, show that the IPN\textsubscript{90} construct with the higher methacrylation degree is characterized by significantly higher compressive moduli, (p < 0.05). This observation is consistent with our SEM, rheology and swelling behavior studies corroborating that hydrogel mechanical properties are enhanced with higher crosslinking density due to an increase in the methacrylic substituent along hyaluronic acid backbone.

In order to assess the tunability of the IPNs, rheology studies were carried out to determine hydrogel storage and loss moduli within the frequency range 0.1-100Hz (Figure 5). In all cases storage moduli (Figure 5 A) exceeded loss moduli (Figure 5 B), indicating “elastic dominant” behavior of all the experimental IPN groups (p < 0.05, n=5-7 gel samples). These studies showed that S-IPNs are structurally looser and
mechanically weaker relative to IPNs, due to their less dense structure (as demonstrated in SEM analysis) and smaller storage moduli, which is a result of smaller crosslinking density. Increasing mechanical properties along with more integrity and higher stiffness was achieved by increasing the percentage of methacrylation in HA polymer. IPN$_{90}$ presented better viscoelastic properties than IPN$_{32}$. Discrete values of storage moduli of

Figure 5. Storage moduli (G') and loss moduli (G'') of the gels were measured to study the viscoelastic behavior of the IPNs and S-IPNs. A) G' and (B) G'' of the gels as a function of frequency. C) Discrete values of G' plotted for different IPNs, S-IPNs and PM at a frequency of 10 Hz. The G’ of the IPN with %90Me was significantly higher than that of the IPN with %32Me. Also, S-IPNs show smaller values than IPNs.
hydrogels were plotted in comparison with swelling behavior and \textit{in situ} compressive moduli. The results demonstrate that the increase in \%Me of IPNs correlates to an increase in compressive moduli and decrease in swelling ratio.

3.3. Incorporation into Micropatterned Hydrogel Model

The primary objective of implementing the dual hydrogel system in our analysis was to improve our \textit{in vitro} model to better mimic the structural and mechanical properties of the nervous system. Formerly, PM showed a very robust neuronal outgrowth but it required careful handling as submergence in media or other movements would result in its detachment from the PEG mold. [17] We have found that our dual hydrogel system is significantly more integrated into the PEG mold. Notably, all IPNs remained within the PEG molds after 48 hours of vigorous shaking (300 rpm) and submersion in PBS. This represented a 9-fold increase in hydrogel integration within the PEG mold over our previous model. Overall fluorescence decreased after 48hrs, possibly due to loss of surface microspheres into the solution over time (Figure 6).
Figure 6. Integration of the dual hydrogel system is confirmed by mixing $1.75 \times 10^7$ beads/ml fluorospheres with GMHA/PM before crosslinking. The constructs were incubated overnight (300 rpm, 37°C). Constructs which were irradiated with UV light (IPN) A) before incubation B) after incubation. Constructs which were not irradiated with UV light (S-IPN) C) before incubation D) after incubation. 100% of IPN constructs remained intact compared to none in S-IPN constructs and 10% in previous study. (n= 6 constructs) Scale bar = 500 µm.

The photomicropatterning ability of the system was investigated by utilizing DMD in order to make a patterned structure with regionally distributed IPNs and S-IPNs (Figure 7). The novel micropatterned 3-D hydrogel was designed with a star formation (containing an S-IPN network on the inside and an IPN region outside. Fluorescein o-acrylate was used to confirm the micropatterning of both IPN and S-IPN regions through the use of DMD. The full hydrogel fluoresced green outside of the star figure that was used, while inside of the region the non-fluorescent, verifying the location of IPN and S-IPN patterns, respectively. The results showed that in this hydrogel, different zones with different mechanical properties, swelling behaviors and porosity could be introduced using photopatterning tools. This can be utilized in applications where slight changes in physical properties in microenvironment are required. The patterns chosen to demonstrate
micropatterning occupied the entire DMD array, which resulted in uneven irradiation and incomplete crosslinking around the edges. Figure 7 is simply used for demonstration of the ability to form micropatterns in the IPN systems.

**Figure 7.** Micropatterned hydrogels with IPN and S-IPN regions. The photopatterned (i.e., IPN) part (outside of the stars, the more bright part) incorporated fluorescein o-acrylate after photopolymerization. Inside of the stars (darker part) contains the S-IPN. A,B) PM/GMHA$_{32}$ system C) PM/GMHA$_{90}$ system. Scale bar = 0.1 mm

### 3.4. Influence of Microenvironment on Neurite and Schwann Cell Behavior

Immunostaining was done for neurites in bipolar microengineered constructs. The main growth was positive for β III tubulin, which is a neuronal marker. The constructs were also stained for glial cell markers and DAPI. The confocal images demonstrated that very few glial cells migrated into the matrix and the outgrowth is mainly axonal and dendritic development along the channels after 7 days (Figure 8).
Figure 8. DRG neurite growth and cell migration in dual hydrogel constructs. DRG explants cultured in hydrogel constructs for 7 days, indicated by β III tubulin-positive neurites (red), DAPI-stained nuclei (blue) and glial cell markers (green). (A-top) Depth coding showed cells are reluctant to migrate in this 3-D environment and the most growth can be assigned to axonal and dendritic elongations. The color scale represents the depth. (A-bottom) 3-D representation of growth near the center, showing both a top view (B), and a side view to demonstrate thickness (C).

Depth coding was done for DAPI and the glial cell marker to show the distribution of nuclei and glial cells. This demonstrated that cell migration stayed confined near the support surface. Figure 8 depicts the 3-D neurite outgrowth in the hydrogel. Confocal images confirmed that the growth is in 3-D and that the average thickness for neurite extension that was labeled with β III tubulin was 103±27µm. This measurement demonstrates that although the growth is not throughout all the gel’s depth, it is still in a 3-D space within the confines of the gel. Confocal imaging also confirmed that neurites are more prone to grow in an environment with lower mechanical properties. Neurite outgrowth and extension was also evaluated (n=7-9 cultured DRGs). Neuronal extension is a general term that refers to the total amount of axonal and dendritic
processes. As explained above, the migration of glial cells into the hydrogel was not noticeable. This leads us to the conclusion that outgrowth was indeed neurite extension.

DRGs were first stained for β III tubulin. The lengths of the longest β III tubulin-positive neurites were then assigned as the length of neurite extension in each side of the bipolar constructs. In other words, the maximum outgrowth in each hydrogel was considered as the neurite extension. Images of neurons in bipolar constructs were taken using fluorescent microscopy and images were interpreted using ImageJ software. According to data shown in Figure 9, neurites appear to extend more in a less stiff environment, and neurite elongation measurements were inversely correlated to the mechanical stiffness of the hydrogels. IPN₉₀ has a higher storage modulus, compressive modulus, crosslinking density and smaller swelling ratio, according to rheometry, tribometry, swelling ratio analysis and SEM, due to higher degree of methacrylation, which showed less neurite elongation. (p < 0.05)

To evaluate the impact of IPN stiffness on neurite outgrowth, DRGs were cultured in between two different hydrogels with different mechanical properties. The neurites were then allowed to pick a side to grow in the bipolar constructs (n=7-9 cultured DRGs). In order to measure the amount of cell growth in 3-D, confocal microscopy was utilized. ImageJ software and an in-house Matlab program were used in order to quantify these results, which depicted in Figure 9.
Figure 9. A) Analysis of length of neurite extension in constructs IPN\textsubscript{90} and IPN\textsubscript{32}, demonstrating that the less stiff substrate allowed longer growth. Extension was longer, with some neurites extending up to 3.3 mm after 7 days in IPN\textsubscript{32} compared to 2.6 mm in IPN\textsubscript{90}. B) Analysis of amount of neurite growth by averaging the lengths of the 5 longest neurites in constructs IPN\textsubscript{90} and IPN\textsubscript{32}, demonstrating that the more compliant substrate allowed a larger amount of total growth. E15 rat DRGs were cultured for 7 days before fixing and staining for the neurite microtubule protein $\beta$III tubulin. DRGs preferentially grew within the more compliant material. Neurite growth was denser in IPN\textsubscript{32}. ($n=7$-9 cultured DRGs). ($p < 0.05$)

After 7 days, outgrowth was seen throughout the length of both sides of the constructs, despite the difference in mechanical properties. The volume of growth for IPN\textsubscript{32} was $0.58 \pm 0.01 \times 10^{-3}$ mm$^3$ and for IPN\textsubscript{90} was $0.11 \pm 0.02 \times 10^{-3}$ mm$^3$. Measurements show a significant difference ($p < 0.05$) between 3-D growth in IPN\textsubscript{90} and IPN\textsubscript{32}. According to the results, neurite outgrowth in 3-D was more likely to happen in a less stiff environment. Significantly more growth was detected in IPN\textsubscript{32}, as compared with the apparently limited growth seen in constructs with IPN\textsubscript{90} ($p < 0.05$). The difference in mechanical properties and microstructure has been discussed in other sections.

The migration studies (Figure 10) demonstrated that Schwann cells do not travel substantially with the axonal extension in this system. In general, the Schwann cell migration in IPN hydrogels was mostly constrained to the area of the initial DRG tissue mass after 7 days. Hydrogel mechanical properties did influence migration. The Schwann
cells were more reluctant to migrate into the stiffer environment, as in IPN₃₂ they traveled 0.31±0.06 mm compared to 0.08±0.02 mm in IPN₉₀ (p < 0.05).

**Figure 10.** Analysis of Schwann cell migration in IPN constructs. Binary images of S100-stained constructs represent migration from day 0 to day 7. Bar plot shows the average minimum distance changes from day 0 to day 7 of the 8 farthest Schwann cells for each group. The results demonstrated that the Schwann cells are more encouraged to migrate in the more compliant environment (IPN₃₂: 0.31±0.06 mm) compared to the stiffer hydrogel (IPN₉₀: 0.08±0.02 mm) after 7 days (n = 4, p < 0.05). Scale bar = 0.2 mm.

4. Discussion

The reconstruction of soft tissue, such as that which is found in the nervous system, is sensitive to the mechanical and chemical cues of the growth environment. To increase the rate of axonal regeneration, it is necessary to understand how neurons behave in a 3-D model with varying mechanical properties. [29-33] This allows optimization of the system in order to find the most favorable values for neurite proliferation.

This research describes an *in vitro* environment that mimics the ECM. Due to its simple design, our model allows us to evaluate how different variables, such as mechanical or chemical signals, individually affect neurite growth. To this end, the effect of mechanical stiffness on neurite growth is evaluated in these models. By varying stiffness, we can
identify parameters that would be ideal for 3-D scaffolds for future neural tissue engineering applications.

HA is found throughout the ECM, including in the central nervous system (CNS), where it assists in both function and structure. [34,35] Furthermore, HA does not generate an immune response, and is in fact part of the wound healing process. [36] HA has unique binding sites that allow for variation of its structure and function, and therefore is available for a wide range of crosslinking groups and biologically active molecules. HA has been modified in a variety of manners that allow the attachment of photocrosslinkable moieties.

Recently, peptide biomaterials have become important for tissue engineering applications. These peptide scaffolds are able to self-assemble, and have been used to mimic ECMs in a variety of *in vitro* scenarios, making them incredibly useful for 3-D cell growth. PM shares many of the characteristics of the ECM, such as fiber size relative to ECM proteins. These properties all lead to strong support of cell proliferation and differentiation in a 3-D architecture. PM has been utilized in applications of neuronal growth and differentiation, and has been shown to promote synapse formation and the growth of a wide variety of primary neurons. [16,37,38] An IPN is made of two or more networks, which are interlaced but not covalently bound to each other. This helps improve the mechanical properties and take advantage of the benefits of each polymer network at once. [9,11,14,15]

The dynamic mask projection photolithography apparatus that was utilized in this study provided an easy fabrication technique for the purpose of producing micropatterned hydrogels. These hydrogels were created on permeable cell culture inserts, which provide
the basis for our neural regeneration study. In order to generate these constructs, we utilized a DMD device, which provided a dynamic bipolar mask. This mask irradiated the PEG to create the mold and IPNs on each side while a negative dynamic mask for the channels assisted in making the methacrylate groups of HA crosslink.

In our studies, we have exploited the self-assembling properties of PM and photocrosslinking of HA to obtain IPN hydrogels. IPN systems similar to the one we have developed are collagen/HA systems. Collagen and HA are major components of the ECM in many tissues, and collagen is a natural polymer that can be purified and solubilized, though it can be difficult to work with. On the other hand, PM is synthetic, which will reduce batch-related variations, easily lyophilized and solubilized, and is optically transparent in gel form. Furthermore, although collagen may help create favorable microenvironment, it can complicate investigations due to the potential risk of other unknown material contaminations, thus raising issues about cell signaling, protein content, and reproducibility. For some cell types, PM has provided an ECM-like environment while promoting more tissue growth than even collagen. [11,14,16] We applied this newly developed material in our in vitro model to study neurite growth. For this work, the IPN’s mechanical properties, morphology, swelling ratio and component distribution were characterized in order to evaluate the material properties of the scaffold. Furthermore, in order to see how neurons behave in environments with different mechanical properties, we varied the hydrogel’s stiffness in our model in order to investigate its effect on neural outgrowth and extension.

IPN hydrogels with different degrees of methacrylation of HA provided a new system with variable biophysical characteristics. The complete IPNs were then examined
to assure the entanglement of the two networks using SEM and gel distribution tests. Both tests confirmed that the gels are well enmeshed and two networks are interconnected. SEM images also confirmed that IPNs, S-IPNs and GMHA with higher degrees of methacrylation have a better well-connected structure. Furthermore, the density of crosslinking is higher since there are more active binding sites available. The density of crosslinking and methacrylation degree of the hydrogel correlates inversely with pore size.

Fluorescein o-acrylate was added to the pre-crosslinked solution prior to gel distribution studies, and since it only binds to acrylate groups of HA after irradiation, it showed the regions with hyaluronic acid in them. [11] It was concluded from the test that the well-mixed composition had an average intensity in between GMHA, which had the highest average intensity, and PM, with intensity close to zero. They all followed a uniform plateau pattern since they all have methacrylated HA uniformly distributed within the gel while the unmixed IPN had a unique decreasing pattern throughout the depth. No phase separation in the areas of interest was observable in the mixed gel since hyaluronic acid is highly hydrophilic and PM after formation of nanofibrils tends to form hydrophilic cavities before creating the ultimate 3-D structure. This is a confirmation of the uniform crosslinking of GMHA chains. This study established the uniform formation of the HA network, and that the two networks homogeneously entangled within the IPN hydrogels.

Higher crosslinking density of GMHA networks in the IPN hydrogel augments the elastic modulus and compressive modulus since it reinforces the network on the molecular scale. The higher degree of methacrylation leads to an improvement in
mechanical properties that inversely correlates with decrease in swelling ratio. The higher crosslinking density leads to more entangled structures, and the structures with these characteristics tend to have a smaller swelling equilibrium. However, in S-IPN structures only the PM network is holding the structure, and that is the reason for a mechanically unstable assembly. The PM’s 3-D structure partly entraps the HA chains in its network, while some chains may diffuse away. SEM also images showed a more discrete crosslinking regions. The microstructure is loose and weak, which is a cause of lacking in one polymer network in comparison with IPNs.

Rheology tests proved that the structures have a smaller storage modulus than IPNs. There is a decrease in S-IPN$_{90}$ and S-IPN$_{32}$ that can be attributed to the mechanism of formation of PM nanofibers and GMHA composition. GMHA forms through an ester formation. In this reaction the epoxide group on GMHA attacks the carboxylic groups of HA and forms an ester bond that ends at the methacrylate group. [7,24,36,38] As a result, an increase in methacrylate groups leads to a more hydrophobic composition. On the other hand, PM has multiple phases to form a 3-D structure. Initially, RADA strands form β sheets. After this, the double bond helical ribbons form followed by nanofiber formation. The final step is the development of hydrophilic cavities with a pore size of approximately 250nm. The first phase in which β sheets form is a result of interplay of various parameters including: ionic bonds, hydrogen bonds and hydrophobic interactions. [16] We suspect that differences in overall hydrophobicity and/or acidity of GMHA$_{32}$ compared to GMHA$_{90}$ may hinder β sheet formation, leading to lower modulus and crosslinking density.
IPNs also showed a superior integration with the PEG molds in the dual hydrogel system. 100% of all samples remained intact after 48hrs of submerging at 300rpm and 37°C. This can be a result of the interconnection of methacrylate groups on the hyaluronic acid backbone by possibly unreacted acrylate groups on PEG chains. Comparing the results of using IPN inside of the PEG mold with previous published results from our group where PM alone was used as the permissive part for cell growth, there is a significant improvement in the system. Moreover, dual hydrogel constructs have been maintained for more than one month in culture medium under physiological conditions, and fixed constructs have been maintained for over a year in PBS at 4°C without visibly noticeable degradation over time (data not shown). These observations make this hydrogel system a suitable option for in vitro studies.

Utilizing the dynamic mask projection photolithography we also fabricated hydrogels with areas that are different in mechanical properties. Using the photolithography method we created a hydrogel with different IPN and S-IPN regions. Depending on the 2-D photomask, some parts of the hydrogel can be exposed to the UV light, IPN, and some parts can stay intact, S-IPN. The simplicity of the lithography method gives the opportunity of micropatterning hydrogels with any kind of configurations in order to create hybrid scaffolds. This property also makes it possible to introduce spatial characteristics into regenerative therapies. [17,20,25,26]

Immunostaining verified that the outgrowth was more axonal and dendritic elongation. The main part of the growth showed a positive response to the neuronal marker; however, glial cell marker and nuclei staining showed that migration of these cells was not prominently involved in the outgrowth of the cells within the channels,
which can be mainly attributed to the culture system and biomolecular signaling that is only optimized for axonal growth in this study. Migration studies demonstrated that even with no stimuli to promote Schwann cell migration, a more compliant environment could encourage Schwann cells to migrate faster. Regulating glial migration and proliferation can play major roles in therapeutic purposes. In the central nervous system it may be beneficial to influence astrocyte migration and glial scar formation, [29,40] while in the peripheral nervous system, Schwann cell stimulation is required to encourage axonal regeneration. [29,41]

Confocal imaging showed that the growth was in 3-D, while neuronal outgrowth does not happen only in a 2-D plane and it is dispersed within the gels. The pore size is ultimately a function of crosslinking density, which in turn affects the swelling ratio and the mechanical properties of the hydrogel. Higher crosslinking density, indicated by lower swelling ratio and higher modulus, expresses a smaller pore size. Based on SEM images we can roughly estimate that the average pore size for IPN$_{90}$ is 50µm and for IPN$_{32}$ is 150µm. Since axon diameter averages between 0.5 and 30µm, we reason that the hydrogel porosity will not form a physical barrier to hinder the growth in any of the developed IPN systems. [42] However, a smaller porosity range would still provide an overall more restrictive environment for cell migration and growth. The results showed that this IPN hydrogel could be a perfect option to be utilized as a 3-D scaffold for neural tissue engineering with a governable mechanical property.

In conclusion, there are multiple benefits with using the IPN systems compared to using PM alone. The mechanical tunability of IPN in our in vitro model is achieved as a result of incorporating GMHA into the system. We showed that simply by controlling the
degree of methacrylation we are capable of introducing a range of mechanical properties. Furthermore, the structure and the properties of the natural ECM can be more closely mimicked and reproduced by generating composite hydrogels since the natural ECM is a hydrogel-like scaffold comprised of different biopolymers. The IPN also demonstrated a better integration between the two compartments of the dual hydrogel system. This model provides us with a simple, reproducible *in vitro* environment to generate different mechanical properties and study neurite growth and extension. It is well accepted that neurite extension is dependent on the mechanical properties of the environment. The results demonstrated that neurite outgrowth in 3-D was significantly higher in the environment with less crosslinking density, therefore more compliant and less interconnected. It is also evident that the storage and compressive moduli, in other words the stiffness of the hydrogel, is a reliable indicator of the ability of the GMHA/PM IPN to encourage neurite extension.
5. References


CHAPTER 4: To Evaluate the Cytotoxicity of Ultraviolet A (UVA) and Visible Light on Cultured Neurons in a 3-D Dual Hydrogel Model For Tissue Engineering (Specific Aim 2)

Abstract: Photopolymerization provides a favorable method for hydrogel formation due to the simplicity, convenience and adjustability of this technique. However, photopolymerization may have a cytotoxic effect due to light exposure. We used this method to establish a biomimetic 3-D in vitro model of the nervous system through the use of a dynamic photolithography apparatus. This setting allows us to investigate neuronal responses to different light wavelengths and exposure times as a result of photoencapsulation. We performed extensive cellular studies, including neurite growth, neurite viability and DNA fragmentation, for the most common spectra used in biological photomicropatterning applications: Ultraviolet A (UVA) (315-400 nm) and visible light (400-700 nm). These studies agree that utilizing visible light, while keeping the exposure low, is optimal for nervous system in vitro models due to it being less damaging to neuronal cells than UVA light.
1. Introduction

The central nervous system (CNS) has limited ability to regenerate after disease or injury events. Clinical techniques for treating CNS impairments are oftentimes unsuccessful due to a limited understanding of the fundamental events that block regeneration. [1]–[4] In addition to this, disruption of the peripheral nervous system (PNS) necessitates regeneration. [5], [6] In vitro platforms that can mimic the complex anatomical structure of nerve injury sites can be utilized to study the important factors that influence the CNS and PNS, leading to the development of preventive and regenerative strategies to restore function. These experimental approaches simplify the nervous system in order to isolate individual variables that have an influence in vivo, leading to a less costly and high-throughput design.

Accurate portrayal of the nervous system requires 3-D matrices that can mimic the extracellular matrix (ECM) and cell microenvironment. Recently, headway has been made in the development of highly reproducible and tunable structures that take advantage of the unique properties of hydrogels. [7]–[16] In vivo physiological structures have been recreated in 3-D hydrogels by applying various techniques such as rapid prototyping, [17]–[19] molecular printing [20]–[24] and photolithography. [7], [12], [15], [25], [26] Furthermore, optical techniques have been widely applied to form and spatiotemporally manipulate functionalized hydrogels.

Photopolymerization is a favorable method for hydrogel formation due to the simplicity, convenience and adjustability of photocrosslinking techniques. [26], [27] This approach has been used in tissue engineering applications such as cell and islet
microencapsulations, controlled platelet deposition in blood vessels and hard tissue reconstructions. Photomicropatterned hydrogels, which are spatially and temporally controllable in order to investigate cell signaling and neuroactivation, have been fabricated previously. [12]–[15], [26]

Despite these broad reaching applications, a limitation of using photosensitive hydrogels is the possible harmful effect of irradiation on the cells. [28], [29] This technique utilizes light in order to transform initiator molecules to a radical form, beginning the polymerization process. The two principal wavelength spectra utilized for this purpose are UVA (315-400 nm) and visible light (400-700 nm). It has been shown that both UVA and visible light can induce cell toxicity, leading to cell death. [30]–[32] However, there is little research that compares the cytotoxic effects of these two wavelengths in a single hydrogel system.

Light irradiation may also have a cytotoxic effect on the nervous system. For example, the PNS is exposed to irradiation throughout the day, as the spectral distribution of solar energy at sea level comprises 3-7% of UV (290-400nm), 44% visible light (400-700nm) and 53% of Infrared (IR) radiation (700-1440nm). [33] Visible light and UVA wavelengths penetrate deep into the dermis, and absorption may produce heat and induce reactive oxygen species, matrix-degrading enzymes, and enzymatic activity. These can cause nuclear DNA damage in human cells. In contrast to the extensive research on the damaging influence of UVB on different cell types, mainly skin cells, there are a few studies that have looked at the possible effects of visible light and UVA on neuronal cells whose behavior may or may not be affected by light exposure. [34] Therefore data from
studies which analyze and compare these spectra can provide an early understanding of the influence of UVA and visible light on peripheral nervous system.

To study the influence of these wavelengths on neuronal behavior, including cytotoxicity and cellular apoptosis as a result of photo-encapsulation, we utilized 3-D hydrogels that incorporate \(-\text{(-)}\) Riboflavin (RF), a photoinitiator that has similar light absorption quantities in UVA and visible region. [28], [29], [35]–[37] Functionalized Dextran has been shown to have the potential of forming a hydrogel network upon addition of RF as a photoinitiator. [28], [29] This design allows a unique platform to study the effect of visible and UVA light on cytotoxicity due to irradiation. We have previously developed a facile and rapid technique that uses a digital micromirror device (DMD) incorporated with a simple microscope objective to photopattern desired 3-D hydrogels. DMDs are capable of structural and molecular 3-D micropatterning. We used this approach to photomicropattern functionalized Dextran and encapsulate neurons in order to study and compare their viability upon UVA or visible light irradiation. These studies will be performed in a 3-D setting that more closely mimics the natural cellular microenvironment than previously used 2-D tissue cultures.

2. Materials and Methods

2.1. Dextran Synthesis and Characterization

Dextran (MW = 70 kDa) was grafted by Glycidyl methacrylate (GMA) based on a published protocol. Initially, 1 g dextran was weighed and added to 9 ml dimethylsulfoxide (DMSO) under nitrogen. 0.2 g 4-dimethylaminopyridine (DMAP) was dissolved in 1 ml of DMSO. Subsequently, the DMAP solution was added dropwise to
the dextran solution followed by addition of 232 µl GMA under nitrogen. The final solution stirred for 48 hrs at room temperature. In order to quench the reaction after 48 hrs, 280 µl 37% hydrochloric acid (HCl) was added to the solution then the resulted product was dialyzed against deionized water for 3 days and lyophilized for 2 days. The resulted product was a glycidyl methacrylate-dextran (MeDex), and the addition of methacrylate groups to dextran was confirmed using $^1$H NMR [(D$_2$O) $\delta$ 6.1-5.7 (m, 2H, CH$_2$), $\delta$ 5.2 (m, 1H, CH), $\delta$ 4.9 (m, 1H, CH), $\delta$ 1.9 (s, 3H, CH$_3$)] with substitution degree of 42%. [14], [38], [39]

2.2. Gel Composition

The gel composition was selected based on the time of gel formation upon UVA and visible light irradiation and relevant time to form a gel. A 50% MeDex was tested with variable concentrations of RF as the photoinitiator for the free radical polymerization, L-Arginine (Arg) as the co-initiator and electron donor and Tetramethylethylenediamine (TEMED) as the catalyst for the radical formation. The composition was tweaked until achieving a consistent gel in 3 time points: 30, 60 and 90 seconds. The final composition was MeDex 50% (w/v), Arg 0.1% of MeDex (w/w), RF 0.001% of MeDex (w/w), TEMED 0.2% of the final solution (v/v). [28], [29]

2.3. Photolithography Setup

The apparatus that was used for irradiating the desired shape on the photosensitive solution is made of two collimated light sources, a UV (OmniCure 1000 with 320–500 nm filter, EXFO, Quebec, Canada) and a visible light source (SOLA light engine with
375-650 nm filter, Lumencor, OR, USA), a digital micromirror device (DMD) (Discovery™ 3000, Texas Instruments, Dallas, TX) that acts as a dynamic photomask, and a 2X objective lens (Plan Fluor, Nikon Instruments, Tokyo, Japan). [12], [13], [15] The light source can work simultaneously or individually based upon the required spectra.

The UV and visible light were filtered using a dichroic (409 nm BrightLine Dichroic Beamsplitter, Semrock, NY, USA) and an ND filter (Unmounted Ø2" Absorptive ND Filter, Optical Density: 0.3, Thorlab, NJ, USA) so that the UV light source was not passing any wavelengths beyond 409 nm and the visible light was not passing any wavelength below 409 nm. The ultimate wavelength ranges for the visible and the UV light were 409-650 nm and 375-409 nm respectively. The surface power density of the lights on the irradiation area for both spectra is 85 mW/cm² that was measured by a radiometer (306 UV Powermeter, Optical Associates, San Jose, CA). The lithography apparatus is used to pattern UV or visible light onto photocrosslinkable polymer solutions in order to shape a desired hydrogel geometry, which is spatially controllable.

2.4. Photomicropatterning

A gel solution was prepared as follows. First, 200 µl of the gel solution was added on a coverslip. An even layer of gel solution was formed. Star-shaped micropatterns were then produced upon exposure to visible light using the dynamic photolithographic device as explained previously. The gel layer was irradiated for 30, 60 and 90 seconds to determine the micropatterning ability of the gel upon UVA and visible light exposure. In order to form micropatterns, a star-shaped dynamic photomask was loaded on the DMD
device and the pattern was irradiated directly on the gel solution surface. Bright field images were acquired using a Nikon AZ100 62 stereo zoom microscope. All of the steps were performed in the dark.

2.5. Mechanical Properties

The viscoelastic properties of the hydrogels were measured using a rheometer (TA Instruments, AR2000, New Castle, DE). Three samples of each experimental group were prepared. The groups consisted of gels forming upon visible or UV light irradiation in 3 time points: 30, 60 and 90 seconds. The samples were loaded between the rheometer cone (1° steel cone, 4 cm diameter) and plate. Storage (G’) and loss (G”) moduli for each sample were evaluated at room temperature over a frequency sweep range of 0.1-10 Hz under a constant strain rate.

2.6. Neurite Growth

In order to study the effect of visible or UVA light on neuronal growth, dual hydrogel culture systems were fabricated via digital projection photolithography. The dual hydrogel system is constructed from two compartments: cell permissive and cell restrictive sections. The latter will provide a mold to contain neuronal projections. In order to make the cell restrictive mold, a 10% solution of polyethylene glycol (PEG)-diacrylate (MW 1000; Polysciences Inc., Warrington, PA) and 0.5% (w/v) Irgacure 2959 in PBS was contained in a collagen-coated PTFE Transwell® Permeable Support (Corning Inc., Corning, NY) (0.1 ml/cm²) insert and was irradiated for 38 seconds with UV light. Following fabrication of the cell restrictive section, the cell culture inserts were
washed three times with wash buffer (1% antibiotic-antimycotic in DPBS). Then the constructs were immersed in an adhesion media consisting of neurobasal medium supplemented with B27, L-glutamine, nerve growth factor (NGF), fetal bovine serum (FBS) and penicillin/streptomycin (P/S) (all provided from Life Technologies, MA, USA) and incubated (37°C, 5%CO₂) overnight. The constructs were then cultured with Long Evans rat embryo dorsal root ganglion (DRG) tissue. All of the animal procedures were accomplished according to the guidelines of the Institutional Animal Care and Use Committee. The DRGs were harvested from rat embryos day 15 (E15) (Charles River, Wilmington, MA). The DRG explants were removed from the cervical section of the spinal cord, trimmed and plated in the mold. The explants were incubated in adhesion media for 2 hrs. Consequently, the adhesion media was aspirated from the mold and MeDex solution was added and irradiated for 30, 60 and 90 seconds with UVA or visible light. Utilizing this procedure, the DRGs were encapsulated into the hydrogel and could encourage three-dimensional growth. The constructs were washed three times to avoid any contamination and also to remove the TEMED in the hydrogel network in order to minimize the effect of it as the only cytotoxic component of the gel system. 1500 µl of neurite growth media consisting of Neurobasal Medium (NBM), 2% v/v B27, 1% v/v penicillin/streptomycin, 1% v/v Glutamax and 0.02 µg/ml NGF was added to each well. The media was changed every hour for the initial 6 hrs to remove any residual TEMED from the gel. The constructs were incubated in 37°C for 7 days, with media changed every 24 hrs after the first 6 hrs of culture. (Figure 1)

Neurite outgrowth was measured using standard immunohistochemical techniques. After fixation of the tissue with 4% paraformaldehyde for 2 hrs, neurites were
tagged with mouse monoclonal [2G10] neuron-specific beta III tubulin primary antibody and Cy3.5 conjugated goat antimouse immunoglobulinG (H + L) secondary antibody (AbCam, Cambridge, England). Each step of the labeling procedure was carried out in 0.1% Saponin and 2.0% Bovine Serum Albumin (BSA) solution.

The volume of growth into the 3-D hydrogel was measured utilizing confocal microscope (Nikon A1, Tokyo, Japan). The number of neurites growing into the model is difficult to count due to the entwined structure that is formed in the system as it extends along the length. The bundle is too complex to identify all of the individual neurons. Therefore, it is optimal to take the volume of cellular mass in the system in order to quantify, in 3-D, the growth of the system. Each sample was imaged in 3-D with an optical slice not greater than 11 µm depth and an average of 20 slices per sample, a resolution of 1024 × 1024 pixels and with a 10X objective. Pre-processing steps including thresholding and transformation into a binary representation were applied uniformly across all images. Data analysis was performed using ImageJ and a custom algorithm in Matlab (Mathworks, Natick, Ma). Neurite growth was quantified using pixel counts of the threshold slices throughout the depth of the gel.
Figure 1. A: Depiction of the methodology for gel formation for TUNEL and Ethidium Homodimer-1 assays. Step 1: Formation of PEG mold; Step 2: DRGs dissociation followed by mixing with the gel solution at a specific cell count; Step 3: Addition of the gel solution to the void; Step 4: Irradiation using the negative mask and gel formation.
B: Depiction of the photocrosslinkable solution being added to the mold for neurite growth studies. Step 1: PEG mold formation; Step 2: DRG insertion; Step 3: Gel solution addition to the mold; Step 4: Irradiation using the negative mask.
2.7. DRG Dissociation

In order to dissociate the DRG explants, 50 E15 DRGs were harvested from 8-10 spinal cords and were incubated for 20 min in 200 µl Trypsin/EDTA 0.05%. 2 ml Neurobasal medium supplemented with B27 was added to neutralize Trypsin and the solution was pipetted up and down followed by centrifuging for 3 mins at 300 rcf and room temperature. The supernatant was then aspirated and desired amount of MeDex solution was added to the cells and agitated until achieving an evenly distributed single cell solution. Cells were counted after dispersing in the MeDex solution using a hemocytometer.

2.8. Ethidium Homodimer-1 Assay

To perform an ethidium homodimer-1 assay, initially a PEG hydrogel mold was formed as described in Section 2.6, followed by addition and irradiation of MeDex single cell solution. Initially, to prevent meniscus formation, 12-well plate inserts with pore size of 0.4 µm were treated with filtered Rain-X (SOPUS Products, Houston, TX) then 120 µl of photocurable PEG solution (10% (w/v) PEG and 0.5% (w/v) Irgacure 2959 in PBS) was added to the insert. A proper photomask was loaded on the DMD and the solution was irradiated for 38 seconds and to photomicropattern a cylindrical void. The hydrogel constructs were washed with wash buffer to prevent contamination. Following this step, the constructs were incubated in adhesion media as described in Section 2.6 (37°C, 5% CO₂) overnight. Dissociated DRGs were prepared according to Section 2.7 and an even single cell MeDex solution of $3 \times 10^6$/ml was prepared. 2 µl of gel solution was then added to each mold and was irradiated for 30, 60 and 90 seconds. The constructs were
washed three times with wash buffer to avoid any sort of contamination and to remove residual TEMED from the system. 600 µl of neurite growth media (as explained in Section 2.6) was added to each well. In order to validate that TEMED left the system the media was changed with fresh growth media every hour for the initial 6 hrs. After 30 hrs a 1:400 solution of ethidium and neurite growth media was prepared and filtered and the constructs were immersed and incubated in the labeling media for 10 mins (37°C, 5% CO₂). The constructs then were washed with sterile PBS twice under aseptic condition and imaged immediately. Images were acquired using 4X objective on an Olympus IX70-CoolSNAP microscope-camera combination. Images were captured in 20 µm intervals through a 200 µm depth and using z-stack image acquisition with NIS-Elements AR (Advanced Research) software (Nikon, Tokyo, Japan) and maximum projection was obtained and used for final image analysis. After imaging the constructs were washed once with the washing buffer, soaked in fresh growth media and placed back in the incubator. The same staining and imaging process was repeated after 48 hrs. The image was processed using ImageJ and a custom algorithm in Matlab. (Figure 1)

2.9. TUNEL Assay

The constructs were prepared as described in Section 2.6 and 2.8. After formation of the PEG mold, the constructs were washed 3 times with wash buffer to avoid any contamination. MeDex single cell solution was prepared according to section 2.7. The remaining liquid was aspirated from the PEG mold. 2 µl of the single cell gel solution (3 × 10⁵/ml) was added to each mold and each group was irradiated for 30, 60 or 90 seconds. The constructs were washed three times with wash buffer to prevent
contamination and any cytotoxic effect of TEMED. The media was changed hourly for the first 6 hrs to remove remaining TEMED. TUNEL assay was performed in order to measure the amount of DNA fragmentation 30 hrs after irradiation with visible light and UV light. An In situ direct DNA fragmentation (TUNEL) assay kit (AbCam, Cambridge, England) was used to study the apoptosis. The process was done according to the manufacturer’s protocol and was slightly tweaked to fit our specimens. Briefly, cultured neurons were fixed in 4% (v/v) paraformaldehyde after 30 hrs and were washed with PBS three times. The constructs were then soaked in ice-cold 70% (v/v) ethanol for 30 mins and were stored in -20°C overnight. The ethanol was aspirated and the constructs were washed three times with the wash solution provided by the manufacturer, each wash for 10 mins, at room temperature and 50 rcf to remove ethanol. The constructs were then incubated in staining solution for 3 hrs (37°C, 50 rpm) followed by three washing steps with the rinsing buffer which comes with the kit. The inserts were then incubated in propidium iodide/RNase at room temperature for 2 hrs. All of the steps were carried out in the dark. The imaging procedure was performed according to Section 2.7. The image was processed using ImageJ and a custom algorithm in Matlab. The total number of cells was measured using DAPI staining. (Figure 1)

3. Results

3.1. Photomicropatterning Ability

Figure 2 shows a selected image for photomicropatterning after 90 seconds of irradiation. The hydrogel system demonstrated the ability to form micropatterns through our lithography apparatus at various time points. The star-shaped dynamic photomask
was projected on the gel solution in order to form a pattern to show the photomicropatterning ability of MeDex solution. The results demonstrate the unique property of this gel composition’s micropatternability after irradiation with UV and visible light.

![Image of photomicrographs](image)

**Figure 2.** Photomicropatterned hydrogels. Star-shaped micropatterns were produced upon exposure to A) UVA and B) visible light using the dynamic photolithographic device. The photopatterned part (outside of the stars) was formed around the star. Scale bar = 0.1 mm.

### 3.2. Correlation of Irradiation Time with Mechanical Properties of MeDex Gel

We have shown previously that microenvironment mechanical properties will affect neuronal behavior such as axonal extension and growth. [My paper if it is published] In order to minimize the impact of these properties in our models such that the light spectra is the only variable, we require hydrogels with similar mechanical properties. Here we measure the mechanical properties of the gels. (n = 3 gel samples)

We utilize the fact that RF has a similar absorption rate in both the UVA and visible
ranges. This leads to the expectation that the mechanical behavior would be only dose-dependent. Figure 3 shows the storage and loss moduli in a frequency range of 0.1-10 Hz and the average G’ for each hydrogel at a frequency of 10 Hz. G’ exceeded G” in all samples, demonstrating the “elastic dominant” behavior of every experimental group (p < 0.05, n=3 gel samples). G’ represents the energy which is stored in the material and elasticity of the hydrogel while G” provides information about the energy that is lost in the form of heat and the vicious behavior. The G’ values are between 10-20 times less than G”. This ratio is a measure of energy stored against energy lost in the cyclic deformation and is referred to as the dissipation factor, which also describes the elastic behavior of the gels for all experimental groups.

**Figure 3.** Storage moduli (G’) and loss moduli (G”) of the hydrogels were measured to study their viscoelastic behavior. A) G’ and B) G” of the gels as a function of frequency, with the legend or A) and B) lying in between them. C) Discrete values of G’ plotted for different exposure lights and light sources at a frequency of 10 Hz. The G’ of the
hydrogels with 90 seconds of exposure time was around five times that of the other groups. (P<0.05)

G’ and G” were almost equivalent for the gel samples after similar irradiation times. The mechanical properties appear to be dose-independent after 30 and 60 seconds of UVA and visible light irradiation and their values were statistically equivalent within and among the experimental groups. This demonstrates that increases in irradiation time after the first 30 seconds to 60 seconds would not substantially increase the formation of the neutral free radicals of RF as the photoinitiator in the presence of Arg as an electron donor. Therefore, methcrylation does not change, resulting in almost analogous G’ and G” after 60 seconds. The storage and loss moduli after 90 seconds is significantly different with 30 and 60 seconds while it is similar for UV and visible light irradiated samples.

3.3. The Effect of Visible and UV Light on Neuronal Viability

To measure cell damage caused by visible and UVA light exposure, cellular viability after 30 and 48 hrs of irradiation was examined with an ethidium homodimer-1 assay. (n= 5-6 constructs) This assay can be utilized to detect dead or dying neurites. During this assay the dye gets in to the cell through the compromised membrane and binds to DNA. The cytotoxicity of these samples were observed after 30 hrs and 48 hrs to see if the quantity of cell damage would change between the two time points. Therefore, every sample is measured at both 30 and 48 hrs.

As shown in Figure 4, there is a statistically significant increase in cytotoxicity when there is an increase in irradiation time for both UVA and visible light. Therefore,
we conclude that the 30 seconds exposure duration is substantially less harmful for photopatterning applications than the 60 or 90 seconds exposure. For each experimental group, there is no significant increase in cellular death at 48 hrs when compared to the same experimental group at 30 hrs. The measurements taken 30 hrs after irradiation all demonstrate showed that there is a significant increase in damage when UVA light is used instead of visible light. This statement holds true for all exposure lengths after 48 hrs as well.
Figure 4. Depiction of the number of dead cells as stained by an Ethidium Homodimer-1 assay. The samples were observed at 30 hrs and 48 hrs after light exposure. Exposure to UVA light is more damaging than visible light for all experimental groups. There is a statistically significant increase in cytotoxicity when there is an increase in irradiation time for both UVA and visible light. For each experimental group, there is no significant increase in cellular death at 48 hrs when compared to the same experimental group at 30 hrs. Representative images depict stained cells for visible light exposure for 30 secs (B) as well as UVA light exposure for 90 secs (C), with each red dot representing a cell stained by Ethidium Homodimer-1. (p < 0.05). (n = 4-6). Scale bar = 100 µm.
3.4. The Influence of Visible and UV Light on DNA Fragmentation

TUNEL assays were performed in order to study DNA fragmentation resulting from UVA and visible light irradiation for 30, 60 and 90 seconds. (n = 5-6 constructs) This assay detects nicks in DNA in order to measure the number of cells that have undergone apoptosis or have severe DNA damage, though not necessarily total cell death. As a result the data shows that there is a growing trend in the number of DNA-damaged cells when there is an increase in exposure time, similar to the results from our ethidium homodimer-1 analysis when damage is measured after 30 hrs. Furthermore, more cells undergo apoptosis or have severe DNA damage using UVA compared to visible light for the same irradiation dosage. [41] (Figure 5)

**Figure 5.** Depiction of the studies that describe the number of apoptotic cells as stained by a TUNEL assay. There is an increase in the number of DNA-damaged cells when there is an increase in exposure time. Furthermore, more cells undergo apoptosis using UVA compared to visible light for the same irradiation dosage. Representative images depict stained cells for UVA light exposure for 90 secs as well as (B) visible light exposure for 30 secs (C). TUNEL stained cells are green while DAPI cells are red. (p<.05). (n = 4-6). Scale bar = 75 µm.
3.5. The Influence of Visible and UV Light on Neurite Growth

DRGs were cultured in hydrogel scaffolds utilizing UVA or visible light in order to study the influence of light on neuronal 3-D outgrowth. (n = 5-6 constructs) Pixel counts of the threshold images throughout the depth of the gel were calculated to determine 3-D neurite growth. As demonstrated in Figure 6, the volume of neuronal growth is suppressed by increasing the time of irradiation in each experimental group. When comparing UVA and visible light exposure, similar to what was demonstrated in our Ethidium Homodimer-1 and TUNEL analyses, UVA is more harmful to the cells when compared to visible light after 30 and 60 seconds of exposure. However, after 90 seconds of exposure, there was no significant improvement over choosing visible light compared to UVA.
Figure 6. Depiction of the volume of cell growth. Visible light exposure contained more growth than irradiation with UVA at 30 and 60 secs. There was no difference in growth after 90 secs of exposure between the two groups. Exposure of the hydrogels to visible light for 30 secs resulted in no statistical difference with the sham. Representative images depict cells after (B) visible light exposure for 30 secs as well as C) UVA light exposure for 90 secs, demonstrating that B resulted in more cell growth than C. (p < 0.05) (n = 3-5). Scale bar = 500 µm.

The volume of growth for UVA light exposed samples after 30, 60 and 90 seconds were $0.078 \times 10^{-3}$, $0.048 \times 10^{-3}$ and $0.045 \times 10^{-3}$ mm$^3$, respectively. There was no significant difference in damage between the 60 and 90 seconds groups. A similar trend was observed in samples after visible light irradiation: $0.119 \times 10^{-3}$, $0.076 \times 10^{-3}$ and
0.041 \times 10^{-3} \text{ mm}^3 \text{ growth after 30, 60 and 90 seconds of irradiation, but here there was a significant difference in damage between the 60 and 90 seconds groups. In comparison to the sham, a 3-D control where gel formation was completed using ambient light, exposure of visible light for 30 seconds had no impact on neurite growth, while every other experimental group had decreased growth. This demonstrates that 30 seconds of visible light can have almost no effect on neuronal growth and extension, which does not apply for other groups.}

4. Discussion

In recent years synthetic hydrogels have become an important tool in tissue engineering as they have the ability to mimic the 3-D structure of the ECM. Engineered hydrogels give scientists the capability to reproduce the cellular microenvironment in a spatial and temporal manner. Recent developments in the engineering of biomimetic scaffolds have led to the utilization of light that can modify the properties of hydrogels by changing a gel solution from a liquid state to a 3-D network in the presence of cells and tissues. This method allows gel formation in situ and under physiological conditions. It has been shown that light can be utilized to either create or even cleave photosensitive bonds in order to alter gel crosslinking density. Both approaches lead to a dynamic change in hydrogel properties that heavily influence cellular response. [12], [14], [26]

Although photocrosslinking techniques have many advantages, including convenience, flexibility and the in situ cell encapsulation ability, there are two main concerns regarding their utilization. First is the chemical cytotoxicity of the synthetic photoinitiators. [28], [42], [43] Many studies have looked into the cytotoxicity of the
photoinitiator system. In an extensive study, Bryant et al. investigated the effect of photoinitiating systems using chondrocytes as a well-characterized cell line. They showed that radical associated with initiators could react with many different cell parts such as cell membrane, proteins and nucleic acid. They performed a comprehensive study on the effect of the photoinitiators on cytotoxicity of encapsulated fibroblasts. [26] In a recent study Mironi-Harpez et al. investigated different aspects of curing kinetics of hydrogels that are being photopolymerized, including covalent chemical crosslinks and their cytotoxicity and mechanical properties during photopolymerization. [44] The second downside of photopatterning in in situ cell encapsulation applications is the effect of light on cellular damage. The two principal wavelength spectra utilized for this purpose are UVA (315-400 nm) and visible light (400-700 nm), which have both been shown to induce cell toxicity, leading to cell apoptosis. [30]

The effect of UVA and visible light, which are the most common lights applied for photopatterning, have been evaluated on different cell types such as skin, retinal or oral cells. [34], [45]–[49]. While many studies show that UV-B is the main cause for DNA damage because of its absorption by cellular DNA, there is evidence that UV-A will have similar impact. UV-A wavelengths will not be absorbed by native DNA, but they are capable of initiating secondary photoreactions in existing DNA photoproducts. The biological responses to UV-A include decreases in both the survival rate and proliferation of cells, as well as protein structure disruption. [48] From the action of UV-A and endogenous photosensitizer such as flavins and porphyrins, single oxygen species (ROS) can be produced that can generate oxidative damage to DNA. [46], [50] Similar to UVA, the effect of visible light on cellular damage has not been vastly researched.
Research has demonstrated the influence of visible light irradiation on cellular responses. Liebel et al. investigated the effect of visible light on ROS and matrix metalloproteinase (MMP) responses in skin grafts. They reported that visible light will induce ROS production. [34] Keilbassa et al. demonstrated that short wavelengths of visible light can generate DNA damage. [51] In a different study, Li et al. determined whether visible light could directly trigger the death pathway by DNA damage. Their study showed that the exposure of retinal ganglion cells (RGCs) to visible light directly affects nuclear DNA damage. [45] Little research compares the magnitude of cytotoxicity in a single 3-D specimen utilizing UVA and visible light.

Photomicropatterning has been previously applied to studies the nervous system, as it allows proper translation of the biomimetic neuronal microenvironment in 3-D. [12], [15], [16], [27] Despite the need for in vitro models for further analysis of the nervous system and the importance of the photopolymerization techniques in developing these systems, there are few studies which look at the effect of light on neuronal behavior. In this study we investigated the effect of UVA and visible light on neuronal behavior in a single photocurable model with analogous physical properties after gelation upon either UV or visible light. Previously Kim, et al. described the photocrosslinking ability of MeDex using a RF photoinitiator system upon UVA or visible light exposure and in the company of Arg as a co-initiator. We utilized this system along with TEMED, which pairs with RF as a catalyst of polymerization. This novel system is capable of gel formation upon either UVA or visible light irradiation.

The mechanism of action behind the formation of our hydrogels involves the reaction of RF and Arg. RF is known to not commence free radical photocrosslinking in
similar systems after being exposed to UV or visible light irradiation, and therefore it is necessary to use an electron donor such as Arg, which has amine groups. [52] TEMED was also used as a free base to accelerate the development of the free radical from RF. TEMED is a tertiary amine that acts as a catalyst for this reaction. [53]

We modified our previously developed photolithography system to work in the spectra ranges of 409-650 nm and 375-409 nm, where RF has analogous absorption peaks and surface power densities for UV and visible light, respectively. [28], [29] Although the intensities of the peaks for RF in the UV region are greater than the visible light region, the peaks in the latter are wider than those in former. This suggests that the quantity of light absorption is almost equivalent. [28], [29], [52]

To validate our model, we scrutinized our system for its micropatternability properties. The results show that the system has the ability to form micropatterns at different time points. Therefore, our photolithography apparatus provides a dynamic mask which allows the formation of desired hydrogel patterns. Since RF behaves as a photoinitiator, the gel solution has the potential to form a micropattern with both UV and visible light. UV and visible light micropatterning have been previously reported separately for biological applications, but there are few hydrogel systems with the potential to form micropatterns under both wavelengths. [13], [54] We demonstrated that with the current composition we were able to form a gel within 30 seconds of irradiation. In order to evaluate the mechanical properties of the hydrogel systems after irradiation with two different spectra, rheology studies were performed (Figure 3). The mechanical properties in these hydrogels are dependent on the photoinitiator system and its concentration, the precursor concentration, and the UV light intensity during
photopolymerization, as demonstrated by Mironi-Harpaz et al. In these cases, if the light intensity and photoinitiator concentration are held constant, raising the precursor concentration (for instance, in our system the amount of functionalized acrylate groups) leads to a corresponding increase in maximum storage modulus and gelation times. [44] In other studies it has been confirmed that light intensity, temperature and irradiation time affect mechanical properties. For example, higher curing light intensity has been demonstrated to raise elastic modulus values in polymer composites, though there is not a linear relationship between the two variables. Other research has demonstrated that the quantity of free radicals formed during irradiation processes heavily influences the degree of monomer conversion. Therefore the energy delivered to the system, and thus the light intensity and irradiation time, are key factors. [44], [55], [56]

Since, as mentioned above, RF has a similar absorption rate in UVA and visible light and we kept all other variables constant, we were expecting to have similar mechanical properties within each group. Figure 3 shows that the storage moduli at 10 Hz are not statistically different for each irradiation dosage. The results indicate that the storage moduli after 30 seconds and 60 seconds of UV and visible light exposure are not statistically significant, demonstrating that the increase in irradiation time will not influence the mechanical properties in a linear manner. This result may be due to the development of insufficient free radicals for chemical bond formation, leading to no difference in the mechanical properties between the 30 and 60 seconds group.

In our experiments we analyzed the amount of cellular growth and cellular damage in 6 different hydrogel systems. These 3-D gel networks were formed using either visible light or UVA light for exposure times of 30, 60 or 90 seconds. We provide
data in Figure 6, which describes the amount of neurite growth, and Figures 4 and 5, which depict the amount of cell damage found by using Ethidium Homodimer-1 or TUNEL analysis. For the groups with 30 and 60 seconds of exposure, the results can be compared because the difference in mechanical properties is not substantial (as demonstrated in Figure 3) and the only factor that is changing is the time of irradiation. One can argue that the results of 90 seconds of irradiation cannot be associated to the influence of light alone. Within the groups (30, 60, 90 seconds) the stiffness is always statistically equivalent after UVA or visible light irradiation. Therefore this method provides us with a suitable model where the only factor that varies is the spectrum wavelength.

In our analysis of neurite growth, shown in Figure 6, we demonstrate that hydrogels, which are formed using visible light with an exposure time of 30 seconds, had no decrease in neurite growth versus our sham. However, the 5 other groups all exhibited decreased cellular growth versus the control group. For 30 seconds and 60 seconds of exposure, UVA had a larger negative impact on growth than visible light. However, there was no difference between the two methods if the exposure time was for 90 seconds. This can be attributed to both the influence of the stiffness on neuronal projection as well as the longer time of irradiation.

Our data trends regarding neuronal extension are supported by previous research into spectra with $\lambda > 400$ nm. Other research has demonstrated the formation of neuronal networks through the use of patterned visible light ($\lambda = 525$ nm). In these experiments it was reported that increasing amounts of light intensity resulted in a decreased percentage of cells with neuronal projections more than 25 $\mu$m in length. Furthermore, they
investigated the influence of different intermittent light patterns on axonal extension. The results showed that changing light intensity, the interval and total time of light irradiation influenced the ratio of outgrowth. [58], [59] In another study, researchers demonstrated that irradiation at 470 nm promotes neuronal growth compared to the broad range of wavelengths used. [59] All of these studies demonstrate that visible light will not drastically hinder neuronal extension and growth, which supports our results.

The results from the neuronal growth study are similar to our Ethidium Homodimer-1 and TUNEL assay analyses of cell viability and DNA fragmentation, which are shown in Figures 4 and 5. Our Ethidium Homodimer-1 data demonstrates that, when measurements are taken 30 and 48 hrs after exposure, UVA is more damaging to the cells than visible light. There is no increase in damage between the two groups, 30 and 48 hrs after exposure, for all hydrogel constructs. This indicates that the damage occurs before 30 hrs. The results showed that there is an increasing trend when the duration of exposure is raised. The UV light cell death was statistically significant compared to visible light within each group.

We studied the influence of light after 30, 60 and 90 seconds of exposure on DNA fragmentation in our TUNEL analysis. The experiment was done after 30 hrs when the DNA damage has its maximum expression. [49] It has been demonstrated that UV exposure induces apoptosis in keratinocytes as a protective mechanism for highly damaged cells to prevent the formation of malignant transformations. The UV damage becomes detectable 8 hrs after exposure and will reach maximum after 24-48 hrs. It is theorized that this maximum damage occurs during this period because the cells have
established repair mechanisms in order to counteract the DNA lesions that are caused by light or any other stressor, including excision repair, photoreactivation. [48], [49]

The results for TUNEL assay show that within each group the influence of visible light on the DNA damage is less than UVA. The findings also verify the previous studies on the influence of visible light on DNA damage when compared to UVA, as the cells at each time point experience less DNA damage, but they are still substantially deteriorated. The number of apoptotic or damaged cells increase with longer exposure times. The same trend was observed for the group that underwent UVA light exposure.

In conclusion, we developed a 3-D in vitro model with biomimetic properties through our dynamic photolithography apparatus. This experimental design allows us to take advantage of photomicropatterning in order to design studies for neuronal responses to different light wavelengths. The physical properties of these hydrogels were investigated in order to describe any possible influence of our design on neuronal behavior. We performed extensive cellular studies including neurite growth, neurite viability and DNA fragmentation. These studies agree that utilizing visible light is more practical for hydrogel formation and cellular encapsulation for photocrosslinking applications as it is less damaging to the cells in our studies than UVA light. However, visible light still causes damage to our cells. These data also confirm that increasing the irradiation dosage through raising the exposure time will result in more cellular damage and DNA fragmentation.
5. References


CHAPTER 5: To Investigate Myelination of Neuronal Cells Incorporating with Schwann Cells in an \textit{in vitro} 3-D Model (Specific Aim 3)

\textbf{Abstract:} The interaction between Schwann cells (SC) and neurons during axonal development and regeneration affects the direction of growth and the ability to generate myelin. Experimental SC/neuron co-cultures provide insight into these processes. We developed a novel \textit{in vitro} 3-D co-culture model that allows incorporation of SCs and neurons through the use of a dynamic photolithography apparatus. Using this design, we photomicropattern functionalized Dextran and encapsulate DRGs and SCs into the culture system and investigate factors which lead to the formation of myelin. We analyzed two culturing processes along with the influence of collagen on neuronal growth and myelination. We demonstrate that this co-culture setting provided us with aligned, highly fasciculated neuronal growth with myelin sheaths wrapped along them. Myelination was confirmed through immunohistochemistry and TEM. This platform provides us with a robust tool for drug discovery and myelination studies.
1. Introduction

Successful axonal regeneration in the PNS is dependent upon properly targeting neuronal growth towards a chosen location along with forming functional synapses for signal propagation. [1] Schwann cells (SCs) native to the peripheral nervous system (PNS) play a major role in this process. [2], [3] SCs wrap developing axons in myelin and produce extracellular matrix (ECM) components, cell adhesion molecules and neurotrophic factors. [2], [4]–[6] These events rely on a complex network of signals, including SC-to-neuron, SC-to-SC, and SC-to-ECM communications, from the local microenvironment. [7] Experiments containing SC/Neuron co-cultures provide insight into these processes and may lead to new clinical approaches to nervous system ailments. Primary neurons and SCs have been previously co-cultured in 2-D and 3-D systems in order to study the mechanisms involved in SC/neuron incorporation. [8]–[12] It has been demonstrated that SCs play an important role in orienting developing axons toward their desired targets, leading to functional re-innervation in these models regardless of the number of dimensions. [6] However, many properties involved in SC/neuron incorporation, such as morphology and gene expression, are dramatically affected by system architecture. [13] 3-D systems offer a stronger representation of the structure and function of the neuronal microenvironment as well as a better understanding of cell-cell and cell-ECM mechanisms. For example, it has been shown that resting potential, action potential propagation and the function of voltage-gated channels are significantly different in 2-D vs. 3-D models. [14], [15] Although the importance of utilizing 3-D biomimetic nervous system microenvironments has been demonstrated, few studies
investigate SC/neuron interactions in a co-culture and their impact on myelin formation. [6], [16]

We have previously developed a facile and rapid technique that uses a digital micromirror device (DMD) incorporated with a simple microscope objective to photopattern desired 3-D hydrogels. DMDs are capable of structural and molecular 3-D micropatterning. This \textit{in vitro} model provides a setting to mimic the support and 3-D architecture of the ECM, with the ability of introducing immobilized or soluble chemical biomolecules, mechanical cues and drugs independently and evaluates their effects on neuronal behavior. [17]–[21] Herein we demonstrate that our system provides us with a unique platform that allows us to 3-D co-culture different cell types in one specimen in order to study them in a more biomimetic environment. We used this approach to photomicropattern functionalized Dextran and encapsulate DRGs and SCs in a 3-D co-culture system in conditions closer to their natural environment and investigate factors which lead to the formation of myelin.

2. Materials & Methods

2.1. Fabrication of Dual Hydrogel System

The dual hydrogel culture system was fabricated using a digital projection photolithography, as previously described. [17], [22] A schematic of the process has been depicted in Figure 1.
Figure 1. Depiction of the methodology for co-culturing SCs and DRGs. Step 1: Formation of PEG mold; Step 2: DRG insertion; Step 3: Mixing SCs with the gel solution at a specific cell count and addition of the gel solution to the void; Step 4: Irradiation using the negative mask and gel formation.

In brief, a photolithography apparatus comprised of a collimated UV light source (OmniCure 1000 with 320–500 nm filter, EXFO, Quebec, Canada) and a visible light source (SOLA light engine with 375-650 nm filter, Lumencor, OR, USA), a digital micromirror device (DMD) (Discovery™ 3000, Texas Instruments, Dallas, TX) as a dynamic photomask and a 2X Plan Fluor objective lens (Nikon Instruments, Tokyo, Japan) was utilized to irradiate the photocurable hydrogel solution that was contained in a permeable cell culture insert with 0.4µm pore size. [17]–[19], [22] The inserts were either collagen-coated PTFE Transwell® Permeable Support or Transwell™ Clear Polyester Membrane Inserts (Corning Inc., Corning, NY, USA) to investigate the influence of collagenated substrates on SC/neuron incorporation. The dual hydrogel system consists of two compartments: a cell permissive part that contains neurons and a cell restrictive part that acts as a hydrogel mold. In order to make the cell restrictive part, a solution of 10% (w/v) PEG-diacylate (Mn 1000; Polysciences Inc., Warrington, PA) and 0.5% (w/v) Irgacure 2959 in PBS was irradiated with 85 mW/cm² UV light as measured by a
radiometer (306 UV Powermeter, Optical Associates, San Jose, CA), for 38 s to make a PEG micromold (Figure 1). The insert was treated with filtered Rain-X® Original Glass Treatment (RainX, Houston, TX) prior to addition of the gel solution in order to avoid meniscus behavior. 0.5 ml of solution was added to each 6-well plate insert. In our previous studies we have demonstrated that addition of 0.5 ml solution will result in a gel thickness of 480 µm. [17] Hydrogel constructs were washed in DPBS with 1% antibiotic-antimycotic additive to inhibit contamination.

2.2. Dextran Synthesis and Characterization and Gel Composition

Dextran (MW = 70 kDa) was grafted by Glycidyl methacrylate (GMA) based on a published protocol. Initially, 1 g dextran was weighed and added to 9 ml dimethylsulfoxide (DMSO) under nitrogen. 0.2 g 4-dimethylaminopyridine (DMAP) was dissolved in 1 ml of DMSO. Subsequently, the DMAP solution was added dropwise to the dextran solution followed by addition of 232 µl GMA under nitrogen. The final solution stirred for 48 hrs at room temperature. In order to quench the reaction after 48 hrs, 280 µl 37% hydrocholoric acid (HCl) was added to the solution then the resulted product was dialyzed against deionized water for 3 days and lyophilized for 2 days. The resulted product was a glycidyl methacrylate-dextran (MeDex), and the addition of methacrylate groups to dextran was confirmed using $^1$H NMR [(D$_2$O) $\delta$ 6.1-5.7 (m, 2H, CH$_2$), $\delta$ 5.2 (m, 1H, CH), $\delta$ 4.9 (m, 1H, CH), $\delta$ 1.9 (s, 3H, CH$_3$)] with substitution degree of 42%. [18], [23], [24] A gel composition of MeDex 50% (w/v), Arg 0.1% of MeDex (w/w), RF 0.001% of MeDex (w/w), TEMED 0.2% of the final solution (v/v) was prepared. [25], [26]
2.3. Primary Tissue Culture in the Dual Hydrogel System

As the first step of the co-culture, the primary tissue culture was performed accordingly. The PEG constructs were prepared and immersed in the adhesion media and incubated (37° C, 5%CO2) overnight prior to the tissue culture. The adhesion media is comprised of Neurobasal medium supplemented with B27 (2%v/v), L-glutamine (0.25%v/v), nerve growth factor (NGF) (0.02 µg/ml), fetal bovine serum (FBS) (10% v/v) and penicillin/streptomycin (1% v/v) (all provided from Life Technologies, CA, USA). The constructs were then cultured with Long Evans rat embryo dorsal root ganglion (DRG) tissue, in keeping with the guidelines of the Institutional Animal Care and Use Committee. The DRGs were isolated from embryonic day 15 rat embryos and trimmed prior to the culture. In each construct a single DRG explant was placed. The DRGs were then incubated in fresh adhesion media overnight to allow the tissue to adhere to the insert.

2.4. Schwann Cell Culture

SC cell line (ScienCell Research Laboratories, CA, USA) isolated from neonatal rat sciatic nerves were purchased. The cryopreserved vial with > 5 x 10^5 cells/ml was thawed in a 37° C water bath. The contents of the vial were then gently re-suspended and dispensed into the equilibrated poly-L-lysine-coated culture vessel to encourage cell attachment with a seeding density of ≥ 10,000 cells/cm^2. The culture was not disturbed for at least 16 hrs afterwards. To remove the residual DMSO and unattached cells, the culture medium was changed after 24 hrs initially and every other day thereafter. The
culture medium was composed of SC medium with FBS (5% v/v), penicillin/streptomycin (1% v/v) and SC medium supplement (1% v/v) (all from ScienCell Research Laboratories, CA, USA). The culture was passaged every time it reached 90% confluence and was not used after the 3rd passage.

2.5. SC Encapsulation and Incorporation in the Dual Hydrogel System

The SCs were dispersed in 50% MeDex solution in SC medium (described above) to reach a cell count of $20 \times 10^6$ cell/ml. In order to achieve an evenly distributed single cell solution, the gel mixture was pipetted up and down vigorously. The adhesion media was aspirated from the channels gently to avoid disturbing the adhered DRGs and 2 µl of the MeDex single cell solution was added to each PEG micromold. A negative photomask was loaded on the DMD and the gel solution in the channel was crosslinked with 85 mW/cm² visible light as measured by a radiometer (306 UV Powermeter, Optical Associates, San Jose, CA) after 30 s of irradiation using a visible light source (SOLA light engine with 375-650 nm filter, Lumencor, OR, USA). From here the constructs were gently washed using the wash buffer described in Section 2.1 three times.

2.6. Media Regimen for the DRG/SC Co-culture in 3-D Hydrogel System

In order to understand the influence of various media regimens on the behavior of DRGs and SCs in a 3-D co-culture, two different culture systems were applied. The culture systems have been described in Table 1. Culture System 1 has two phases where Medias 1 (10 days) and 2 (15 days) are applied in that order. This media regimen has been previously used by Eshed et al. to promote growth and neurite extension as well as
encouraging endogenic SCs of the DRG bulk to incorporate in myelination process. [27] Culture system 2 only applies Media 2 that is specialized to induce myelin. The media was changed every other day for each specimen in each experimental group.

### 2.7. Immunohistochemistry

To evaluate neurite growth and myelin formation, immunohistochemistry techniques were utilized. Initially, the tissue was fixed with 4% paraformaldehyde (PFA) for 2 hours at 37°C followed by 3 washing steps prior to each staining procedure. All of the reagents were provided from AbCam, Cambridge, MA, unless otherwise is stated.

Neurites were labeled with mouse monoclonal [2G10] neuron-specific beta III tubulin primary antibody and Cy3.5 conjugated goat antimouse immunoglobulinG (H + L) secondary antibody (AbCam, Cambridge, MA). The labeling steps were completed in 2% bovine serum albumin (BSA) and 0.1% saponin in PBS, overnight at 4°C and every step were followed by 3 washing steps with PBS.

To assess myelin formation, constructs were labeled for three myelin proteins: Myelin Basic Protein (MBP), Protein Zero (P0) and Myelin Associated Glycoprotein (MAG). For this to occur, primary antibody chicken polyclonal anti-Myelin Basic Protein, mouse monoclonal anti-Myelin Associated Glycoprotein and rabbit polyclonal Anti-Myelin Protein Zero antibody were utilized. The stains were diluted in 2% BSA/PBS solution with a concentration of 1:500. The constructs were stored at 4°C overnight in primary antibody solution and were washed 3 times with PBS. Prior to this step, constructs were immersed in 5% goat serum at room temperature for 30 mins in
order to avoid any non-specific protein binding. After three washing cycles the hydrogel systems were incubated at 4°C in the secondary antibody solution. The secondary solution was prepared as follows: 1:500 antibody solution in 2% BSA solution Goat Anti-Chicken IgY H&L, Goat Anti-Mouse IgG H&L and Goat Anti-Rabbit IgG H&L, respectively.

2.8. Image Processing, Neurite Growth, Myelin Formation

The volume of growth into the 3-D hydrogel was measured utilizing confocal microscope (Nikon A1, Tokyo, Japan). Because of the entangled and dense neurite outgrowth in the model it is difficult to count the number of individual neurons as it extends along the length. Therefore, in order to measure the growth of the system in 3-D, it is optimal to take the volume of cellular mass in the dual hydrogel culture systems. Each sample was imaged in 3-D with optical slices no greater than an 11 µm depth with an average of 20 slices per sample, a resolution of 1024 × 1024 pixels and with a 10X objective. Pre-processing steps including thresholding and transformation into a binary representation were applied uniformly across all images. Data analysis was performed using ImageJ and a custom algorithm in Matlab (Mathworks, Natick, Ma). Neurite growth was quantified using pixel counts of the threshold slices throughout the depth of the gel. Furthermore, after 25 days myelin was dense and entwined and therefore same image processing procedures was utilized in order to evaluate the volume of myelin throughout the depth. This process allows us to measure the volume throughout the depth considering the 3-D nature of the cultures. In addition, since the size of the constructs was larger to be imaged at once for both imaging processes above a large image z-stack
was taken (1 × 5). For demonstration pictures, samples were imaged in 3-D with an optical slice not greater than 11 µm depth and an average of 20 slices per sample, and a resolution of 1024 × 1024 pixels and with a 20X objective. (Figure 2)

![Image of nerve fibers with B-III, MBP, and merged views for NCol-15, NCol-25, Col-15, and Col-25 groups.]

**Figure 2.** Development of myelin protein (MBP) after 25 days. DRG/SC co-cultured with neurons fixed and immunolabeled with anti-MBP and beta-III tubulin antibodies for compact myelin and neurofilaments. Objective 20X; scale bar represents 25 µm. SCs completely envelop axons after 25 days, forming MBP-positive axons in all experimental groups.

A maximum projection acquisition was used in order to form 2-D images of the total growth. For the volume of growth the same procedure was utilized and the 3-D volume acquisition was used in order to confirm that the growth and myelination occurs throughout the depth (Figure 3).
Figure 3. 3-D rendering of confocal images. Immunohistochemistry for MBP protein depicted in green and MAG depicted in red. The culture thickness is 190 µm confirming 3-D myelin formation ability of the in vitro system.

2.9. Transmission Electron Microscopy

In order to investigate the nanoscale structure of neuronal processes and SCs and their spatial crosstalk, distribution and morphology in the hydrogel cultures TEM was utilized. All of the reagents used for this procedure were provided from Electron Microscopy Sciences, Hatfield, PA unless otherwise stated. The hydrogel constructs were fixed after submerging in 4% PFA solution for 2 hrs at 37° C. The samples were then washed three times for 15 min intervals with PBS. The post-fixation steps included staining with 1% osmium tetroxide (OsO₄) in 100 mM phosphate acetate for 2 hrs followed by 4 washing steps with PBS. The tissue was then stained with 2% aqueous uranyl acetate for 30 min at room temperature in the dark. The procedure was followed by the dehydration step including immersing the samples in 50% and 70% ethanol for 10 mins, then 95% ethanol overnight. The samples were then soaked in 100% ethanol that was filtered with Molecular Sieves, 4 Å (Sigma-Aldrich, St. Louis, MO) for two 30 min intervals. After this step, the constructs were cut to only maintain the regions of interest.
followed by resin embedment. An infiltration step was performed using a 1:1 propylene oxide-spurr resin for 45 minutes. The samples were then embedded in 100% spur resin at 70°C for 48 hrs in order to allow the resin polymerization to complete. Embedded samples were trimmed and sliced with thicknesses varying from 80 nm to 100 nm using a Reichert Ultracut S ultratome (Leica Microsystems, Buffalo Grove, IL) and Ultra 45° diamond knife (Diatome, Fort Washington, PA). After this step the slices were loaded on copper grids (Formvar carbon-coated, 200 mesh). Thereafter, the grids were floated on droplets of 2% uranyl acetate for 20 mins, and rinsed by floating on deionized water droplets 3 times, for 1 min. After mounting the grids on a single-tilted stage they were imaged using a FEI Tecnai G2 F30 Twin transmission electron microscope (FEI, Hillsboro, OR) with an accelerator voltage of 100-200 kV. Images were taken at 3,000-20,000x magnifications with 4000 x 4000 pixel resolution.

3. Results

3.1. 3-D Dual Hydrogel System and DRG/SC Co-culture

In this study we developed a 3-D model to investigate the use of our dual hydrogel platform for co-culture applications. We have previously developed a 3-D hydrogel system using a DMD as a dynamic photolithography tool. Utilizing this model we were able to investigate the influence of mechanical stimuli and chemical cues, either repulsive or attractive biomolecules, on neuronal outgrowth in vitro. [17], [19], [22] This model mimics the 3-D structure of the ECM and translates neuronal microenvironment more accurately. In this work we studied the ability of this system to handle two cell types in single culture in order to investigate their behavior. We co-cultured SCs and
neurons to scrutinize the myelination processes in conditions closer to their natural environment. We also showed (discussed in the upcoming sections) that our model allows myelin formation as a result of SC-neuron co-cultures in three dimensions. The methodology behind the dual hydrogel system has been depicted in Figure 1.

3.2. The Influence of Collagen on Neurite Growth in 3-D Co-cultures

Figure 4 demonstrates that there is a significantly higher volume of neuronal outgrowth in the cultures with collagen compared with the constructs without collagen. (n = 15-18 constructs). The amount of growth was not substantially different between the two media regimen. We also showed that the growth in both cultures were robust, fasciculated, aligned and in 3-D.

**Figure 4.** Quantification of the amount of neuronal growth in each of the four culture models in 3-D. More neuronal growth in the two systems with collagen was observed. No significant impact was detected on neuronal outgrowth due to the change in media regimen.
This characteristic differentiates our system from previously developed in vitro models as the growth is directed within a channel. Although the growth is highly dense after 25 days, it is mostly contained in the cell permissive section of the 3-D hydrogel system.

The beta-III tubulin positive neuronal filaments are depicted in Figures 5.A, 6.A and 7.A.

Figure 5. A) The immunohistochemistry for neurofilaments (beta-III) demonstrated in red and MBP depicted in green. The figures are acquired using z-stack acquisition with confocal microscopy. A maximum projection was obtained subsequently. A dense fasciculated growth can be observed after 25 days. Scale bar = 500 µm. B) Volume of myelination is shown. The amount of MBP positive myelin increased in the presence of collagen. NCol-15 with lesser AA exposure has the least amount of myelin. C) The ratio of the volume of MBP positive myelin to the volume of neurofilaments shows that cultures with longer exposure to AA form more compact myelin. In all experimental groups the percentage of myelin formation drastically decreases in the control groups demonstrating that the exogenic SCs has a major role in myelination process.

3.3. Myelin Development in 3-D Co-culture Model in Our Dual Hydrogel System

Here we demonstrate that our co-culture system promotes myelin formation in 3-D. We utilized immunohistochemistry and TEM in order to prove the formation of myelin. The cultures were stained with 3 antibodies: MBP, MAG and P0. The constructs were positive for MAG, MBP and P0, confirming the formation of compact and non-compact myelin. Figure 5.B and 6.B, show neurofilaments, stained for beta-III tubulin,
and MBP (5.B) and P0 (6.B) positive mature myelin sheath and the merged images that confirm the formation of MBP and P0 segments along the axonal extensions.

Figure 6. A) The immunohistochemistry for neurofilaments (beta-III) demonstrated in red and P0 depicted in green. Scale bar = 500 µm. B) Volume of myelination is shown. The amount of P0 positive myelin increased in the presence of collagen. P0 exists in the PNS compact myelin and therefore P0-positive myelin (red) represents the PNS compact myelin. Col-25 with higher AA exposure and incorporation of collagen has the most amount of compact myelin. The decreasing trend shows that removing both factors, the collagen existence and the longer exposure to AA, will result in the least myelin formation in the 3-D cultures after 25 days. C) The percentage of P0 positive myelin to neurofilaments shows the productivity of the system only in myelin formation despite the volume of neuronal production. Excluding the volume of the neuronal growth shows in the presence or absence of collagen (Col or N-Col), the exposure to AA plays an important role in myelin formation in 3-D. However, Col-15 is statistically equivalent with NCol-25, showing that the efficiency of the constructs after 25 days of AA exposure in absence of collagen is similar to that after 15 days of AA exposure in the presence of collagen. Note that the amounts are substantially different as shown in section B.

As mentioned previously, all images were taken through z-stack acquisition.

Confocal imaging confirmed that neurite growth occurred in 3-D throughout the channel.

The depth of growth and myelination for these constructs was 88±15 µm.
Figure 7. A) The immunohistochemistry for neurofilaments (beta-III) demonstrated in red and MAG depicted in green. MAG is one of the main proteins that is present in the non-compact myelin and thus, in all of these images, red represent non-compact myelin. Scale bar = 500 µm. B) Volume of compact myelin in all four experimental groups. Col-25 with higher AA exposure and incorporation of collagen has the most amount of non-compact myelin. C) The ratio of the volume of MAG positive myelin to neurofilaments shows that NCol-15 with the shortest time of AA exposure and in the absence of collagen has the least efficiency in non-compact myelin formation regardless of the volume of nerve fibers in the system.

TEM images confirmed myelin formation. (Figure 8) Slices taken in the neural tract show high density of parallel, highly fasciculated and myelinated neurites, presence of Schwann cells, and Schwann cell encapsulation of neurites. Myelin segments were consistently identified in TEM images agreeing the compact myelin formation. These findings demonstrate that our 3-D in vitro model enables SCs to form mature myelin layers around neurites.
Figure 8. Transmission electron microscope of neural culture cross-sections demonstrate myelin sheaths around individual nerve fibers in 25-day cultures. A) NCol-25 B) NCol-15 D) Col-25 E) Col-15. C) High density of parallel, fasciculated neurites in channel. Neurons are either myelinated or the SCs have started to sheath around the nerve fibers which explains the high amounts of myelin protein positive in immunohistochemistry staining. F) An enlargement of a thick myelin sheath. A: Axons, M: Myelin, S: Schwann cells throughout.

3.4. The effect of Ascorbic Acid (AA) on Myelin formation in 3-D

We used two media regimen for our cultures. For NCol-25 and Col-25, 25 days of media containing AA resulted in a considerable increase in the amount of myelin. The amount of myelin demonstrates the ability of the culture to form myelin sheath regardless of the amount of neuronal growth. The ratio of myelin to neuronal growth was measured. The results show that the percentage of myelin in the constructs would increase by longer exposures to AA. This observation was confirmed through 3 immunohistochemistry
antibody stains for MBP, MAG and P0 demonstrating that this is accurate for both compact and non-compact myelin.

3.5. The Impact of Collagen on Myelin Development

The influence of collagens I and III on compact and non-compact myelin development was evaluated in our system. The myelin proteins we investigated followed similar trends as shown in Figures 5, 6 and 7. Addition of collagen increased the amount of myelin formation in the system. The ratio of myelin to neurite growth was similar value for Col15 and NCol-25. This demonstrates that increased quantities of myelin in Col-15 compared with NCol-25 is due to increases in the amount of neuronal growth. Furthermore, the efficiency of the two systems in developing myelin is dependent on AA exposure. In Figure 4 it is depicted that collagen augments neuronal growth drastically. NCol-15 shows that in the absence of collagen and with a shorter exposure to AA, myelin forms the least.
Table 1. Media components for co-culture systems over a 25-day period

<table>
<thead>
<tr>
<th>Components</th>
<th>Media 1</th>
<th>Media 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Eagle’s Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamax (1% v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITS supplement (1% v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA (0.2% w/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-glucose (0.4% w/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ug/ml NGF - 10ul</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin/Streptomycin (1% v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBS (15% v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-ascorbic acid (0.004 w/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture System 1</td>
<td>10 Days</td>
<td>15 Days</td>
</tr>
<tr>
<td>Culture System 2</td>
<td></td>
<td>25 Days</td>
</tr>
</tbody>
</table>

Table 2. Nomenclature of the experimental groups

<table>
<thead>
<tr>
<th>Culture Name</th>
<th>Type I and III Collagen</th>
<th>Media Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCol-15</td>
<td>No</td>
<td>Media 1 (10 Days) and Media 2 (15 Days)</td>
</tr>
<tr>
<td>NCol-25</td>
<td>No</td>
<td>Media 2 (25 Days)</td>
</tr>
<tr>
<td>Col-15</td>
<td>Yes</td>
<td>Media 1 (10 Days) and Media 2 (15 Days)</td>
</tr>
<tr>
<td>Col-25</td>
<td>Yes</td>
<td>Media 2 (25 Days)</td>
</tr>
</tbody>
</table>
4. Discussion

The myelin sheath is a specialized cell membrane with a multi-lamellar spiral structure that surrounds the axon and reduces nervous system capacitance. Well-myelinated nerves are completely surrounded by myelin sheaths except for small, periodic gaps known as nodes of Ranvier that are exposed to the extracellular environment. [28]–[31] Myelin exists in two forms: compact and non-compact. The compact myelin ultrastructure is made of a spiraled cellular sheath that lacks cytoplasm as well as extracellular spaces while containing two plasma membranes. Non-compact myelin is the channel-like segment of myelin and is non-condensed and is made of Schmidt-Lanterman incisures, periodic interruptions in the myelin layer, as well as paranodal regions. [29], [32]–[36]

Compact and non-compact myelin contain various proteins such as Myelin Basic Protein (MBP), an essential component of CNS and PNS compact myelin. MBP is located on the cytoplasmic surface of the myelin sheath and is extremely charged. Another vital myelin protein in the PNS is P0, which is a transmembrane glycoprotein that affects cell adhesion, maintains the main dense line of PNS compact myelin, and plays an important role in keeping the space between compact myelin consistent. One of the major components of non-compact myelin is Myelin Associated Glycoprotein (MAG), which does not exist on the outer layer of myelin but is present in the inner layer. It is in contact with the axon, connecting it to compact myelin. These three proteins are essential for myelin formation and maintenance and have been vastly utilized to detect myelin in cultures. [34], [29], [37]
A co-culture system of SCs and neurons, either derived from a primary tissue source or a cell line, can accurately portray the events of the native PNS and the complex myelin architecture. Sango et al. used PC12 cell lines and SCs with the aim of establishing motor neuron/SC co-culture models in order to study motor neuron diseases. [12]

In a different study an *in vitro* model of sensory neurons and SCs was used in order to understand the mechanisms behind myelination, and the model may be useful for drug screening. [38] Many of these studies employ DRGs, as they are well studied and are recognized as strong *in vitro* models that allow the development of neuron/SC co-cultures to evaluate myelination processes in the PNS. [38], [39]

The *in vitro* co-culture models described above as well as other similar studies have been mostly performed in 2-D cell cultures and 3-D tissue slices. [16] There are few studies that investigate the incorporation of neuron/SC and their influence on myelin formation in 3-D cultures. In one study, a co-culture system using aligned electrospun polycaprolactone fiber scaffolds analyzed the influence of SCs on neurite fiber diameter in a 3-D model. In this investigation they demonstrated that the presence of SCs could improve neurite extension. [40] Other researchers have developed a 3-D setting where spinal cord motor neurons were seeded on a collagen sponge and followed by a SC and fibroblast culture in order to mimic *in vivo* phenomena. They achieved myelin formation in a 3-D microenvironment in long-term cultures (28 days) with these co-culture models. [9] Suri et al. demonstrated the synergistic effects of ECM components within a 3-D setting that utilized photopatterning. While they showed that their hydrogel system was capable of supporting neuronal and SC survival, they did not look into the myelination processes. [6]
To design a 3-D biomimetic polymer network in order to study myelination in neuron/SC co-cultures, we utilized our previously designed photomicropatterning setting. Photopatterning has been previously applied to study the nervous system as it allows proper translation of the biomimetic neuronal microenvironment in 3-D. [19], [22], [41], [42], [6] The dynamic mask projection photolithography apparatus that was utilized in this study provided an easy fabrication technique for the purpose of producing micropatterned hydrogels. These hydrogels were created on permeable cell culture inserts that provide the basis for our neural regeneration study. In order to generate these constructs, we utilized a DMD device that provided a dynamic photomask. This mask irradiated PEG solution to create the mold into which DRGs are initially adhered, followed by the addition of a photocurable single cell MeDex solution. A negative dynamic photomask was utilized to encapsulate SCs in 3-D, incorporating them with the DRGs. Previously we performed extensive cellular studies, including neurite growth, neurite viability and DNA fragmentation, in an investigation to understand the effect of light spectra on neuronal survival and extension. These studies agree that utilizing visible light with short (30 sec) exposure lengths is the most practical for hydrogel formation and cellular encapsulation in order to decrease cytotoxicity, and thus these findings were utilized for this design. This model provided us with a long-term (25 days) in vitro platform that ensures the survival of neurons and their elongation and myelination in 3-D environment.

In our models we use two different cell culture media as described in Table 1. Media 1 is composed of factors that have been well-characterized and are known to support DRG and SC growth. [27] This media contains BSA, which SCs have been
shown to migrate in the presence of. [43] However, this system is not specialized to promote myelin formation. Media 2 contains FBS in conjunction with ascorbic acid, which has been demonstrated to promote myelination in 2-D cultures. Previous studies of SCs in the presence of neurons show that they are able to create a complete ECM with a basal lamina and collagen fibrils in vitro. [44] Researchers have demonstrated in SC/DRG co-cultures that ascorbic acid may promote SCs to generate myelin by enabling them to form a basal lamina. [45] The media also contain ITS (insulin, transferrin and selenium) which has been shown to promote myelination in rat cell lines. [46]

We utilized laminin in every experimental group, as it has been previously demonstrated in neuron/SC co-cultures to be necessary for myelination. In vivo, the absence of laminin has been shown to lead to peripheral neuropathy in both mice and humans. [47] Research has also confirmed that in mutant mice that are deficient in laminin there will be disruption of the endoneurium basal lamina, which subsequently reduces nerve conduction velocity. [48]

Our systems also investigate the presence of collagen on neuronal growth in our 3-D model through the use of collagen-coated substrates. For these studies we utilized Type I and Type III collagen. Researchers have demonstrated that Type III collagen binds to and activates an adhesion g-protein coupled receptor on Schwann Cells, Gpr56, which may lead to the activation of Gpr125 to initiate myelination. [49] Furthermore, Type I and Type III collagen are key components of the epineurium, which is the outermost layer of dense tissue that supports and surrounds peripheral nerves and myelin. [6]

To investigate the ability to form myelin in 3-D, we will evaluate the influence of our two different media as well as the impact of collagen. Our four culture systems are
differentiated by the presence of collagen and the media regimen the co-cultures were exposed to. We utilized two media regimens: Media 1 for 10 days and then Media 2 for 15 days (Culture System 1), and a different regimen where the cells were only exposed to Media 2 for 25 days (Culture System 2). Table 2 describes the groups. In order to see if the myelination was influenced by exogenic SCs, we performed the above experiments without the addition of encapsulated SCs to the dual hydrogel system while holding all other variables constant.

The formation of myelin was confirmed using immunohistochemistry and confocal imaging and was further validated by TEM. 2-D images of 20X magnification show the formation of myelin segments that wraps around the neuronal projections (MBP/beta-III tubulin positive cultures). (Figure 3) The 3-D development of myelin, stained for both MBP and MAG, due to the formation of compact and non-compact myelin is depicted in Figure 4. TEM images also confirmed the occurrence and abundance of mature myelin layers in all of the experimental groups. (Figure 8 (A-D)) In Figure 8.F a magnified image of myelin layers has been depicted. Figure 8.E shows that after 25 days in culture, SCs had formed myelin sheaths around many of the neurites and some SCs have begun to roll cytoplasmic layers around the nerve fibers. From this image we can conclude that the amount of myelin is significant and the cultures can also be utilized for long-term studies such as long-lasting drug evaluations in 3-D. This figure also shows the high density of aligned, highly fasciculated neurons in our culture.

The first set of analyses performed, as described in Figure 2, quantify the amount of neuronal growth in each of our four culture systems in 3-D. It is well established that collagen and their receptors promote neurite outgrowth. [48] These data demonstrate that
there is significantly more neuronal growth in the two systems where collagen is present. However, there was no significant impact on growth due to the media regimen that was utilized, demonstrating that it had little impact on the amount of neurite extension after 25 days in our contained system.

The amount of myelin was measured by two different approaches. The first approach was to look at myelination as an independent variable and scrutinize the total amount of the myelination regardless of the amount of neuronal development in the system. The second approach was a calculation of the ratio of myelin to neurite extension and normalizing the amount of the myelin development. This gives us an understanding of the myelination efficiency and describes the percentage of neuronal projections with myelin sheaths wrap around. To investigate the amount of myelin produced by our four experimental groups, we utilized stains for MBP, MAG and P0.

Figure 5.C describes the percentage of myelin formed in our culture systems. For these data we utilized an MBP antibody. While all four samples were positive for MBP after 25 days of culture, there were significant differences between the groups. As mentioned above, MBP is one of the proteins that exist in compact myelin and its expression in the culture verifies the formation of compacted membrane segments of mature myelin sheath. Increased myelination occurs in our systems when there is increased exposure to ascorbic acid, which has been previously demonstrated to promote the formation of myelin in DRG/SC co-cultures. These results were achieved in a 3-D in vitro model that mimics the environment of the nervous system more closely than typical 2-D cultures or tissue sections. The data indicates that there is a significant increase in the ratio of myelin to neuronal outgrowth in our systems when exposed to myelination media.
for 25 days. We also note that the media regimens result in increased myelination when they are in the presence of collagen for the same exposure length. Based on these data, we conclude that two factors are playing role in these cultures: the presence of collagen and the longer exposure to ascorbic acid. The constructs lacking both of these factors (NCol-15) are the least myelinated. The percentage of myelin to neuronal growth for our cultures showed that the same AA exposure would have a similar effect despite the amount of neurons that have been produced. However, Figure 5.B shows that when both factors are present in the experiment (Col-25), a synergistic response is observed which results in a significant increase in myelin magnitude. Maximum projections of z-stack planes are included to support these data.

DRGs contain neurons and SCs. [50] In order to confirm that exogenous SCs significantly alter myelination, a control group with no additional SCs were scrutinized. The data in Figure 5.C reveal that every experimental group had a significant increase in myelination versus their corresponding control, demonstrating that exogenous SCs had a large impact on the system. The results show that collagen significantly increases myelination in the control groups, but AA exposure duration has a lesser impact.

Myelination was also measured in the 3-D cultures using P0 protein antibody. 70% of the total proteins in PNS myelin consist of P0 and a lack of this protein will verify a lack of uncompact myelin. The ratio of P0 expression to beta-III tubulin positive neurofilaments was evaluated. The results that are depicted in Figure 6.C demonstrate that the NCol-15 presents the least amount of P0. The percentage of P0 expression is substantially higher in cultures in the presence of AA for 25 days, which is in line with the results from MBP staining where the expression of MBP for Col-25 group shows the
most expression. This is interesting as P0 and MBP both are signature proteins of compact myelin in PNS but have different responsibilities. P0 retain the organized recurrence of both the ECM and cytoplasmic spacing of myelin membrane while MBP plays a role in cytoplasmic fusion. This value is equivalent for NCol-25 group showing that the efficiency of the cultures after 25 days of Media 2 was the same regardless of having collagen in the cultures.

Figure 6.B shows the amount of myelin in the cultures that was labeled with P0. Col-25 shows the maximum amount of compact myelin P0 development regardless of the amount of the neuronal growth. The results show that the samples with collagen in the culture will lead to more neurons resulting in a higher amount of myelin. Between the two collagen-containing samples the exposure to AA increases the amount of P0 occurrence. This is also accurate where we normalize the volume of myelin values in collagen-containing samples by calculation the ratio of myelin to volume of the neurofilaments. (Figure 6.C) The images demonstrate that the amount of P0 will decrease drastically in constructs with no collagen, NCol-15 and NCol-25. The volume of neuronal growth also decreases, and as a result the percentage of compact myelin formation will not show any significant variance from the Col-15. Therefore, in our long-term 3-D constructs if the culture is exposed to AA for 25 days the percentage of compact myelin that expresses P0 would not be substantially different from the cultures with collagen in the presence of AA for 15 days. It has been previously established that AA is necessary for myelination in serum containing media, such as ours, for 2-D cultures. [51] We have demonstrated here that the duration of using AA plays an important role in efficiency of the formation of myelin. Collagen I and III, as discussed above, support neuronal growth
and can help in initiating the myelination process. The presence of collagen in the system increases the neuronal 3-D extension, and as a result augments the amount of myelin formation in a 3-D setting.

A different measure for Myelin is MAG protein. As mentioned above MAG is a protein that is abundant in non-compact myelin. The ability of rat DRG/SC co-cultures to form myelin in our 3-D construct was evaluated by MAG immunostaining. All of the constructs were MAG positive while following the same pattern as P0 and MBP. High levels of myelin synthesis were demonstrated by confocal microscopy analysis of MAG similar to P0 and MBP. MAG indicates the Schmidt-Lanterman incisures and paranodes that are characteristics of non-compact myelin. The amount of non-compact myelin, regardless of the volume of neuronal growth, was higher in the Col-25 group in the presence of collagen with longer AA exposure. AA helps the system form basal lamina and encourages myelin formation. The percentage of the MAG labeled structures is not substantially different in the cultures with the same exposure to AA (Col-25 and N-Col 25). However, the amount of growth substantially decreases when collagen is not added to the system.

In this work we developed a novel in vitro 3-D co-culture model that allows incorporation of SCs and neurons. We utilized a facile high-throughput photolithography method that previously provided us with a unique 3-D setting to replicate neuronal phenomena in controlled microenvironments, in order to introduce mechanical and chemical cues with highly-resolved spatiotemporal precision. Here, we demonstrated that this co-culture setting provided us with aligned, highly fasciculated neuronal growth with myelin sheath nicely wrapped along them. Myelination was confirmed through
immunohistochemistry and TEM. We used two culture systems and also studied the influence of collagen on neuronal growth and myelination. This platform provides us with a great simple tool for drug discovery and myelination studies.
5. References


CHAPTER 6: CONCLUSION

In these studies we developed a facile and high throughput 3-D *in vitro* model that provides us with a platform to study the nervous system. We fabricated these *in vitro* scaffolds utilizing a digital micromirror device acting as a dynamic photomask to create three dimensional neuronal study platforms. Our system is a dual hydrogel model that is made of two segments: a cell permissive and a cell restrictive part. The cell permissive part allows neuronal proliferation and outgrowth while the cell restrictive section contains the growth in order to achieve aligned, highly fasciculated neuronal growth.

For the first specific aim, we developed a novel hydrogel system composed of hyaluronic acid (HA) and Puramatrix (PM) that forms an interpenetrating network of polymers (IPNs). Using an IPN system we were able to take advantage of both HA and PM characteristics. Extracellular matrix (ECM) has a composite structure that was recreated using our IPN system. We also demonstrated that through functionalizing HA we were able to change the mechanical properties of the hydrogel system and study the influence of the microenvironment stiffness on neuronal outgrowth in a single specimen.

For the second specific aim we utilized our photolithography system to investigate one of the main drawbacks of photomicropatterning neuronal encapsulation. We employed a functionalized Dextran and Riboflavin as photoinitiators and L-Arginine, an amino acid, as a co-initiator. This unique system has the potential to form a hydrogel upon UVA and visible light irradiation. Our results showed that both UVA and visible light will result in cytotoxicity and will influence neuronal extension. However, visible spectra are less harmful than UVA, and decreasing exposure time subsequently decreases cytotoxicity.
For the third specific aim we utilized the optimum system from the second specific aim to develop a co-culture system of DRG/Schwann cells. We established a 3-D platform to encapsulate two cell types and study their incorporation. We investigated the process of myelin formation as a result of neuron/glial cell co-culture and the influence of collagen and ascorbic acid on overall cellular growth and myelination in a 3-D setting. The results demonstrated that our 3-D co-culture system provided us with aligned, highly fasciculated neuronal growth with myelin sheaths wrapped along them.

Herein we have utilized the previously developed dual hydrogel system to study the effect of mechanical cues and influence of light on neuronal behavior and incorporate different cell types in a single 3-D model. These findings show the broad range of applications that our simple high throughput 3-D dual hydrogel system provides us with for future applications. This model may be an ideal setting for the study of myelination processes in 3-D as it is a closer recreation of *in vivo* phenomena compared with existing *in vitro* models.
BIOGRAPHY

Parastoo Khoshakhlagh was born on March 4th, 1983 in Rasht, Iran to Maryam and Eddie, who raised her along with her brother Pedram and sister Pooneh. Her career in science began when she got accepted to the National Organization for Development of Exceptional Talents (NODET) in Iran for her middle school and high school. Afterwards, she moved to Tehran and attended Iran University of Science and Technology (IUST) for her undergraduate studies and Amirkabir University of Technology (Tehran Polytechnic) for her master's research. Following her master’s degree, she pursued a Ph.D. in Biomedical Engineering at Amirkabir University, but she decided to continue her studies in the United States. She began her Ph.D. research in Biomedical Engineering at Tulane University in August 2010 and the work that is presented here demonstrates her contributions to the study of neural microengineering under Dr. Michael Moore’s supervision.

Following her Ph.D. research at Tulane University, Parastoo will pursue a post-doctoral fellowship at Harvard University under Dr. Ali Khademhosseini, where she will work on business development regarding new medical therapies.