RELEASE OF URIC ACID FROM POLY(Ɛ-CAPROLACTONE) NANOFIBERS AS POTENTIAL TREATMENT FOR SPINAL CORD INJURY

By

SALMAN KHALIQ

A thesis submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Biomedical Engineering

written under the direction of

Bonnie L. Firestein, Ph.D.

and approved by

_____

_____

_____

New Brunswick, New Jersey

May 2020

ABSTRACT OF THE THESIS

Release of uric acid from poly(ε-caprolactone) nanofibers as potential treatment for spinal cord injury

by SALMAN KHALIQ

Thesis Director:

Bonnie L. Firestein, Ph.D.

Spinal cord injury (SCI) is characterized by two phases: the primary phase involves a traumatic event, which can be external or internal injury, and a secondary phase, which entails a number of biochemical processes, eventually resulting in inflammation, neuronal death, and axonal demyelination. Glutamate-induced excitotoxicity (GIE) is the major contributor to this secondary SCI pathway. GIE is mediated by the release of excessive glutamate into synaptic clefts, overstimulating N-methyl-D-aspartate channels, which increases intracellular Ca2+, and results in cell swelling and mitochondrial dysfunction. Furthermore, GIE increases the production of toxic reactive species, leading to DNA and mitochondrial damage, and eventually, cell death. Currently, there is no clinical treatment that specifically targets GIE after SCI, and emergence of a therapeutic target for secondary damage in SCI patients is of utmost need. Uric acid (UA), a product of purine synthesis, acts as an antioxidant by scavenging free radicals and preserves neuronal viability in several *in vitro* and *in vivo* SCI models. However, high systemic UA concentrations can be detrimental and lead

to hypertension, kidney disease, and gout. Thus, there is need to develop a drug delivery system that can deliver UA locally to the target injured region. Natural polymers show high biocompatibility but lack the ability to be fabricated in such a way that the rate of drug release is controlled. In contrast, use of the synthetic polymer, poly (ε-caprolactone; PCL), offers an advantage over natural polymers since it is not only biodegradable and biocompatible, but it also has a controllable degradation rate and is compatible with a vast number of drugs. As such, it has been studied and used extensively in the context of drug delivery applications. Here, using the electrospinning technique, we developed a PCL-UA nanofiber mat containing UA, which has the potential as an implantable drug delivery carrier for UA. We then optimized delivery of UA via this PCL nanofiber mat in short bursts of 2 hours by coating the mats with PEGDA. We then optimized the effective dose of UA released from PCL nanofibers to protect neurons from GIE in organotypic spinal cord slice culture. We show that the mats decrease reactive oxygen species generation and cell death. The long-term goal of this project is to extend these studies *in vivo*, and ultimately, optimize use for SCI patients. This approach is therapeutically viable since PCL is an FDA approved polymer currently used to deliver multiple drugs and fully excreted from the body upon degradation without any toxic effects.

ACKNOWLEDGMENTS

There are a lot of people I came across during my journey in this master's program in BME, who influenced me and made a positive contribution in my research. First, I would like to acknowledge and thank my thesis advisor, Dr. Bonnie L. Firestein, for giving me the opportunity to work in her lab, providing me continuous guidance, and supporting me all the way. Thank you for your patience as I learned all the skills needed to complete this work. I admire your strong work ethics and ability to remain calm and understanding in times of stress.

Next, I would also like to thank my committee members, Dr. Joseph Freeman, and Dr. Li Cai. To Dr. Freeman, thank you for personally showing me how to do electrospinning and all the subsequent steps for my research regarding nanofibers. I would also like to thank you for being kind and understanding as a Graduate Director of BME and guiding me along the way. I also enjoyed your lectures for the course of Biomechanics and Biomaterials and learned a great deal in that regard. To Dr. Cai, your professionalism and expertise are impressive and much appreciated. I also enjoyed your approach for the course of Dynamics and Stem Cell Biology and learned a great deal.

Furthermore, I am grateful for the encouragement and assistance from my lab mates in the Firestein Lab including Christen Crosta, Harita Menon, Marina Lopez, Nisha Singh, and especially Anton Omelchenko for training me on most of the techniques I learned during my time in Firestein lab, Mihir Patel for training me on managing cre mouse colony, Przemyslaw Swiatkowski for training me on

patch-clamp electrophysiology, and Kate M. O'Neill for guiding me during my troubleshooting of MEA rig. I would also like to appreciate the help of undergraduate students in the lab.

Additionally, I appreciate all the help I got from BME administrative staff, especially Lawrence Stromberg. I am also very grateful to the Department of Biomedical Engineering and Cell Biology and Neuroscience for granting me teaching assistantship to support my graduate studies.

In addition, I would not be here without the constant support of my Parents, Abdul Khaliq and Samina Khaliq, my siblings and all the friends and family. Ultimately, the most important contribution was from my wife, Dr. Ayesha Aijaz, who was my backbone during my course in Rutgers University, supporting me emotionally and mentally day in day out and mentoring me through every step of the way. This journey would not be complete without my son, Zoraiz Salman, who was the best medicine during stress and made this voyage very happy and eventful.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER 1: INTRODUCTION

Spinal cord injury (SCI) occurs with the initial physical damage or primary insult followed by biochemical damage or secondary insult that may continue for days or months post-injury. Glutamate, a common excitatory neurotransmitter in the central nervous system, is a significant mediator of secondary damage that occurs after spinal cord injury. [1, 2] Excessive glutamate released by injured neurons overstimulates N-methyl-D-aspartate glutamate receptors (NMDAR), which causes ionic imbalances as intracellular concentrations of Na^+ and Ca^{2+} start to rise, resulting in cell swelling and mitochondrial dysfunction. [3-5] Decreased ATP production, due to mitochondrial damage, increases the production of reactive oxygen species. [6] Furthermore, high intracellular concentrations of Ca^{2+} trigger the formation of nitric oxide (NO), which reacts with O_2^- (superoxide) and forms toxic peroxynitrite ($ONOO^-$), damaging mitochondria and eventually leading to cell death. [7-10] Currently, there is no safe and effective treatment available to address damage due to glutamate-induced excitotoxicity (GIE) caused by SCI. [11]

Uric acid (UA), the most abundant and important antioxidant in the plasma [12-16], plays neuroprotective roles in reducing GIE as a peroxynitrite scavenger [17-19] and [20] by upregulating glutamate transporters on astroglia. [21] However, administration of UA can lead to hyperuricemia, which has been linked to kidney disease, hypertension, and gout. [22] Detrimental systemic effects and

advantageous local effects of UA treatment makes UA an ideal molecule for controlled release to the wounded area. UA is highly insoluble in most solvents, except for heated basic solutions, making it technically challenging to integrate into biocompatible nanofibers, [23, 24] and sustained release of UA via biocompatible nanofibers has not yet been achieved. Designing a nanofiber mat for local delivery of UA will open new avenues for treating GIE as a result of SCI.

Electrospinning, a simple technique often employed to produce non-woven nanofiber mats, [25] has the potential for creating implantable drug delivery carriers. [26] The nanofibers produced by this process have comparatively higher surface area to volume ratio and enhanced mechanical functions than do large fibers. [27-31] This technique is versatile and allows for adjustment of fiber diameter distribution, pore size, porosity, release kinetics, and spatial arrangement by manipulating parameters, such as solution composition, polymer solution feed rate, applied voltage, and cathode distance. [27, 32-35] Furthermore, three-dimensional electrospun nanofibers mimic the extracellular matrix (ECM), making it an ideal drug delivery system. [36] Complete control of design over nanofiber morphology, dimensions, and porosity enables the delivery of a variety of drugs from these nanofibers for controlled release.

Various natural and synthetic polymers are used as drug delivery systems for different clinical applications. Natural polymers have advantages over synthetic polymers, such as high biocompatibility, [37] but they lack mechanical strength,

suitable controlled degradation rates, and the ability to be fabricated from various materials. Degradation of polymers *in vivo* or *in vitro* determines the release rate of the drugs entrapped in the polymers. The degradation chemistry of polymers is dependent on several factors, such as the type and nature of polymer mixtures (pristine/copolymer), architectural scale (micro scale/nano scale), and presence of hydrolytic accelerators or suppressors. [38] Of the many synthetic polymers, polycaprolactone (PCL) possesses optimal mechanical properties and high compatibility with a vast range of drugs and is extensively used in drug applications because of these characteristics. [39, 40] Therefore, we used PCL nanofibers as a drug delivery system for this research. PCL has a slow degradation rate, and adding a coating of polyethylene glycol diacrylate (PEGDA), which is a common polymer used to deliver various FDA approved drugs and therapies, can slow degradation of PCL even further. Hence, we used PEGDA coating to slow the rate of release of UA from UA-PCL nanofiber mats.

The use of isolated and homogeneous cells *in vitro* reduces the number of experimental animals needed for study. [41] This technique allows for the examination of a wide array of cellular processes and the effects of compounds on toxicity and cellular viability in primary cultures. However, for neuronal studies, isolated cells do not reflect the true physiological conditions of an organism and lack supporting cells and electrophysiological connections with surrounding cells. [42] Organotypic slice culture can mimic *in vivo* conditions by preserving several aspects of extracellular and synaptic organization of primary tissue. Several

organotypic cultures have been proposed and include organotypic culture chamber [43] and roller tube technique, [44-51] which was modified and optimized by placing the slice on a semipermeable membrane. [52] It is for this reason that this culture system is used to assess the efficacy of our UA fiber mats on neuronal viability in response to GIE.

CHAPTER 2: LITERATURE REVIEW

Spinal cord injury is a devasting neural pathology with drastic effects on the social and financial aspects of the affected person, their families, and the healthcare system. There are many new advancements in rehabilitation strategies, but still SCI remains a major contributor in disability and morbidity cases worldwide. [53] According to an estimate, the incidence rate of SCI in the United States alone is between 43 and 71 per million, [54, 55] and approximately 12,500 cases of SCI are registered in North America every year. [56] Currently, it costs approximately $2.35 million per patient for medical, acute care, hospitalization, and rehabilitation costs along with lost earnings over the lifetime of the injury. [56] Therefore, it is critical to understand the pathways that lead to the loss of motor function after SCI and to develop new therapeutic treatments.

2.1 Phases of Spinal Cord Injury

SCI is characterized by two phases, primary and secondary. Primary damage is the first initial physical trauma to the spinal cord, which leads to spontaneous cell death in the region of impact. There are four main characteristic mechanisms of primary injury: (1) impact plus persistent compression; (2) impact alone with transient compression; (3) distraction; (4) laceration/transection. [57-59] These mechanisms occur independent of the type of primary injury and damage the afferent and efferent spinal cord pathways, blood vessels, and nerve cell membranes, [58, 60] implicating imbalances in ionic environment and build-up of

neurotransmitters. [61] Thus far, early surgical decompressions have been the gold standard for limiting the damage on the spinal cord due to primary injury. [62, 63]

Secondary injury is the series of biochemical events that start within minutes of initial physical trauma and can continue for weeks or even months, resulting in gradual loss of functionality due to the spread of the diffusible signals in the tissue surrounding the lesion site. [64] These biochemical events are characterized into acute, sub-acute, and chronic phases. The acute phase initiates instantaneously after SCI and leads to ionic imbalances as intracellular concentrations of Na+ and Ca2+ start to rise, resulting in cell swelling and mitochondrial dysfunction, eventually leading to cell death due to the production of reactive oxygen species (ROS). [3-5, 59, 64-66] The sub-acute and chronic phases follow and further damage neurons in the injury site and their surroundings, ultimately leading to extreme deterioration of motor functions. [59, 64] Currently, there is no therapeutic to counter the effects of acute and sub-acute secondary injury that leads to ultimate chronic damage. Therefore, this research is focused on these phases of secondary damage, which involves glutamate-induced excitotoxicity.

2.2 Mechanism of Glutamate-Induced Excitotoxicity

Glutamate is the most abundant excitatory neurotransmitter in the brain and contributes in virtually all activities of the CNS. Normally, the extracellular

concentration of glutamate is in the low micromolar range, [67, 68] but after SCI, the extracellular glutamate concentration increases drastically and leads to excitotoxicity. [69, 70] During normal neurotransmission, glutamate is stored in vesicles in the presynaptic terminal, and upon depolarization, the glutamate is released into the synaptic cleft and binds to ionotropic glutamate receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid (AMPA), kainate, and N-methyl-D-aspartic acid (NMDA)), which eventually results in membrane depolarization of the postsynaptic neuron. The glutamate released into the synaptic cleft is taken up by excitatory amino acid transporters (EAATs) present on astroglia ensheathing the glutamatergic synapse. [71] Astrocytes convert glutamate into glutamine using an enzyme, glutamine synthetase, and release the glutamine into the extrasynaptic extracellular space. The glutamine is taken up by neurons, converted back to glutamate, and is used again for neurotransmission. [72]

Minutes after spinal cord injury, extracellular glutamate concentrations increase to neurotoxic levels [73-77], which causes membrane depolarization and release of the Mg2+ block on NMDA channels. [78] NMDA and neuronal nitric oxide synthase (nNOS) are linked by the postsynaptic density protein (PSD-95). [7, 8] Activation of NMDA channels leads to the influx of Ca2+, which eventually results in production of nitric oxide (NO) via Ca2+, and calmodulin binding to nitric oxide synthase. [79, 80] Furthermore, higher concentrations of Ca2+ in the cell causes mitochondrial membrane depolarization, resulting in mitochondrial dysfunction,

which subsequently generates high concentrations of ROS, including super oxide (O2-). [81, 82] Interaction of NO and O2- forms the toxic peroxynitrate (NO3-), [10] which interferes with mitochondrial respiration, causes lipid peroxidation, damages mitochondrial and cellular DNA, and eventually leads to cell apoptosis (Fig.2.1). [83-85] Additionally, expression of EAATs following SCI decreases due to the loss of astrocytes and downregulation of EAATs on surviving cells, causing decreased glutamate uptake by astrocytes. [86, 87]

Several strategies have been used to counter the effects of glutamate-induced excitotoxicity, but no current treatment has been effective thus far. Uric acid (UA), displays neuroprotection from the excitotoxic effects of glutamate by scavenging ROS and upregulating EAATs, [17-21] in a rodent SCI model. Therefore, UA has a potential to be a therapeutic for SCI.

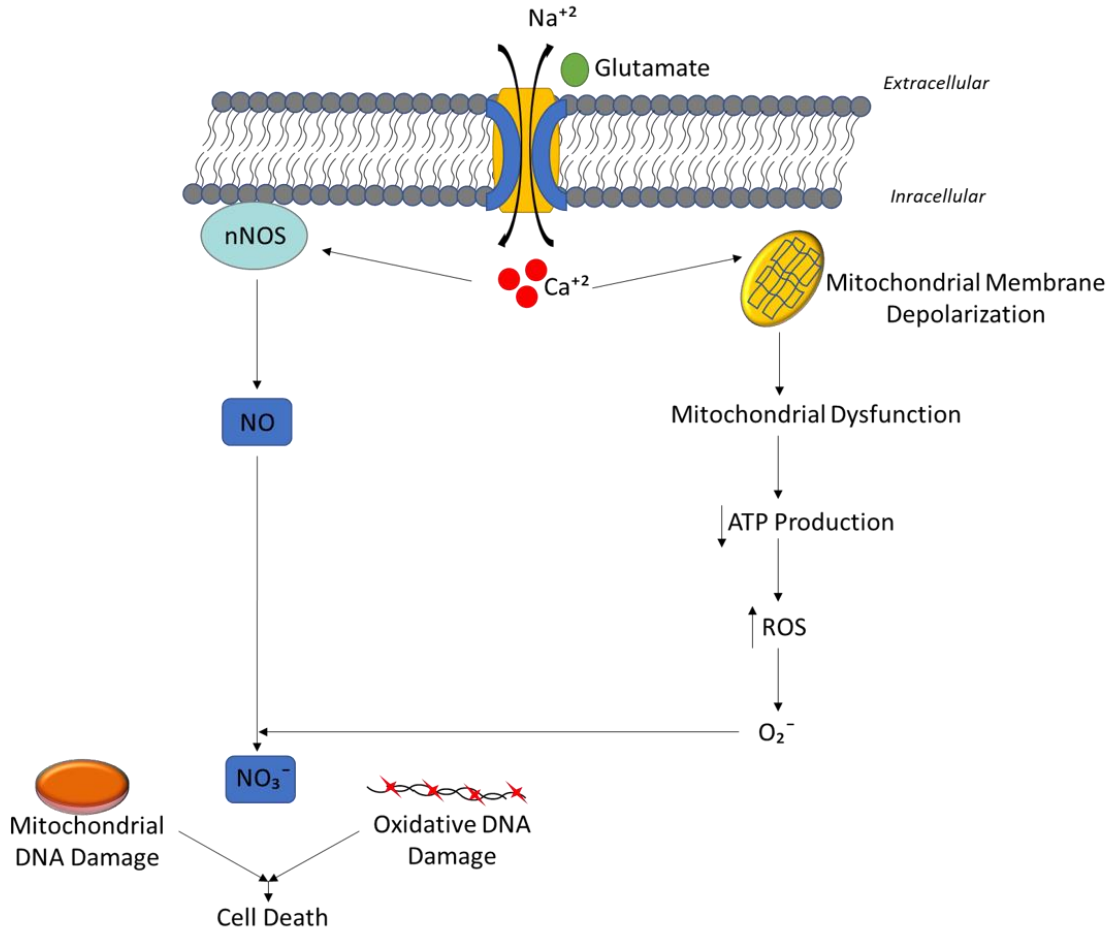

Figure 2.1: Mechanism of Glutamate-induced Excitotoxicity. Glutamate-induced excitotoxicity results in the production of reactive oxygen species (ROS), which eventually leads to cell death.

2.3 Uric Acid: A Free Radical Scavenger

Uric acid is an end product of purine synthesis. Cellular purines are mainly derived from large animal proteins and from live and dying cells. Deamination of adenine and dephosphorylation of guanine yields inosine and guanosine, respectively, which are further converted to hypoxanthine and guanine, respectively, via purine nucleoside phosphorylase. The oxidation of hypoxanthine and deamination of guanine results in the formation of xanthine, which is then subsequently oxidized to uric acid via xanthine oxidase (Fig.1.2). [88-90]

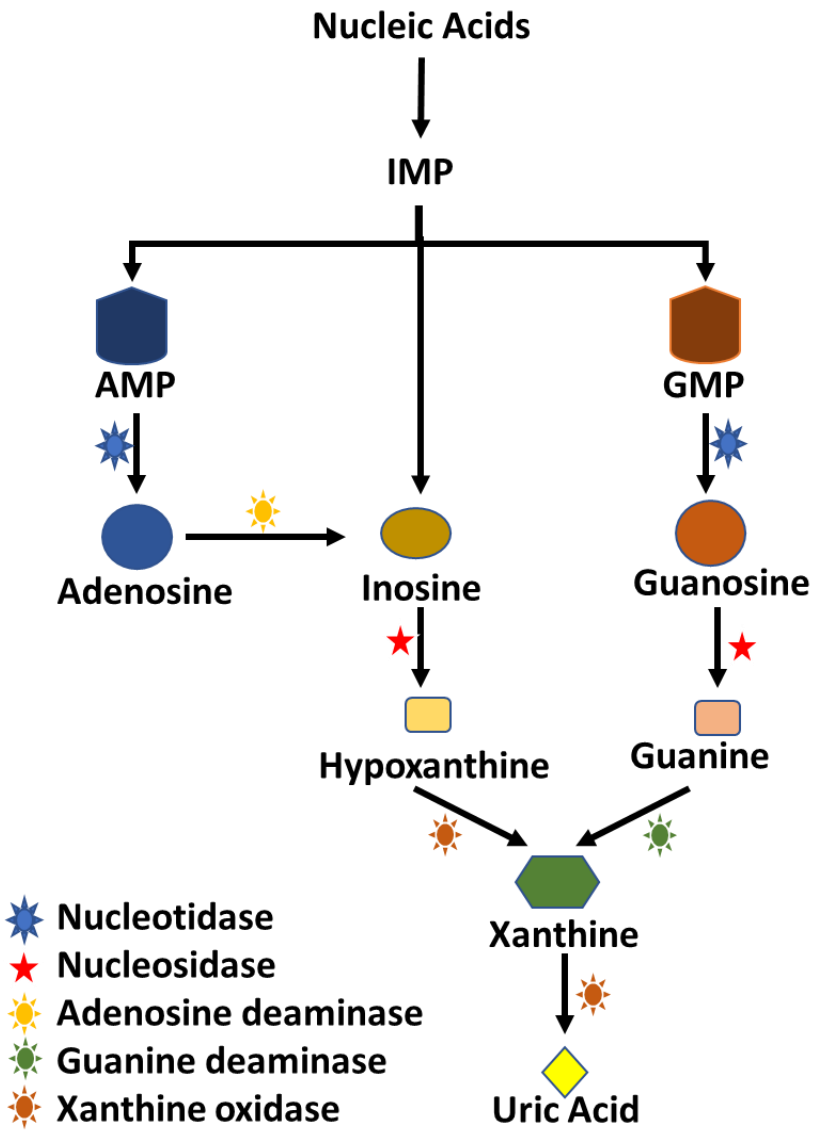

Figure 2.2: Purine synthesis pathway. Uric acid is the end product of purine metabolism.

The physiological importance of uric acid is underscored by the fact that 90% of serum uric acid filtered by kidney glomeruli is reabsorbed [16, 89]. Uric acid can be further oxidized into the more soluble 5-hydroxysourate, which is subsequently degraded into allantoic acid and ammonia by uricase, and allantoic acid can be excreted by the kidneys. However, due to the presence of two

premature stop codons in uricase mRNA, the gene encoding uricase is a pseudogene in some primates, including humans and apes. [91, 92]

Due to this fact, humans cannot metabolize uric acid into a more soluble form, and hence, serum uric acid levels in humans and apes are 3 to 10 times higher compared to mammals that possess functional uricase. Recent studies have shown that UA protects CNS neurons from excitotoxic and metabolic injuries. [17-21, 93] This suggests that increased UA concentration is a compensatory mechanism for longevity due to evolution.

Uric acid plays a vital role in scavenging ROS and inducing an inflammatory response, both of which are necessary for tissue healing. Uric acid upregulates EAAT-1 in astrocytes, which helps regulate excessive extracellular glutamate after SCI. [21] However, high systemic concentrations of uric acid have been linked to gout [94], metabolic syndrome [95-99], cardiovascular disease[98-100], and is one of the best independent predictors of diabetes. [101, 102] Nanofiber release of UA is a potential therapeutic approach to counter the negative effects of bolus injection of uric acid as uric acid is highly insoluble in aqueous solution. Targeted and controlled delivery of UA via electrospun biocompatible nanofibers mats may be more efficacious for treating GIE after SCI.

2.4 Polymer drug delivery systems

Polymer drug delivery systems can be controlled by diffusion (monolithic devices), osmosis (solvent-activated devices), [103] chemical (biodegradable), and external triggers (pH, temperature). [104] Various synthetic and natural polymers have been used as drug delivery carriers and offer advantages over others according to the application. Natural polymers are biocompatible, but compared to synthetic polymers, their mechanical properties, degradation rate, pore size, and loading capacity cannot be controlled. Ideally, incorporation of a drug in polymer offers various benefits, including nontoxicity, nonimmunogenicity, enhanced permeation and retention rate, greater loading capacity, and the ability for targeted and controlled delivery. [105] Among several synthetic polymers, poly (Ɛ-caprolactone) (PCL) is considered as a “go to” biomaterial in the fields of tissue engineering, wound dressing, and targeted drug delivery systems.[106-108] Electrospun PCL nanofibers have been extensively studied and used for various biomedical applications because of their slow biodegradation, high biocompatibility, and thermal stability.[106, 109-113] Hence, we chose synthetic polymers for the delivery of uric acid to injured spinal cord tissue.

2.5 Electrospun UA-PCL nanofibers

The electrospinning setup consists of five components: a DC power supply (kV range), polymer solution, electrically charged spinneret, a syringe pump, and a grounded collector. (Fig. 2.3) The process begins with jet initiation, in which polymer is pumped through the spinneret to make a droplet. The electrostatic

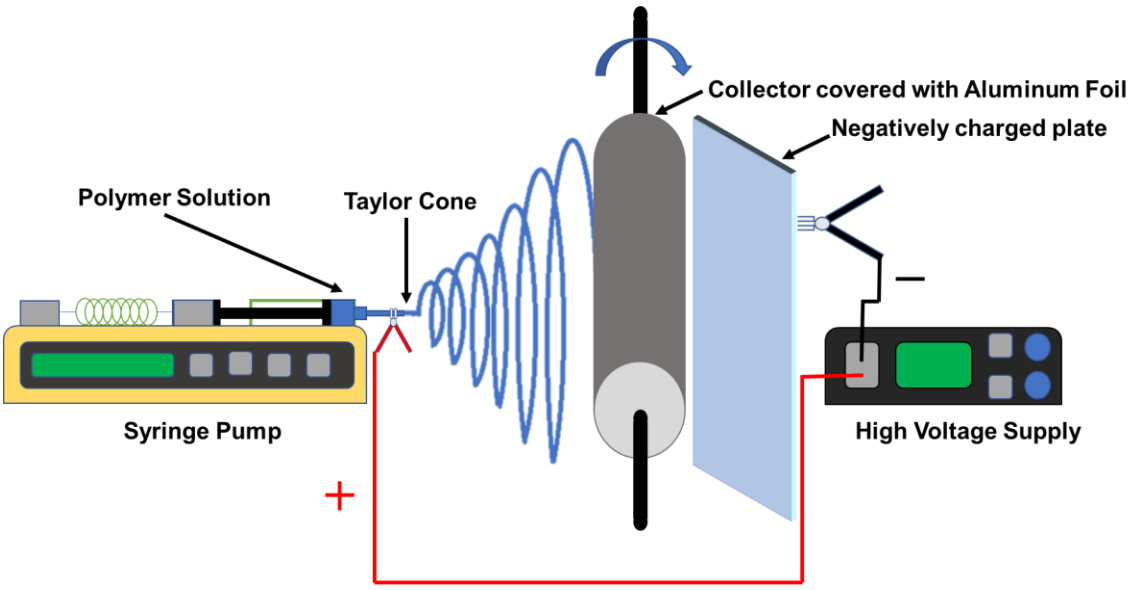

Figure 2.3: Electrospinning Setup. The electrospinning setup consists of five components: a DC power supply, polymer solution, electrically charged spinneret, a syringe pump, and a grounded collector.

field accumulates on the droplet, causing elongation of the droplet and forming a Taylor cone. When electrostatic forces overcome surface tension on the droplet, a polymer solution jet is ejected to the grounded collector, and the solvent is evaporated. This thinning of the polymer jet results in production of fibers of nanometer dimensions. [33, 114, 115] Several parameters can be changed to control the characteristics of the nanofibers, such as type of collector, applied voltage, distance between spinneret and collector, molecular weight, solvent evaporation characteristics, solution conductivity and viscosity, and dispersion flow rate. [33, 114-117] Typically, these parameters have direct effects on the morphological features of the nanofibers, which in turn, can shape the bioactive characteristics of the nanofibers. Therefore, we electrospun UA-PCL polymer solution to form nanofiber mats to achieve the desired characteristics of an ideal drug delivery system for SCI. In applications, which require even slower drug

release, materials, such as poly (ethylene glycol) diacrylate (PEGDA), are used to coat the base nanofiber mats, [118-120] which in addition to slowing drug release, improve the mechanical strength of the nanofiber mats and inhibit protein adsorption. [121, 122]

2.6 PEGDA as a coating polymer

PEGDA is formed by adding two acrylate groups, the crosslinker between the molecular chain polymers, on either side of the PEG chain. (Fig.2.4) PEG hydrogels can form different structures using three types of polymerization: (1) chain-growth, (2) step-growth, and (3) mixed-mode growth. [123] PEGDA follows chain-growth polymerization and uses photopolymerization for cross-linking, and initiation of the reaction occurs with cleavage of the initiation molecule by visible light, eventually leading to covalent crosslinking of PEGDA. The non-ionic and hydrophilic properties of PEGDA provide a lack of binding sites for proteins, and hence, reduce the chance of the material being recognized by the immune system. PEGDA has been shown to be an ideal polymer for drug delivery systems as it improves the pharmacokinetics of drugs and is FDA approved to deliver several enzymes, cytokines, antibodies, and growth factors. [124] We coated UA-PCL nanofiber mats with PEGDA to slow the release of UA from the PCL nanofibers.

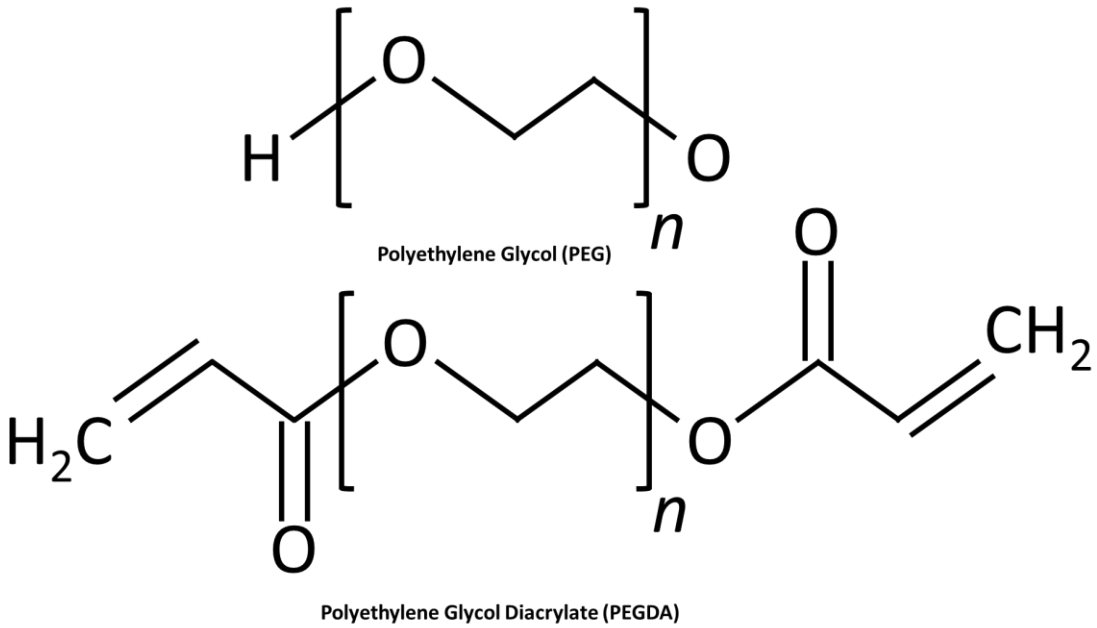

Figure 2.4: Structures of polyethylene glycol (PEG) and polyethylene glycol diacrylate (PEGDA)

2.7 Organotypic slice Culture

The Firestein group reported that UA acts astroglia to protect spinal cord neurons from injury, [21] and this work and the work of others was performed *in vitro using* dissociated neuron cultures. *In vitro* cell culture is crucial and important for studying cellular processes in an isolated environment, and it reduces animal suffering and numbers of animal used for an experiment. However, primary dissociated cells do not reflect the true architecture of the cellular environment. Therefore, organotypic slice cultures, which simulate *in vivo* like conditions, are used for the studies described here.

Organotypic culture includes several cell types and preserves neuronal synaptic organization, which are crucial for understanding the role of different therapeutics for SCI. Large volume organotypic slice culture was first introduced by Boyd [43]

and further optimized by Ansevin and Lipps. [125] Gähwiler's group introduced the roller tube method, [44-46, 49, 51, 126] following optimization of the culture of tissues on semipermeable membranes by the Stoppini group. [52] This method is still used widely to culture organotypic brain and spinal cord slices with some modifications. The lack of the ability to obtain electrophysiological readings from long-term organotypic slices cultures on semipermeable membrane points to a need to grow organotypic slices directly on the culture dish without a semipermeable membrane. Therefore, we optimized organotypic spinal cord slice culture from embryonic day 15-17 rats without using a semipermeable membrane insert, allowing for study of the functional aspects of long-term organotypic slice cultures.

CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental Design

Spinal cord slices were isolated from rat embryos at embryonic day 16 (E16) and sliced at 350μm using vibratome. The slices were then placed into 12 well plates previously coated with poly-D-lysine and laminin. The slices were cultured for 7 days *in vitro* (DIV) in Neurobasal A medium supplemented with B-27, GlutaMAX, and gentamycin. Locke's buffer (NaCl, 154 mM; KCl, 5.6 mM; CaCl2, 2.3 mM; MgCl2, 1.0 mM; NaHCO3, 3.6 mM; glucose, 5 mM; Hepes, 5 mM; pH 7.2) was used as vehicle for glutamate and UA treatments. On DIV7, the slices were treated with 3mM glutamate for 1 hour and different concentrations of UA for 24 hours at 37C and 5% CO_2. For assessment of oxidative stress, phase contrast and fluorescence micrographs were taken under a 4x objective using the EVOS FL Microscope (ThermoFisherScientific) following the incubation of slices in 3.5 μM CellROX™ Green Reagent for 1 hour (Fig.3.1.).

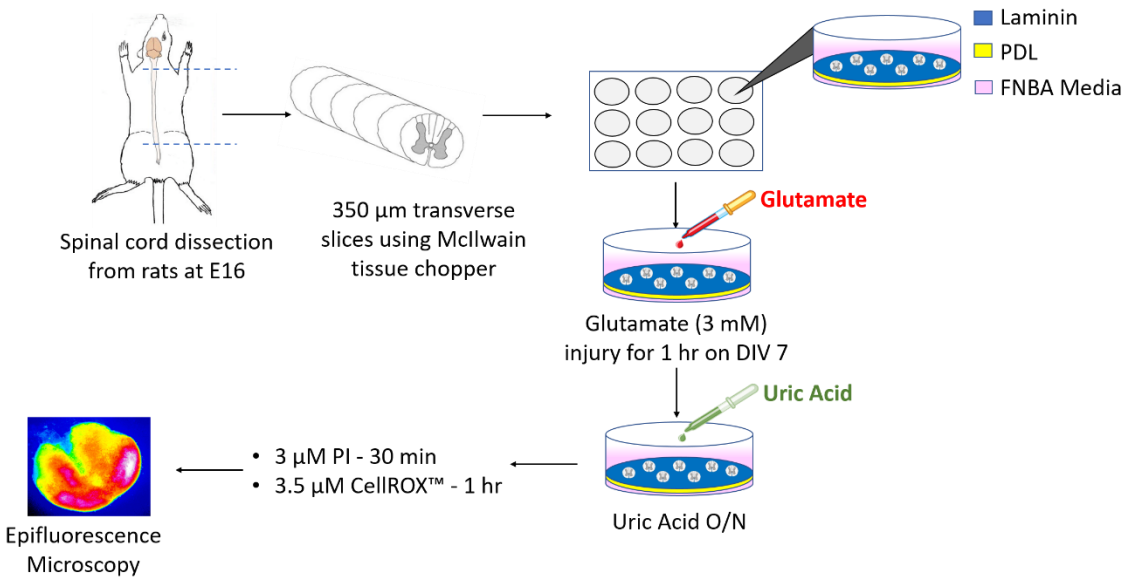

Figure 3.1: Experimental setup for assessment of effects of UA on glutamate-induced cell death (propidium iodide staining) and generation of reactive oxygen species (ROS; CellROX staining).

3.2 Preparation of UA-containing PCL nanofibers

Preparation of UA containing PCL nanofibers was adapted from McKeon-Fisher et. al.. [127] Briefly, PCL and dichloromethane (DCM) were combined for a final solution of 20% PCL (w/v). To load the fibers with UA, 0.1% and 0.2% of UA solution was added to the PCL solution. Solutions were loaded into a 5 ml syringe with a 20 gauge blunt stainless steel needle and placed into a syringe pump set at an extrusion rate of 5 ml/h. A distance of 18 cm was set between the needle tip, and a 3000±200 rpm rotating mandrel with a 5 cm diameter was used to collect the nanofibers. A 2 kV negatively charged plate was placed behind the rotating mandrel to aid in attracting the positively charged solutions. The positive voltage ranged from 13 – 17 kV and was adjusted to produce the most stable Taylor cone during the electrospinning process to produce the most consistent fiber morphology in the scaffold.

3.3 Coating of PEGDA on UA-PCL Nanofibers

All hydrogel solutions were prepared by dissolving poly (ethylene glycol) diacrylate (PEGDA) in phosphate-buffered saline (PBS) using a vortex mixer. Photo-initiator solution comprised of 300 mg/ml 2,2-dimethoxy-2-phenylacetophenone in 1-vinyl-2-pyrrolidinone was prepared and added to the hydrogel solution (50 µL of photo-initiator solution per 1 mL hydrogel solution (5% v/v) just prior to applying UV radiation. The hydrogel solution mixture was poured on top of UA-PCL nanofibers, and UV radiation was applied at a wavelength of 365 nm using a 3UVTM Lamp at 30 s intervals until the solution solidified and failed to flow.

3.4 Release profile of UA

UA-PCL nanofibers were cut into 1 inch x 1 inch cross section mats and were incubated in 10 ml phosphate-buffered saline (PBS). The pH of the PBS, which decreases with increasing concentration of UA released from the nanofiber mats, was measured over time with a pH meter.

3.5 Organotypic Spinal Cord Slice Culture

Sprague Dawley rats at 16 days of gestation (E16) were extracted via Caesarian section in Gey's balanced salt solution supplemented with 1% of 30µM kynurenic acid and 0.6% glucose solution. A dorsal incision was made in the embryos to isolate spinal cords, which were sliced at 350 µm using a McIlwain tissue chopper. The slices were placed into 12 well plates previously coated with 0.1%

poly-D-lysine for 24 hours and 0.1mg/ml laminin for 2 hours at 37C and 5% CO_2. The slices were cultured in serum-free medium (FNBA; Neurobasal A medium supplemented with B-27, 0.5mM GlutaMAX, 25µg/ml gentamycin) for 7 *days in vitro* (DIV). Medium (100 µL) was added every other day.

3.6 Glutamate-induced Injury and UA Nanofiber Treatment

On DIV7, cultures were treated with 3mM glutamate dissolved in Locke's buffer (LB; NaCl, 154 mM; KCl, 5.6 mM; CaCl2, 2.3 mM; MgCl2, 1.0 mM; NaHCO3, 3.6 mM; glucose, 5 mM; HEPES, 5 mM; pH 7.2), or with LB alone as control for 1 hour followed by treatment with nanofiber mats for 15 minutes, after which, the mats were removed. The slices were then placed into an incubator at 37C and 5% CO_2 overnight.

3.7 CellROX, H2DCFDA, PI Treatment, and Fluorescence Imaging

For assessment of oxidative stress, phase contrast and fluorescence micrographs were taken under a 4x objective using the EVOS FL Microscope (ThermoFisher Scientific) following the incubation of slices in 3 µM propidium iodide (PI) for 30 minutes and 5 µM CellROX™ Green Reagent or H2DCFDA for 1 hour. Images were quantified using ImageJ software, and statistical analysis was performed using GraphPad Prism 6 software.

3.8 Image Processing

Images of organotypic slices that were exposed to PI for quantifying % dead cells and CellROX/H2DCFDA for quantifying % ROS were taken under a 4x objective using the EVOS FL Microscope (ThermoFisherScientific). Fluorescence micrographs were quantified using ImageJ by converting to 16-bit, then the region of interest was traced and mean grey value (intensity) was measured.

CHAPTER 4: RESULTS

4.1 Uric Acid is Neuroprotective and Scavenges Reactive Oxygen Species in Organotypic slice culture

To determine whether UA reduces levels of reactive oxygen species (ROS) in organotypic slice cultures incubated with 3 mM glutamate, we stained the slices with CellROX Green Reagent. We found that treatment with UA at concentrations of 200, 400, and 800 μM after glutamate-induced injury resulted in decreased ROS, suggesting that UA scavenges ROS (Fig. 4.1 and 4.2).

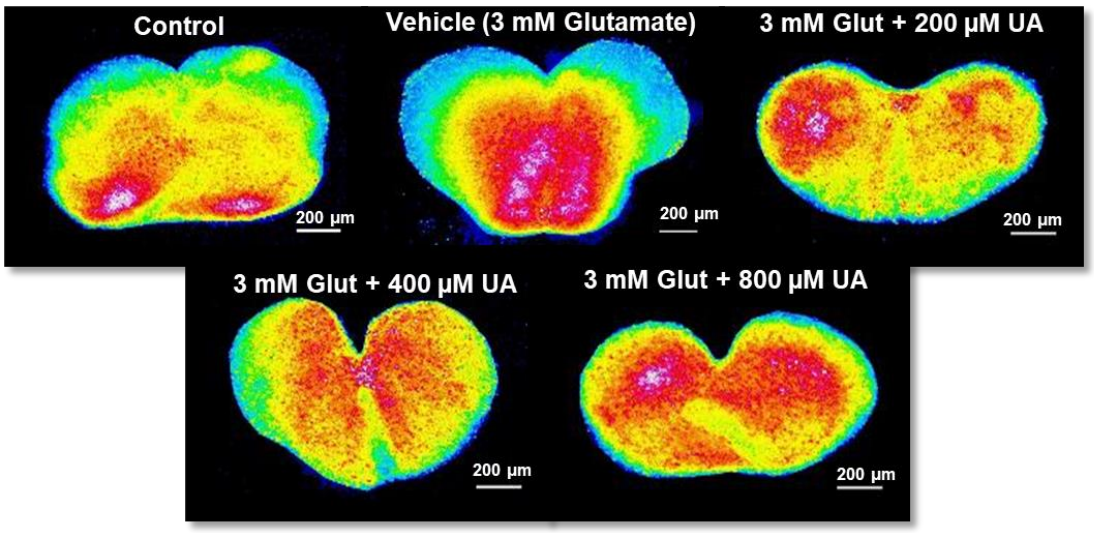

Figure 4.1: Representative heat maps demonstrating ROS in organotypic spinal cord slices following 3 mM glutamate treatment and post-treatment with (A) Control, (B) Vehicle, (C) 200 μM UA, (D) 400 μM UA and (E) 800 μM UA.

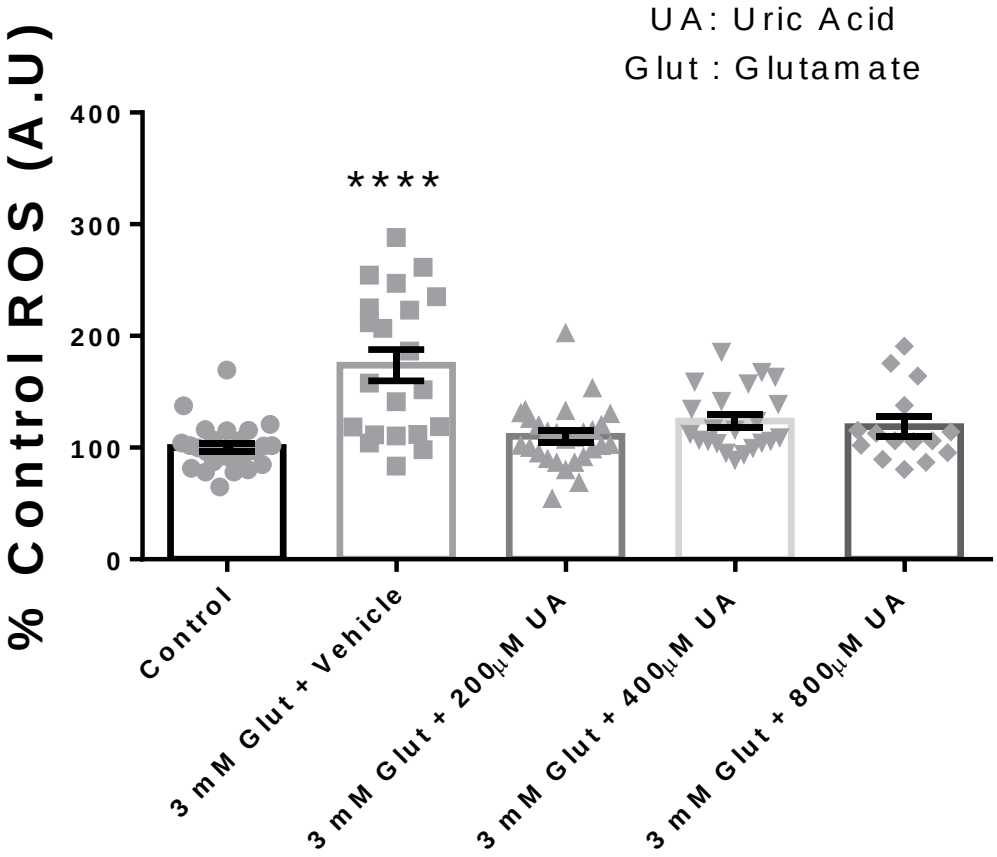

Figure 4.2 Quantitation of % Control ROS from heat maps after 1 hour of 3.5 μM CellROX Green Reagent exposure the n(CTL) = 28, n(VEHICLE) = 21, n(200 μM UA) = 27, n(400 μM UA) = 22, and n(800 μM UA) = 27. Statistics calculated by one-way ANOVA followed by Tukey's multiple comparisons test. ****p<0.0001. Error bars indicate SEM.

4.2 Sustained Release v/s Burst Release of UA from UA-PCL Nanofibers

To determine whether the protective effects of UA are mediated over time, we constructed nanofiber mats that release UA as a burst or over time. We incorporated UA into PCL and electrospun UA-PCL nanofiber mats for burst release of UA (Fig. 4.3A). We also coated UA-PCL nanofibers with PEGDA for sustained release. Using a decrease in solution pH as an assay for UA release from the nanofiber mats, we found that UA was released into PBS solution within

20 minutes from UA-PCL nanofibers (burst) whereas PEGDA coated UA-PCL nanofibers released in UA in a sustained manner over a 2 hour period (Fig. 4.3B). We calculated the concentration of UA in the solution at different time points using the release profile and determined that at the 15 minute time point, the concentration of UA in the solution is approximately 200 μM. Thus, we applied the nanofiber mats to spinal cord cultures for 15 minutes for comparison of effects of the nanofibers to that of 200 μM soluble UA.

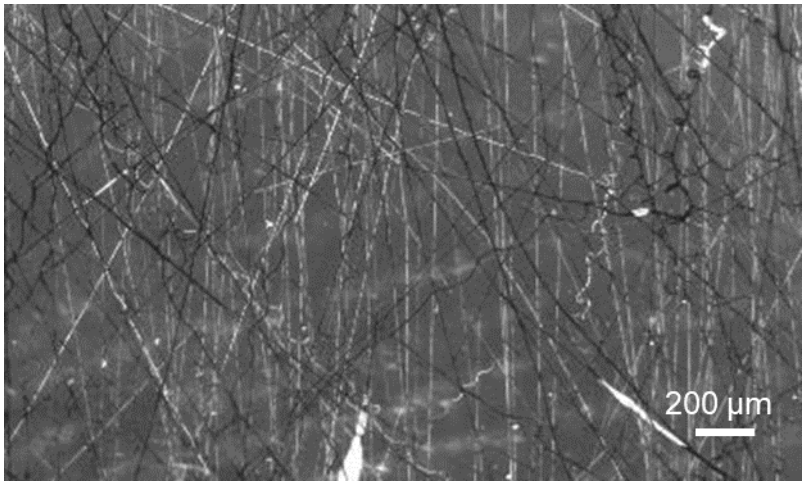

Figure 4.3A: Bright field image of nanofibers, demonstrating that they mimic the extracellular matrix microenvironment

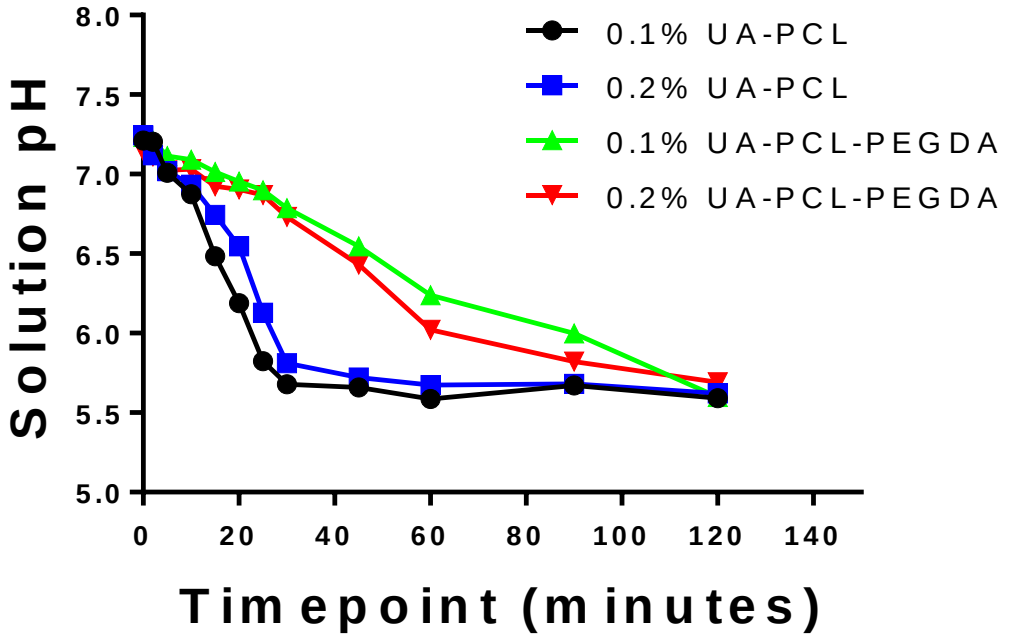

Figure 4.3B: Release Profile of UA-PCL and UA-PCL-PEGDA nanofibers. UA-PCL-PEGDA fibers release UA more slowly than UA-PCL fibers as measured by a change in the pH of the solution (as a proxy for UA release) with respect to time. A 1 inch x 1 inch nanofiber was used for the release profile**.**

4.3 Effects of UA Release from Nanofibers on Cell Death

To investigate the effects of UA release from the two types of nanofibers on cell death in organotypic spinal cord slices after glutamate induced excitotoxicity, we stained the slices with 3 μM propidium iodide (PI) after GIE and treatment. We observed that treatment with UA-PCL and UA-PCL-PEGDA nanofibers after injury increased cell viability as compared to the slices that were not treated with nanofibers. Furthermore, treatment with PCL and PCL-PEGDA nanofibers alone does affect cell death in the cultures (Fig 4.4).

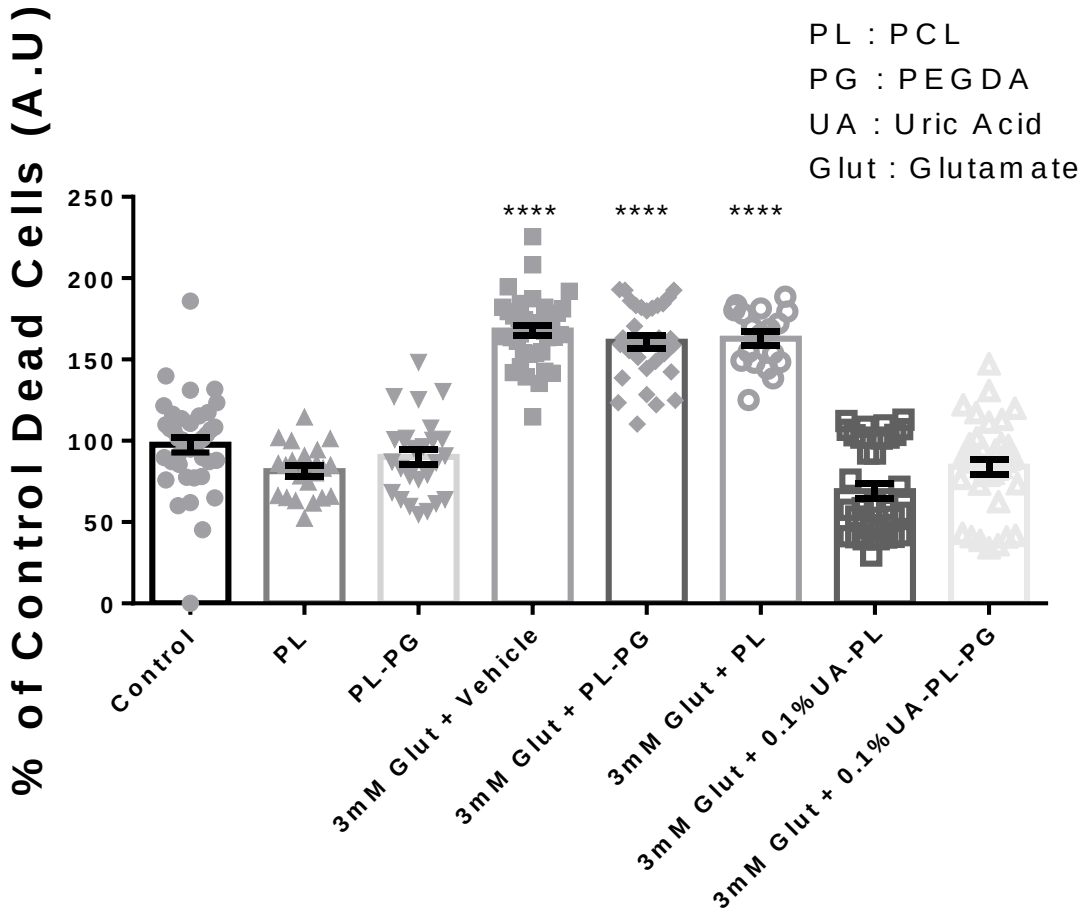

Figure 4.4: Quantitation of propidium iodide staining in response to high levels of glutamate with and without UA nanofiber mat treatment. Statistics calculated by one-way ANOVA followed by Tukey's multiple comparisons test. ****p<0.0001 vs. control. Error bars indicate SEM. n=3 cultures.

4.4 Effects of UA Release from Nanofibers on ROS

Finally, to assess the effects of treatment with UA-PCL and UA-PCL-PEGDA nanofibers on ROS after GIE, we treated the slices with 5 μM of H2DCFDA, a ROS indicator. Treatment with UA-PCL and UA-PCL-PEGDA nanofibers after GIE significantly decreased the % ROS as compared to no treatment.

Furthermore, PCL and PCL-PEGDA nanofibers alone do not affect ROS production in the cultures (Fig 4.5).

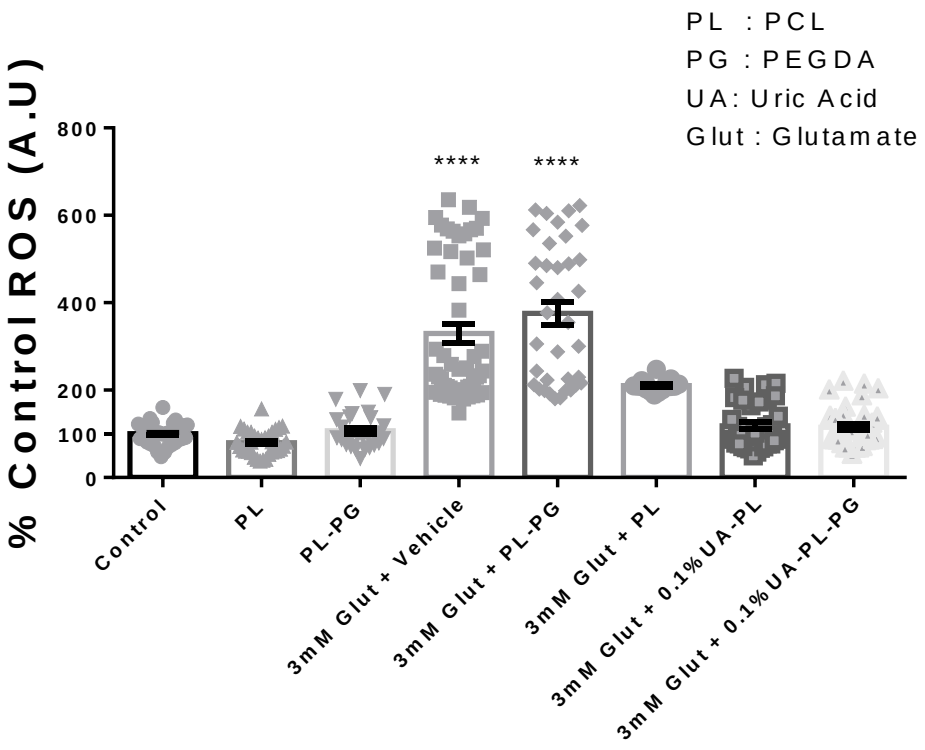

Figure 4.5 Quantitation of % baseline (control) ROS in response to excess glutamate with and without nanofiber mats. Statistics calculated by one-way ANOVA followed by Tukey's multiple comparisons test. ****p<0.0001 vs. control. Error bars indicate SEM. n=3 cultures.

CHAPTER 5: DISCUSSIONS, CONCLUSIONS, AND FUTURE DIRECTIONS

This study investigated the role of PCL nanofibers as a potential drug delivery carrier of UA for the treatment of secondary damage in spinal cord injury due to glutamate-induced excitotoxicity. Experiments were performed to broadly assess the recovery of organotypic spinal cord slices after glutamate-induced injury with the application of UA-PCL nanofibers. Initially, a dose-response curve was constructed for UA treatment of cultured organotypic spinal cord slices injured with 3 mM glutamate for 1 hour. Our results demonstrate that all concentrations of UA tested (200 µM, 400 µM, and 800 µM) significantly reduced the level of glutamate-induced ROS formation in organotypic slice cultures. Additionally, we found that the reduction of ROS levels occurred to the same degree for all concentrations tested. Hence, we treated with 200 µM UA for our experiments, keeping in mind that higher systemic concentrations of UA have detrimental effects on kidneys and heart, [94, 98-100] and the ultimate goal is to design a therapeutic material. We then used our nanofiber mats that release UA within 20 minutes and those that release UA over 2 hours to determine whether bolus vs. slow release results in better outcome after the slices are injured with glutamate. We found that regardless of the rate of release, the nanofiber mats that released UA, but not control mats, resulted in less damage, as evidenced by lower cell death and lower ROS production. These results are consistent with previous finding that UA reduced damage on neurons caused by oxidative stress. [21]

Glutamate-induced excitotoxicity causes the production of highly toxic peroxynitrite, a type of ROS that causes lipid peroxidation, mitochondrial and cellular DNA damage, and ultimately, apoptosis. Since UA is considered a peroxynitrite scavenger and plays a role in mediating damage caused by overstimulation of glutamate receptors after CNS injury, it has been a focus of research. In one study, it was observed that UA protects hippocampal neurons by reducing ROS accumulation after excitotoxic insult. [19] Furthermore, UA scavenges peroxynitrite in mouse spinal cord neuronal culture. [20] Previously, our lab established that UA plays a role in neuroprotection from secondary injury by upregulating EAAT-1 on the astrocytes. [21] The ability of UA to reduce the accumulation of ROS in organotypic spinal cord slice culture has also been demonstrated in our study. (Fig.3.2) It has been established that high systemic concentrations of UA are linked to several diseases, such as gout, hypertension, and diabetes, and so controlled release of UA is a potential therapeutic to promote the beneficial effects of UA without raising serum UA levels.

We decided to use nanofiber mats to deliver UA to injured neurons for the following reasons. Nanoscale polymer fibers display a wide variety of properties, such as very large surface area to volume ratio, flexibility in pore size and volume, and appropriate stiffness and tensile strength to match biological tissue, making it the ideal candidate to be used in a wide variety of applications. [128-131] There are several methods available for producing polymer nanofibers, including drawing, [132] template synthesis, [133, 134] phase separation, [135]

self-assembly, [136, 137] and electrospinning, [138, 139]. Each of these methods allows for production of fibers with different characteristics and applications. However, electrospinning offers a huge advantage over other methods as it allows for various fiber assemblies and scalability, [131] is low cost, allows for control over fiber dimensions, can form long continuous nanofibers, [140] and produces nanofibers from both natural and synthetic polymers. [141]. In fact, the fundamental idea of electrospinning (*i.e.* electrostatic spinning of fibers) dates to 1902, [142, 143] and was modified and perfected over time. [144-146] Our results demonstrate that electrospinning is a viable technique to produce nanofiber mats that release UA for biological applications.

Electrospun nanofibers have been used as composite reinforcements [131, 147, 148] for gas, liquid, and molecule filtration, [149, 150] as thin porous films in prosthetics [151-154], and as tissue templates because its structure and function mimics the natural extracellular matrix (ECM). [155] Furthermore, nanofibers have been used for wound dressing as they promote natural skin growth and eliminate scar tissue formation. [156-159] Electrospun nanofibers are also widely used as drug delivery carriers for antibiotics, [160] anticancer drugs, [161] proteins [162], and DNA. [163] Several drug delivery systems have been developed, such as liposomes, polymer micelles, and nanofibers, and all offer the benefits of cost effectiveness, high loading capacity and encapsulation efficiency, ease of operation, simultaneous delivery of various therapies, and reduction in toxic effects caused by traditional delivery systems. [131] However,

we chose to use nanofibers formed by electrospinning since we had control over the morphology and degradation rate of nanofibers by manipulating the parameters, such as type of collector, applied voltage, distance between spinneret and collector, molecular weight, solvent evaporation characteristics, solution conductivity and viscosity, and dispersion flow rate. [33, 114-117] Our results support the fact that electrospinning provides the ability to control the release of UA from PCL nanofiber mats, as release is controlled by the degradation rate of PCL. This allows for the opportunity to modulate UA delivery by optimizing the percent concentration and molecular weight of PCL. Additional coating with PEGDA provides a barrier to hydrolysis of PCL since PEGDA is non-degradable. The release kinetics can be further tailored by changing the molecular weight of PEGDA utilized. [164] Higher molecular weight is associated with higher pore size of the hydrogel structure. PEGDA is hydrophilic and does not allow adsorption of proteins, making it non immunogenic and biocompatible and inhibiting foreign body response. [165, 166] Our results supports this notion that PEGDA does slow the release of UA from UA-PCL nanofiber mats and has no detrimental physiological effect on the organotypic spinal cord slices.

5.1 Future studies

There has been a large amount of *in vitro* research performed using dissociated neurons as a model for SCI, and these cultures do not offer the architectural and morphological structure of the intact spinal cord, which is important for understanding the roles of neurons and other cell types surrounding the injured

area. Organotypic slice culture has been optimized by many research groups, and currently, the majority of researchers use the semipermeable membrane method optimized by the Stoppini group. [52] However, this method does not allow for recording of electrophysiological signals in long-term culture. We optimized our organotypic slice cultured directly on culture dishes without the use of semipermeable membranes. This culture allows for the recording of electrical signals long-term. In the future, we will culture organotypic spinal cord slices directly on microelectrode arrays (MEAs) and record electrophysiological signals to analyze the effects of UA-PCL nanofibers on preserving neuronal function after glutamate-induced excitotoxicity. The data obtained will further our understanding of the neuroprotective effects of UA from secondary SCI. Functional activity will give information about the strength of synaptic connections.

Another future direction is to research the antioxidant effects of UA for SCI in preclinical *in vivo* studies in rodents and large animals. Additionally, the long-term effects of UA-PCL nanofibers on systemic concentrations of UA, *in vivo* pharmacokinetics and pharmacodynamics of UA released from nanofiber mats, and the extent of recovery after injury can be assessed. Release kinetics of the nanofibers can be further improved by changing the concentration of PCL, flow rate of polymer, distance of spinneret from mandrel, and coating of PCL nanofibers mats with different molecular weights of PEGDA during the

electrospinning process. We can also explore other burst and sustained release polymer drug delivery models.

REFERENCES

1. Faden, A.I., et al., *The role of excitatory amino acids and NMDA receptors in traumatic brain injury.* Science, 1989. **244**(4906): p. 798-800.
2. Yanase, M., T. Sakou, and T. Fukuda, *Role of N-methyl-D-aspartate receptor in acute spinal cord injury.* Journal of neurosurgery, 1995. **83**(5): p. 884-888.
3. Olney, J.W. and L.G. Sharpe, *Brain lesions in an infant rhesus monkey treated with monosodium glutamate.* Science, 1969. **166**(3903): p. 386-388.
4. Choi, D.W., *Excitotoxic cell death.* Journal of neurobiology, 1992. **23**(9): p. 1261-1276.
5. Choi, D.W., *Ionic dependence of glutamate neurotoxicity.* Journal of Neuroscience, 1987. **7**(2): p. 369-379.
6. Prentice, H., J.P. Modi, and J.-Y. Wu, *Mechanisms of Neuronal Protection against Excitotoxicity, Endoplasmic Reticulum Stress, and Mitochondrial Dysfunction in Stroke and Neurodegenerative Diseases.* Oxidative medicine and cellular longevity, 2015. **2015**: p. 964518-964518.
7. Brenman, J.E., et al., *Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α1-syntrophin mediated by PDZ domains.* Cell, 1996. **84**(5): p. 757-767.
8. Nakamura, T., et al., *Aberrant protein s-nitrosylation in neurodegenerative diseases.* Neuron, 2013. **78**(4): p. 596-614.
9. Bredt, D.S., et al., *Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase.* Nature, 1991. **351**(6329): p. 714.
10. Chen, H., et al., *Oxidative stress in ischemic brain damage: mechanisms of cell death and potential molecular targets for neuroprotection.* Antioxidants & redox signaling, 2011. **14**(8): p. 1505-1517.
11. Kim, Y.-H., K.-Y. Ha, and S.-I. Kim, *Spinal Cord Injury and Related Clinical Trials.* Clinics in orthopedic surgery, 2017. **9**(1): p. 1-9.
12. Baillie, J.K., et al., *Endogenous urate production augments plasma antioxidant capacity in healthy lowland subjects exposed to high altitude.* Chest, 2007. **131**(5): p. 1473-1478.
13. Maxwell, S., et al., *Antioxidant status in patients with uncomplicated insulin-dependent and non-insulin-dependent diabetes mellitus.* European journal of clinical investigation, 1997. **27**(6): p. 484-490.
14. Glantzounis, G., et al., *Uric acid and oxidative stress.* Current pharmaceutical design, 2005. **11**(32): p. 4145-4151.
15. Proctor, P.H., *Uric acid: neuroprotective or neurotoxic?* Stroke, 2008. **39**(5): p. e88.
16. Ames, B.N., et al., *Uric acid provides an antioxidant defense in humans against oxidant-and radical-caused aging and cancer: a hypothesis.* Proceedings of the National Academy of Sciences, 1981. **78**(11): p. 6858-6862.
17. Hooper, D., et al., *Uric acid, a natural scavenger of peroxynitrite, in experimental allergic encephalomyelitis and multiple sclerosis.*

Proceedings of the National Academy of Sciences, 1998. **95**(2): p. 675-680.
18. Squadrito, G.L., et al., *Reaction of uric acid with peroxynitrite and implications for the mechanism of neuroprotection by uric acid.* Archives of biochemistry and biophysics, 2000. **376**(2): p. 333-337.
19. Yu, Z.F., et al., *Uric acid protects neurons against excitotoxic and metabolic insults in cell culture, and against focal ischemic brain injury in vivo.* Journal of neuroscience research, 1998. **53**(5): p. 613-625.
20. Scott, G.S., et al., *Uric acid protects against secondary damage after spinal cord injury.* Proceedings of the National Academy of Sciences, 2005. **102**(9): p. 3483-3488.
21. Du, Y., et al., *Astroglia-mediated effects of uric acid to protect spinal cord neurons from glutamate toxicity.* Glia, 2007. **55**(5): p. 463-472.
22. Heinig, M. and R.J. Johnson, *Role of uric acid in hypertension, renal disease, and metabolic syndrome.* Cleveland Clinic journal of medicine, 2006. **73**(12): p. 1059.
23. McCrudden, F.H., *Uric Acid: The Chemistry, Physiology and Pathology of Uric Acid and the Physiologically Important Purin Bodies, with a Discussion of the Metabolism in Gout*. 1905: Samuel Usher.
24. Xinhua, D., *Preparation of uric acid standard stock solution.* Clinical chemistry, 2006. **52**(11): p. 2117-2118.
25. Schiffman, J.D. and C.L. Schauer, *Cross-linking chitosan nanofibers.* Biomacromolecules, 2007. **8**(2): p. 594-601.
26. Ding, Y., et al., *Electrospun Fibrous Architectures for Drug Delivery, Tissue Engineering and Cancer Therapy.* Advanced Functional Materials, 2019. **29**(2): p. 1802852.
27. Beachley, V. and X. Wen, *Effect of electrospinning parameters on the nanofiber diameter and length.* Materials science & engineering. C, Materials for biological applications, 2009. **29**(3): p. 663-668.
28. Hsu, Y.-M., et al., *The effects of fiber size on MG63 cells cultured with collagen based matrices.* Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2009. **91B**(2): p. 737-745.
29. Huang, Z.M., et al., *A review on polymer nanofibers by electrospinning and their applications in nanocomposites.* Composites Science and Technology, 2003. **63**(15): p. 2223-2253.
30. Zhang, Y., et al., *Recent development of polymer nanofibers for biomedical and biotechnological applications.* Journal of Materials Science: Materials in Medicine, 2005. **16**(10): p. 933-946.
31. Keun Kwon, I., S. Kidoaki, and T. Matsuda, *Electrospun nano- to microfiber fabrics made of biodegradable copolyesters: structural characteristics, mechanical properties and cell adhesion potential.* Biomaterials, 2005. **26**(18): p. 3929-3939.
32. Bhardwaj, N. and S.C. Kundu, *Electrospinning: A fascinating fiber fabrication technique.* Biotechnology Advances, 2010. **28**(3): p. 325-347.
33. Reneker, D.H. and A.L. Yarin, *Electrospinning jets and polymer nanofibers.* Polymer, 2008. **49**(10): p. 2387-2425.

34. Greiner, A. and J.H. Wendorff, *Electrospinning: A Fascinating Method for the Preparation of Ultrathin Fibers.* Angewandte Chemie International Edition, 2007. **46**(30): p. 5670-5703.
35. Reneker, D.H., et al., *Bending instability of electrically charged liquid jets of polymer solutions in electrospinning.* Journal of Applied Physics, 2000. **87**(9): p. 4531-4547.
36. Han, D. and P.-I. Gouma, *Electrospun bioscaffolds that mimic the topology of extracellular matrix.* Nanomedicine: Nanotechnology, Biology and Medicine, 2006. **2**(1): p. 37-41.
37. Shields, K.J., et al., *Mechanical Properties and Cellular Proliferation of Electrospun Collagen Type II.* Tissue Engineering, 2004. **10**(9-10): p. 1510-1517.
38. La Mantia, F.P., et al., *Degradation of polymer blends: A brief review.* Polymer Degradation and Stability, 2017. **145**: p. 79-92.
39. Reichert, J.C., et al., *A Tissue Engineering Solution for Segmental Defect Regeneration in Load-Bearing Long Bones.* Science Translational Medicine, 2012. **4**(141): p. 141ra93-141ra93.
40. Dash, T.K. and V.B. Konkimalla, *Poly-ε-caprolactone based formulations for drug delivery and tissue engineering: A review.* Journal of Controlled Release, 2012. **158**(1): p. 15-33.
41. Russell, W.M.S., R.L. Burch, and C.W. Hume, *The principles of humane experimental technique*. Vol. 238. 1959: Methuen London.
42. Humpel, C., *Organotypic brain slice cultures: A review.* Neuroscience, 2015. **305**: p. 86-98.
43. Boyd, W., *A chamber for organotypic culture; adapted for growing large volumes of tissue.* Stain technology, 1971. **46**(2): p. 85-87.
44. Gähwiler, B.H., *Morphological differentiation of nerve cells in thin organotypic cultures derived from rat hippocampus and cerebellum.* Proceedings of the Royal Society of London. Series B. Biological Sciences, 1981. **211**(1184): p. 287-290.
45. Gähwiler, B., *Organotypic monolayer cultures of nervous tissue.* Journal of neuroscience methods, 1981. **4**(4): p. 329-342.
46. Gähwiler, B., *Organotypic cultures of neural tissue.* Trends in neurosciences, 1988. **11**(11): p. 484-489.
47. Gähwiler, B. and F. Hefti, *Guidance of acetylcholinesterase-containing fibres by target tissue in co-cultured brain slices.* Neuroscience, 1984. **13**(3): p. 681-689.
48. Gähwiler, B., et al., *Continuous presence of nerve growth factor is required for maintenance of cholinergic septal neurons in organotypic slice cultures.* Neuroscience, 1990. **36**(1): p. 27-31.
49. Gähwiler, B., et al., *Organotypic slice cultures: a technique has come of age.* Trends in neurosciences, 1997. **20**(10): p. 471-477.
50. Gähwiler, B.H., S.M. Thompson, and D. Muller, *Preparation and maintenance of organotypic slice cultures of CNS tissue.* Current protocols in neuroscience, 1999. **9**(1): p. 6.11. 1-6.11. 11.

51. Victorov, I.V., A.A. Lyjin, and O.P. Aleksandrova, *A modified roller method for organotypic brain cultures: free-floating slices of postnatal rat hippocampus.* Brain Research Protocols, 2001. **7**(1): p. 30-37.
52. Stoppini, L., P.-A. Buchs, and D. Muller, *A simple method for organotypic cultures of nervous tissue.* Journal of neuroscience methods, 1991. **37**(2): p. 173-182.
53. Sekhon, L.H. and M.G. Fehlings, *Epidemiology, demographics, and pathophysiology of acute spinal cord injury.* Spine, 2001. **26**(24S): p. S2-S12.
54. Griffin, M.R., et al., *Mortality, survival and prevalence: traumatic spinal cord injury in Olmsted County, Minnesota, 1935–1981.* Journal of chronic diseases, 1985. **38**(8): p. 643-653.
55. GRIFFIN, M.R., et al., *Traumatic spinal cord injury in Olmsted County, Minnesota, 1935–1981.* American journal of epidemiology, 1985. **121**(6): p. 884-895.
56. Hachem, L.D., C.S. Ahuja, and M.G. Fehlings, *Assessment and management of acute spinal cord injury: From point of injury to rehabilitation.* The journal of spinal cord medicine, 2017. **40**(6): p. 665-675.
57. Dumont, R.J., et al., *Acute spinal cord injury, part II: contemporary pharmacotherapy.* Clinical neuropharmacology, 2001. **24**(5): p. 265-279.
58. Rowland, J.W., et al., *Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon.* Neurosurgical focus, 2008. **25**(5): p. E2.
59. Alizadeh, A., S.M. Dyck, and S. Karimi-Abdolrezaee, *Traumatic spinal cord injury: an overview of pathophysiology, models and acute injury mechanisms.* Frontiers in neurology, 2019. **10**: p. 282.
60. Figley, S.A., et al., *Characterization of vascular disruption and blood–spinal cord barrier permeability following traumatic spinal cord injury.* Journal of neurotrauma, 2014. **31**(6): p. 541-552.
61. Fehlings, M.G. and L. Sekhon, *Restoration of spinal cord function.* Fardon DF. Garfin SR. OKU Spine, 2002. **2**: p. 483-488.
62. Fehlings, M.G., et al., *Early versus delayed decompression for traumatic cervical spinal cord injury: results of the Surgical Timing in Acute Spinal Cord Injury Study (STASCIS).* PloS one, 2012. **7**(2).
63. Wilson, J.R., et al., *Timing of decompression in patients with acute spinal cord injury: a systematic review.* Global spine journal, 2017. **7**(3_suppl): p. 95S-115S.
64. Oyinbo, C.A., *Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade.* Acta Neurobiol Exp (Wars), 2011. **71**(2): p. 281-99.
65. Michael Fehlings, A., et al., *Burns Essentials of Spinal Cord Injury Basic Research to Clinical Practice.* Denver, CO: Thieme Medical Publishers Inc, 2013.
66. von Leden, R., et al., *Review: CNS ijury and NADPH oxidase: oxidative stress and therapeutic targets.* Journal of neurotrauma, 2016.

67. Baker, D.A., et al., *Neuroadaptations in cystine-glutamate exchange underlie cocaine relapse.* Nature neuroscience, 2003. **6**(7): p. 743-749.
68. De Bundel, D., et al., *Loss of system xc− does not induce oxidative stress but decreases extracellular glutamate in hippocampus and influences spatial working memory and limbic seizure susceptibility.* Journal of Neuroscience, 2011. **31**(15): p. 5792-5803.
69. Rossi, D.J., J.D. Brady, and C. Mohr, *Astrocyte metabolism and signaling during brain ischemia.* Nature neuroscience, 2007. **10**(11): p. 1377-1386.
70. Niciu, M.J., B. Kelmendi, and G. Sanacora, *Overview of glutamatergic neurotransmission in the nervous system.* Pharmacology Biochemistry and Behavior, 2012. **100**(4): p. 656-664.
71. Chaudhry, F., *Lehre KP, van Lookeren Campagne M, Ottersen OP, Danbolt NC, and Storm-Mathisen J.* Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron, 1995. **15**: p. 711-720.
72. Coulter, D.A. and T. Eid, *Astrocytic regulation of glutamate homeostasis in epilepsy.* Glia, 2012. **60**(8): p. 1215-1226.
73. Liu, D., W. Thangnipon, and D.J. McAdoo, *Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord.* Brain research, 1991. **547**(2): p. 344-348.
74. FAROOQUE, M., et al., *Changes of extracellular levels of amino acids after graded compression trauma to the spinal cord: an experimental study in the rat using microdialysis.* Journal of neurotrauma, 1996. **13**(9): p. 537-548.
75. Xu, G.-Y., et al., *Considerations in the determination by microdialysis of resting extracellular amino acid concentrations and release upon spinal cord injury.* Neuroscience, 1998. **86**(3): p. 1011-1021.
76. Liu, D., et al., *Neurotoxicity of glutamate at the concentration released upon spinal cord injury.* Neuroscience, 1999. **93**(4): p. 1383-1389.
77. McAdoo, D.J., et al., *Changes in amino acid concentrations over time and space around an impact injury and their diffusion through the rat spinal cord.* Experimental neurology, 1999. **159**(2): p. 538-544.
78. Johnson, J. and P. Ascher, *Voltage-dependent block by intracellular Mg2+ of N-methyl-D-aspartate-activated channels.* Biophysical journal, 1990. **57**(5): p. 1085-1090.
79. Schrammel, A., et al., *S-nitrosation of glutathione by nitric oxide, peroxynitrite, and• NO/O2•−.* Free Radical Biology and Medicine, 2003. **34**(8): p. 1078-1088.
80. Brown, G.C., *Nitric oxide and neuronal death.* Nitric oxide, 2010. **23**(3): p. 153-165.
81. Yang, J.-L., et al., *The excitatory neurotransmitter glutamate stimulates DNA repair to increase neuronal resiliency.* Mechanisms of ageing and development, 2011. **132**(8-9): p. 405-411.
82. Cheng, B., et al., *Retinoic acid protects against proteasome inhibition associated cell death in SH-SY5Y cells via the AKT pathway.* Neurochemistry international, 2013. **62**(1): p. 31-42.

83. Radi, R., et al., *Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide.* Journal of Biological Chemistry, 1991. **266**(7): p. 4244-4250.
84. Salgo, M.G., G.L. Squadrito, and W.A. Pryor, *Peroxynitrite causes apoptosis in rat thymocytes.* Biochemical and biophysical research communications, 1995. **215**(3): p. 1111-1118.
85. Szabo, C., *Multiple pathways of peroxynitrite cytotoxicity.* Toxicology letters, 2003. **140**: p. 105-112.
86. Landeghem, F.K.V., et al., *Decreased expression of glutamate transporters in astrocytes after human traumatic brain injury.* Journal of neurotrauma, 2006. **23**(10): p. 1518-1528.
87. Beschorner, R., et al., *Expression of EAAT1 reflects a possible neuroprotective function of reactive astrocytes and activated microglia following human traumatic brain injury.* Histology and histopathology, 2007.
88. Chaudhary, K., et al., *Uric Acid - Key Ingredient in the Recipe for Cardiorenal Metabolic Syndrome.* Cardiorenal Medicine, 2013. **3**(3): p. 208-220.
89. Maiuolo, J., et al., *Regulation of uric acid metabolism and excretion.* International Journal of Cardiology, 2016. **213**: p. 8-14.
90. El Ridi, R. and H. Tallima, *Physiological functions and pathogenic potential of uric acid: A review.* Journal of advanced research, 2017. **8**(5): p. 487-493.
91. Chang, B.S., *Ancient insights into uric acid metabolism in primates.* Proceedings of the National Academy of Sciences, 2014. **111**(10): p. 3657-3658.
92. Kratzer, J.T., et al., *Evolutionary history and metabolic insights of ancient mammalian uricases.* Proceedings of the National Academy of Sciences, 2014. **111**(10): p. 3763-3768.
93. Trackey, J.L., T.F. Uliasz, and S.J. Hewett, *SIN-1-induced cytotoxicity in mixed cortical cell culture: peroxynitrite-dependent and-independent induction of excitotoxic cell death.* Journal of neurochemistry, 2001. **79**(2): p. 445-455.
94. MCCARTY, D.J. and J.L. HOLLANDER, *Identification of urate crystals in gouty synovial fluid.* Annals of Internal Medicine, 1961. **54**(3): p. 452-460.
95. Jalal, D.I., *Hyperuricemia, the kidneys, and the spectrum of associated diseases: a narrative review.* Current medical research and opinion, 2016. **32**(11): p. 1863-1869.
96. Hjortnaes, J., et al., *Serum uric acid levels and risk for vascular diseases in patients with metabolic syndrome.* The Journal of rheumatology, 2007. **34**(9): p. 1882-1887.
97. Choi, H.K. and E.S. Ford, *Prevalence of the metabolic syndrome in individuals with hyperuricemia.* The American journal of medicine, 2007. **120**(5): p. 442-447.
98. Sánchez-Lozada, L.G., et al., *Hormonal and cytokine effects of uric acid.* Current opinion in nephrology and hypertension, 2006. **15**(1): p. 30-33.

99. Kanbay, M., et al., *Uric acid in metabolic syndrome: from an innocent bystander to a central player.* European journal of internal medicine, 2016. **29**: p. 3-8.
100. Mazzali, M., et al., *Uric acid and hypertension: cause or effect?* Current rheumatology reports, 2010. **12**(2): p. 108-117.
101. Dehghan, A., et al., *High serum uric acid as a novel risk factor for type 2 diabetes.* Diabetes care, 2008. **31**(2): p. 361-362.
102. Zou, D., et al., *Analysis of risk factors and their interactions in type 2 diabetes mellitus: A cross‐sectional survey in Guilin, China.* Journal of diabetes investigation, 2017. **8**(2): p. 188-194.
103. Verma, R., B. Mishra, and S. Garg, *Osmotically controlled oral drug delivery.* Drug development and industrial pharmacy, 2000. **26**(7): p. 695-708.
104. Langer, R. and N.A. Peppas, *Present and future applications of biomaterials in controlled drug delivery systems.* Biomaterials, 1981. **2**(4): p. 201-214.
105. Duncan, R., *Polymer conjugates as anticancer nanomedicines.* Nature reviews cancer, 2006. **6**(9): p. 688-701.
106. Takakura, Y., M. Hashida, and H. Sezaki, *Lymphatic transport after parenteral drug administration*. 1992: CRC Press: Boca Raton, FL, USA.
107. Kopeček, J., *Smart and genetically engineered biomaterials and drug delivery systems.* European Journal of Pharmaceutical Sciences, 2003. **20**(1): p. 1-16.
108. Inomata, H., S. Goto, and S. Saito, *Phase transition of N-substituted acrylamide gels.* Macromolecules, 1990. **23**(22): p. 4887-4888.
109. Schmaljohann, D., *Thermo-and pH-responsive polymers in drug delivery.* Advanced drug delivery reviews, 2006. **58**(15): p. 1655-1670.
110. Malmsten, M. and B. Lindman, *Self-assembly in aqueous block copolymer solutions.* Macromolecules, 1992. **25**(20): p. 5440-5445.
111. Dušek, K. and D. Patterson, *Transition in swollen polymer networks induced by intramolecular condensation.* Journal of Polymer Science Part A-2: Polymer Physics, 1968. **6**(7): p. 1209-1216.
112. Duncan, R., *The dawning era of polymer therapeutics.* Nature reviews Drug discovery, 2003. **2**(5): p. 347-360.
113. Liechty, W.B., et al., *Polymers for drug delivery systems.* Annual review of chemical and biomolecular engineering, 2010. **1**: p. 149-173.
114. Garg, K. and G.L. Bowlin, *Electrospinning jets and nanofibrous structures.* Biomicrofluidics, 2011. **5**(1): p. 013403.
115. Lee, J.K.Y., et al., *Polymer-based composites by electrospinning: Preparation & functionalization with nanocarbons.* Progress in Polymer Science, 2018. **86**: p. 40-84.
116. Palangetic, L., et al., *Dispersity and spinnability: Why highly polydisperse polymer solutions are desirable for electrospinning.* Polymer, 2014. **55**(19): p. 4920-4931.

117. Torres-Martínez, E.J., et al., *A summary of electrospun nanofibers as drug delivery system: Drugs loaded and biopolymers used as matrices.* Current drug delivery, 2018. **15**(10): p. 1360-1374.
118. Gong, C., et al., *Synthesis and characterization of PEG-PCL-PEG thermosensitive hydrogel.* International journal of pharmaceutics, 2009. **365**(1-2): p. 89-99.
119. Lin, C.-C. and K.S. Anseth, *PEG hydrogels for the controlled release of biomolecules in regenerative medicine.* Pharmaceutical research, 2009. **26**(3): p. 631-643.
120. Kim, B. and N.A. Peppas, *Poly (ethylene glycol)-containing hydrogels for oral protein delivery applications.* Biomedical Microdevices, 2003. **5**(4): p. 333-341.
121. Zhao, X. and J. Milton Harris, *Novel degradable poly (ethylene glycol) hydrogels for controlled release of protein.* Journal of pharmaceutical sciences, 1998. **87**(11): p. 1450-1458.
122. Underhill, G.H., et al., *Assessment of hepatocellular function within PEG hydrogels.* Biomaterials, 2007. **28**(2): p. 256-270.
123. Wona, G. and H. Janik, *Review: Synthetic polymer hydrogels forbiomedical application.* Chem Chem Technol, 2010. **4**(4): p. 297-304.
124. Alconcel, S.N., A.S. Baas, and H.D. Maynard, *FDA-approved poly (ethylene glycol)–protein conjugate drugs.* Polymer Chemistry, 2011. **2**(7): p. 1442-1448.
125. Ansevin, K.D. and B.V. Lipps, *Tissue plate: A new technique for longterm organotypic culture.* In vitro, 1973. **8**(6): p. 483-488.
126. Braschler, U., et al., *A modified roller tube technique for organotypic cocultures of embryonic rat spinal cord, sensory ganglia and skeletal muscle.* Journal of neuroscience methods, 1989. **29**(2): p. 121-129.
127. McKeon-Fischer, K.D., et al., *Poly (3, 4-ethylenedioxythiophene) nanoparticle and poly (ε-caprolactone) electrospun scaffold characterization for skeletal muscle regeneration.* Journal of Biomedical Materials Research Part A, 2015. **103**(11): p. 3633-3641.
128. Xue, J., et al., *Electrospinning and electrospun nanofibers: Methods, materials, and applications.* Chemical reviews, 2019. **119**(8): p. 5298-5415.
129. Rasouli, R., et al., *Nanofibers for biomedical and healthcare applications.* Macromolecular bioscience, 2019. **19**(2): p. 1800256.
130. Islam, M.S., et al., *A review on fabrication of nanofibers via electrospinning and their applications.* SN Applied Sciences, 2019. **1**(10): p. 1248.
131. Huang, Z.-M., et al., *A review on polymer nanofibers by electrospinning and their applications in nanocomposites.* Composites science and technology, 2003. **63**(15): p. 2223-2253.
132. Ondarcuhu, T. and C. Joachim, *Drawing a single nanofibre over hundreds of microns.* EPL (Europhysics Letters), 1998. **42**(2): p. 215.

133. Feng, L., et al., *Super-hydrophobic surface of aligned polyacrylonitrile nanofibers.* Angewandte Chemie International Edition, 2002. **41**(7): p. 1221-1223.
134. Martin, C.R., *Membrane-based synthesis of nanomaterials.* Chemistry of Materials, 1996. **8**(8): p. 1739-1746.
135. Ma, P.X. and R. Zhang, *Synthetic nano-scale fibrous extracellular matrix.* Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials, 1999. **46**(1): p. 60-72.
136. Liu, G., et al., *Polystyrene-block-poly (2-cinnamoylethyl methacrylate) Nanofibers—Preparation, Characterization, and Liquid Crystalline Properties.* Chemistry–A European Journal, 1999. **5**(9): p. 2740-2749.
137. Whitesides, G.M. and B. Grzybowski, *Self-assembly at all scales.* Science, 2002. **295**(5564): p. 2418-2421.
138. Deitzel, J.M., et al., *Controlled deposition of electrospun poly (ethylene oxide) fibers.* Polymer, 2001. **42**(19): p. 8163-8170.
139. Fong, H., *Electrospinning and the formation of nanofibers.* Structure formation in polymeric fibers, 2001.
140. Hekmati, A.H., et al., *Effect of needle length, electrospinning distance, and solution concentration on morphological properties of polyamide-6 electrospun nanowebs.* Textile Research Journal, 2013. **83**(14): p. 1452-1466.
141. Pakravan, M., M.-C. Heuzey, and A. Ajji, *A fundamental study of chitosan/PEO electrospinning.* Polymer, 2011. **52**(21): p. 4813-4824.
142. Cooley, J.F., *Apparatus for electrically dispersing fluids*. 1902, Google Patents.
143. Morton, W.J., *Method of dispersing fluids*. 1902, Google Patents.
144. Formhals, A., *Method and apparatus for spinning (US, 2349950)*. 1944.
145. Harttig, H., *Test element with nanofibers*. 2010, Google Patents.
146. Taylor, G.I., *Disintegration of water drops in an electric field.* Proceedings of the Royal Society of London. Series A. Mathematical and Physical Sciences, 1964. **280**(1382): p. 383-397.
147. Bergshoef, M.M. and G.J. Vancso, *Transparent nanocomposites with ultrathin, electrospun nylon-4, 6 fiber reinforcement.* Advanced materials, 1999. **11**(16): p. 1362-1365.
148. Park, C., et al., *Polymer-single wall carbon nanotube composites for potential spacecraft applications.* MRS Online Proceedings Library Archive, 2001. **706**.
149. Schreuder-Gibson, H., et al., *Protective textile materials based on electrospun nanofibers.* Journal of advanced materials, 2002. **34**(3): p. 44-55.
150. Groitzsch, D. and E. Fahrbach, *Microporous multilayer nonwoven material for medical applications*. 1986, Google Patents.
151. Athreya, S.A. and D.C. Martin, *Impedance spectroscopy of protein polymer modified silicon micromachined probes.* Sensors and Actuators A: Physical, 1999. **72**(3): p. 203-216.

152. Buchko, C.J., et al., *Processing and microstructural characterization of porous biocompatible protein polymer thin films.* Polymer, 1999. **40**(26): p. 7397-7407.
153. Buchko, C.J., et al., *Mechanical properties of biocompatible protein polymer thin films.* Journal of Materials Research, 2000. **15**(1): p. 231-242.
154. Buchko, C.J., K.M. Kozloff, and D.C. Martin, *Surface characterization of porous, biocompatible protein polymer thin films.* Biomaterials, 2001. **22**(11): p. 1289-1300.
155. Laurencin, C.T., et al., *Tissue engineering: orthopedic applications.* Annual review of biomedical engineering, 1999. **1**(1): p. 19-46.
156. Coffee, R.A., *Method for forming material*. 2007, Google Patents.
157. Jin, H.-J., et al., *Electrospinning Bombyx mori silk with poly (ethylene oxide).* Biomacromolecules, 2002. **3**(6): p. 1233-1239.
158. Lee, S.-H., et al., *Design, synthesis and electrospinning of a novel fluorescent polymer for optical sensor applications.* MRS Online Proceedings Library Archive, 2001. **708**.
159. Smith, D. and D. Reneker, *PCT/US00/27737.* 2001.
160. Bölgen, N., et al., *In vivo performance of antibiotic embedded electrospun PCL membranes for prevention of abdominal adhesions.* Journal of Biomedical Materials Research Part B: Applied Biomaterials: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials, 2007. **81**(2): p. 530-543.
161. Xie, J. and C.-H. Wang, *Electrospun micro-and nanofibers for sustained delivery of paclitaxel to treat C6 glioma in vitro.* Pharmaceutical research, 2006. **23**(8): p. 1817.
162. Kim, T.G., D.S. Lee, and T.G. Park, *Controlled protein release from electrospun biodegradable fiber mesh composed of poly (ε-caprolactone) and poly (ethylene oxide).* International journal of pharmaceutics, 2007. **338**(1-2): p. 276-283.
163. Burger, C. and B. Chu, *Functional nanofibrous scaffolds for bone reconstruction.* Colloids and Surfaces B: Biointerfaces, 2007. **56**(1-2): p. 134-141.
164. Hamid, Z.A. and K. Lim, *Evaluation of UV-crosslinked poly (ethylene glycol) diacrylate/poly (dimethylsiloxane) dimethacrylate hydrogel: properties for tissue engineering application.* Procedia Chemistry, 2016. **19**: p. 410-418.
165. Padmavathi, N.C. and P. Chatterji, *Structural characteristics and swelling behavior of poly (ethylene glycol) diacrylate hydrogels.* Macromolecules, 1996. **29**(6): p. 1976-1979.
166. Alcantar, N.A., E.S. Aydil, and J.N. Israelachvili, *Polyethylene glycol–coated biocompatible surfaces.* Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials, 2000. **51**(3): p. 343-351.