Neuroprotective Drug Delivery to the Injured Spinal Cord with Hyaluronan and Methylcellulose

by

Catherine Elizabeth Kang

A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy
Department of Chemical Engineering and Applied Chemistry in collaboration with the Institute for Biomaterials and Biomedical Engineering

University of Toronto

© Copyright by Catherine Elizabeth Kang 2010
Neuroprotective Drug Delivery to the Injured Spinal Cord with Hyaluronan and Methylcellulose

Catherine Elizabeth Kang
Doctorate of Philosophy

Department of Chemical Engineering and Applied Chemistry in Collaboration with the Institute of Biomaterials and Biomedical Engineering

University of Toronto
2010

Abstract

Traumatic spinal cord injury (SCI) is a devastating condition for which there is no effective clinical treatment. Neuroprotective molecules that minimize tissue loss have shown promising results; however systemic delivery may limit in vivo benefits due to short systemic half-life and minimal passage across the blood-spinal cord barrier. To overcome these limitations, an injectable intrathecal delivery vehicle comprised of hyaluronan and methylcellulose (HAMC) was developed, and previously demonstrated to be safe and biocompatible intrathecally. Here, HAMC was determined to persist in the intrathecal space for between 4-7 d in vivo, indicating it as an optimal delivery system for neuroprotective agents to reduce tissue degeneration after SCI. HAMC was then investigated as an in vivo delivery system for two neuroprotective proteins: erythropoietin (EPO) and fibroblast growth factor 2 (FGF2). Both proteins demonstrated a diffusive release profile in vitro and maintained significant bioactivity during release. When EPO was delivered intrathecally with HAMC to the injured spinal cord, reduced cavitation in the tissue and significantly improved neuron counts were observed relative to the conventional delivery strategies of intraperitoneal and intrathecal bolus. When FGF2 was delivered
intrathecally from HAMC, therapeutic concentrations penetrated into the injured spinal cord tissue for up to 6 h. Poly(ethylene glycol) modification of FGF2 significantly increased the amount of protein that diffused into the tissue when delivered similarly. Because FGF2 is a known angiogenic agent, dynamic computed tomography was developed for small animal serial assessment of spinal cord hemodynamics. Following SCI and treatment with FGF2 from HAMC, moderate improvement of spinal cord blood flow and a reduction in permeability were observed up to 7 d post-injury, suggesting that early delivery of neuroprotective agents can have lasting effects on tissue recovery. Importantly, the entirety of this work demonstrates that HAMC is an effective short-term delivery system for neuroprotective agents by improving tissue outcomes following traumatic SCI.
Acknowledgments

Science is an endeavor in which a single person cannot take complete credit for their work; as scientists and engineers, we stand on the shoulders of scientists and engineers who came before us to pave our way. As graduate students, we stand on the shoulders of those who support and guide us through the many years of diligent hard work. To each of you on whose shoulders I have stood, thank you.

I would like to thank Dr. Molly Shoichet for giving me the opportunity to work with her diverse team of engineers, neurosurgeons and chemists in the lab and for having confidence in me through the last several years. Your collaborative efforts truly shaped and provided breadth to my thesis. Thank you to Dr. Charles Tator for encouraging me to tackle difficult problems and providing invaluable guidance throughout my research. I am grateful to Dr. Michael Sefton for not being afraid to challenge me at each committee meeting, and motivating me to improve my depth of knowledge. I appreciate the patience of Dr. Ivan Yeung for allowing me to explore a new technique in a field outside my expertise. I would also like to thank Dr. Grant Allen for your role in my oral examinations, and for providing extracurricular opportunities to further my professional and academic goals. Finally, thank you to Dr. Ravi Bellamkonda for providing an honest evaluation of my thesis work. I am indebted to all of you who have contributed to my experience and my research here at the University of Toronto.

My experience here has been shaped through research with Team Injectable, fun times in the Cluster, and fruitful discussion with the many members of SBERT. More than anyone in Toronto, I want to thank Karyn Ho for her friendship and moral support when I needed it most – I can never pick up my guitar without thinking of you! I am genuinely appreciative to those who went above and beyond to make my time in Toronto more than enjoyable – Douglas Baumann, Jennifer Hudgins, Tina Bhinder, Ryan Wylie, Howard Kim, Yukie Aizawa, and Michael Conrad, and Peter Poon. I could have never called Toronto home without each of you. Also, thank you to those who have made my time here both profitable and productive – Kat Vulic, Ankeeta Tadkase, Yuanfei Wang, Valerie Peng, and Siang Lee. Your dedication to research was a source of motivation for me.

Finally, my deepest gratitude goes to the one who stayed with me through immeasurable heartache, all the while providing immense support – my husband, Peter Kang. I may never be able to adequately express the profound love and appreciation that I have for you. I never could have made it this far without you and I look forward to the days we will spend together as a family with our little OranguKang. Thanks baby Aravis for letting mommy finish her Ph.D. in the first days of your life! Thank you to my parents Jeff and Cathy Clark and to Mei Wan Kang for helping Peter and I in every way you could. Finally, thank you to Veronica Veale for being such a great friend and always knowing what to say to make me feel better.

Catherine E. Kang
# Table of Contents

Acknowledgments .......................................................................................................................... iv  
Table of Contents .......................................................................................................................... v  
List of Tables ...................................................................................................................................... ix  
List of Figures ...................................................................................................................................... x  
List of Appendices ............................................................................................................................ xiv  

1 Background & Introduction ............................................................................................................ 1  
1.1 Rationale ........................................................................................................................................ 1  
1.2 Hypothesis and Objectives ............................................................................................................. 2  
1.3 Anatomy and Physiology of the Human Spinal Cord ................................................................. 3  
1.4 Pathology and Prevalence of Spinal Cord Injury ........................................................................... 5  
1.5 Animal Models of Spinal Cord Injury ............................................................................................ 8  
1.5.1 Measurement Techniques for Spinal Cord Hemodynamics ................................................... 10  
1.5.2 Motor Function Outcome Measures ....................................................................................... 12  
1.6 Treatment Strategies for Spinal Cord Injury Repair .................................................................... 14  
1.6.1 Clinical Trials and Treatments ............................................................................................... 14  
1.6.2 Experimental Neuroprotective Agents ................................................................................... 16  
1.6.3 Routes of Drug Administration ............................................................................................... 20  
1.7 Polymers for Drug Delivery ......................................................................................................... 22  
1.7.1 Hyaluronan ............................................................................................................................. 24  
1.7.2 Methylcellulose ...................................................................................................................... 25  
1.7.3 Poly(ethylene glycol) ............................................................................................................. 27  
1.8 Historical Context and Motivation for this Research ................................................................. 28  

2 Localized Delivery of Erythropoietin with the Hyaluronan-Methylcellulose Blend ............. 31
3.4.1 PEGylation of FGF2 ................................................................. 58
3.4.2 PEG-FGF2 Bioactivity............................................................. 60
3.4.3 FGF2 Release from HAMC In vitro ........................................ 60
3.4.4 In vivo Distribution of FGF2 and PEG-FGF2............................. 61
3.5 Discussion (Penetration of FGF2) ................................................ 64
3.6 Conclusions (Penetration of FGF2) ............................................. 66

4 Live Animal Imaging of Post-Injury Spinal Cord Blood Flow ........... 67
4.1 Abstract (Hemodynamics with FGF2)......................................... 67
4.2 Introduction (Hemodynamics with FGF2).................................... 68
4.3 Materials & Methods (Hemodynamics with FGF2)...................... 72
  4.3.1 HAMC preparation .............................................................. 73
  4.3.2 In vivo Surgical Procedures .................................................. 73
  4.3.3 Computed Tomography Imaging Acquisition with Dynamic Contrast Enhancement ................................................. 74
  4.3.4 Image Analysis.................................................................. 74
  4.3.5 Statistical analysis............................................................... 75
4.4 Results (Hemodynamics with FGF2).......................................... 77
  4.4.1 SCBF and PS Measurements in the Uninjured Spinal Cord......... 77
  4.4.2 Epicenter of Injury (T1/T2) .................................................... 77
  4.4.3 0.75-1 mm Rostral to Injury Epicenter (T1) .............................. 80
  4.4.4 1.8-2 mm Rostral to Injury Epicenter (T1/C7) ......................... 82
4.5 Discussion (Hemodynamics with FGF2)...................................... 84

5 Discussion .................................................................................. 87
5.1 Localized Drug Delivery............................................................. 87
  5.1.1 In vitro Release of Therapeutic Molecules from HAMC .......... 88
  5.1.2 In vivo Delivery from HAMC ................................................ 91
5.2 Neuroprotection Achieved with Localized Delivery ..........................................................96
  5.2.1 Implications of EPO Crossing the BSCB .................................................................97
  5.2.2 Improvements in SCBF and Permeability with FGF2 Delivery ............................98
5.3 PEGylation for Improving Tissue Delivery .....................................................................100
5.4 Spinal Cord Blood Flow Imaging Strategies ...............................................................101
5.5 Achievement of Objectives .........................................................................................103
5.6 Conclusions ................................................................................................................105

6 Limitations and Recommendations for Future Work ......................................................106
  6.1 Extending Delivery Window for HAMC .................................................................106
    6.1.1 Define Mechanism for HAMC Elimination from CNS .....................................106
    6.1.2 Extend In vivo Lifetime of HAMC .................................................................107
    6.1.3 Controlled Release .........................................................................................107
  6.2 Drug Choice Matrix ..................................................................................................108
  6.3 Development of CT for SC hemodynamics ..............................................................109
  6.4 Limitations for Bench to Bedside Translation ............................................................110

References & Bibliography ..............................................................................................112
Appendix 1: Glossary ....................................................................................................137
Appendix 2: Abbreviations .............................................................................................139
Appendix 3: Additional Data ..........................................................................................142
List of Tables

Table 1.1: Secondary injury timeline ................................................................. 6

Table 1.2: Basso, Beattie and Bresnahan (BBB) locomotor score rankings. ................. 13

Table 1.3: Drugs for SCI treatment in clinical or experimental studies indicating potential benefits and limitations ................................................................. 17
List of Figures

Figure 1.1: Cross section of spinal cord showing the multiple layers of protection in CNS (Copyright Michael Corrin) .................................................................................................................................................. 4

Figure 1.2: Illustration of leading models of spinal cord injury (Copyright Michael Corrin) ..... 10

Figure 1.3: Illustrations of drug delivery routes: (A) Intravenous [201], (B) intrathecal bolus via lumbar puncture [202], and (C) implanted minipump (copyright Michael Corrin) ................. 22

Figure 1.4: Chemical structure of hyaluronan (A), methylcellulose (B), and poly(ethylene glycol) (C) ................................................................................................................................................................. 25

Figure 1.5: Gelation of MC caused by heating via formation of hydrophobic junctions .......... 26

Figure 1.6: Injectable polymeric drug delivery system for intrathecal delivery (copyright Michael Corrin) ........................................................................................................................................... 30

Figure 2.1: (A) EPO release from HAMC over 128 h shows fast release within 16 h. (B) Cumulative release of EPO vs. the square root of time demonstrates a diffusion-mediated release profile for the first 75% of EPO released. (C) EPO released from HAMC is 80% bioactive over time relative to cumulative EPO released. (■=Cumulative EPO released; ●=Bioactive portion of EPO released) .................................................................................................................................................. 40

Figure 2.2 (see next page): (A) In vitro mass degradation is the same for labeled and unlabeled HAMC. (B) Brightfield image of a parasagittal spinal cord section with corresponding fluorescent images of HA (Green) and MC (Red) in the IT space immediately after injection. (C) Loss in the area of fluorescence shows that HA degrades faster than MC in the IT space. Scale bars =1mm. (n=3, mean ± standard deviation plotted) ((■)=mass loss of labeled HAMC; ●=mass loss of unlabeled HAMC) ◆=fluorescence loss of HA; ▲=fluorescence loss of MC) 41

Figure 2.3: (A) Parasagittal section of a representative spinal cord of an animal that received IT administration of EPO-loaded HAMC at maximal cavitation stained with LFB/H&E. (B) Comparison of maximal cavity area measured from parasagittal spinal cord sections. (n=4,
mean ± standard deviation; horizontal bars show statistical significance based on a t-test, *p<0.05)

Figure 2.4: Neuron counts observed in the ventral horns caudal to the injury site based on NeuN staining. (n=3, mean ± standard deviation; horizontal bars show statistical significance based on t-test, *p<0.05)

Figure 2.5: Comparison of maximal inflammation area measured with ED-1 immunostaining of macrophages and microglia from parasagittal spinal cord sections. (n=3, mean ± standard deviation).

Figure 2.6: Open field motor scores as assessed by the BBB scale over six weeks. (*shows statistical significance measured by ANOVA; n=9 to 11, data shown as mean ± standard deviation) (◆=IT administration of EPO-loaded HAMC; ■=IT administration of HAMC alone; ▲=IT administration of EPO; ○=IP administration of EPO)

Figure 3.1: (A) Diagram of injection paradigm shows hydrogel spreading following intrathecal injection. Arrows indicate potential directions for protein flux from HAMC into the CSF or into spinal cord tissue. (B) Tissue sampling paradigm for observing depth of dorsoventral penetration. Spinal cord segments of 2 cm spanning the entire rostro-caudal spread of the hydrogel were removed and then sectioned sagittally into 1mm thick sections. The most medial two sections were then sliced longitudinally into 300 μm sections. These 1mm x 300μm x 2cm sections were used to measure the dorsoventral penetration of FGF2 or PEG-FGF2 from the dorsal aspect to the center of the spinal cord.

Figure 3.2: FGF2 with free cysteine groups reacts with PEG-mal to produce single PEGylated FGF2 and double PEGylated FGF2 (with some unreacted, non-PEGylated FGF2).

Figure 3.3: Representative gel electrophoresis shows molecular weight ladder, control FGF2, products of PEG-FGF2 reaction isolated from heparin binding column, and flow through PEG from heparin binding column.

Figure 3.4: Dose dependent activity of FGF2 (white bars) and PEG-FGF2 (black bars) show that bioactivity based on cell density is maintained after PEGylation and subsequent purification. (n=5, mean ± standard deviation are shown).
Figure 3.5: *In vitro* release of FGF2 (□) and PEG-FGF2 (●) from HAMC drug delivery system shows diffusive release within 24 h in almost identical concentrations. (n=5, mean ± standard deviation are shown).  

Figure 3.6: Cumulative comparison shows a greater amount of PEG-FGF2 (●) in the injured spinal cord than FGF2 (□) when delivered from the intrathecal HAMC drug delivery system.  

B At 3 h post-intrathecal injection, higher concentrations of PEG-FGF2 (●) than FGF2 (□) have diffused into the cord. Lines indicate the predicted concentration profiles of PEG-FGF2 (solid) and FGF2 (dotted).  

C At 6 h post-intrathecal injection, drug depletion from the hydrogel results in a plateau of drug in the tissue, but elevated concentrations of PEG-FGF2 (●) are still observed in the spinal cord. Asterisks indicate statistical significance at p<0.05. (n=5, mean ± standard deviation are shown).  

Figure 4.1: (A) One-compartment and (B) two-compartment model of blood flow.  

Figure 4.2: (A) Representative arterial region of interest at T3 where a cross section of the entire rat is shown. The box outlines the inset on right showing anatomical landmarks, including the contrast filled catheter inside the jugular vein, the aorta (used for determining the arterial input function), the trachea, and the intact T3 vertebra. The corresponding arterial input function is shown below the inset.  

(B) A representative region of interest near the injury epicenter (T1/T2), and the corresponding tissue uptake curve below.  

(C) A representative region of interest 0.75-1 mm rostral to epicenter (T1) where a laminectomy was performed and the corresponding tissue uptake curve below.  

(D) A representative region of interest 1.8-2 mm rostral to the epicenter (T1/C7) and the corresponding tissue uptake curve below.  

Figure 4.3: (A) Spinal cord blood flow (SCBF) and (B) permeability-surface (PS) area products nearest to injury epicenter at T1/T2 for: ▲ = FGF2/HAMC; ◊ = aCSF; = HAMC (n= 8, mean ± standard error of the mean are shown; letters indicate statistical differences as compared to pre-injury values where a = aCSF, b = HAMC, c = FGF2/HAMC).  

Figure 4.4: (A) SCBF and (B) PS at 0.75-1 mm rostral to epicenter at T1 for: ▲ = FGF2/HAMC; ◊ = aCSF; = HAMC (n=8, mean ± standard error of the mean are shown; letters indicate statistical differences as compared to pre-injury values where a = aCSF, b = HAMC, c = FGF2/HAMC).  

...
Figure 4.5: (A) Spinal cord blood flow and (B) permeability-surface area products at 1.8-2 mm rostral to epicenter at T1/C7: ▲ = FGF2/HAMC; ◊ = aCSF; ○ = HAMC (n= 8, mean ± standard error of the mean are shown; letters indicate statistical differences as compared to pre-injury values where a = aCSF, b = HAMC, c = FGF2/HAMC; * indicates statistical significance between aCSF and FGF2/HAMC groups at that time). ................................. 83

Figure 0.1: Protein release from HAMC is linearly related to time^{0.5} for >60% release. ........ 142

Figure 0.2: NBQX and solubilized nimodipine (with ethanol) release from HAMC. Inset shows linear relationship with time^{0.5} for >60% of release. ................................................................. 142

Figure 0.3: Nimodipine release from HAMC for solid nimodipine particulates sonicated to 380nm and 900nm. Biphasic release profile was observed where fast release was observed in the first 8 h (solid line = 380nm, dashed line = 900nm) and was likely due to soluble nimodipine fraction saturating HAMC. Slow release was observed for 3 d (small dashed line = 380 nm, large dashed line = 900 nm) likely due to fraction released as surface of particles were solubilized into surrounding media.................................................................. 143

Figure 0.4: Comparison of nimodipine solubility in MC and HA, with different nimodipine particle sizes: (◆) 100-μm nimodipine particles in 0.25 wt% HA; and (□) 100-μm, (▲) 900-nm, and (○) 380-nm nimodipine particles in 7 wt% MC. The upper shaded concentration range indicates nimodipine solubility values achieved in 7 wt% MC, while the lower shaded range indicates aqueous nimodipine solubility reported in the literature. ........................................... 144

Figure 0.5: (A) Longitudinal echogram imaging of the spinal cord where brightness indicates density of the tissue. The bright spots show the dense bones of the vertebrae surrounding the spinal cord; yellow line indicates where the laminectomy was performed to expose the spinal cord. The spinal cord tissue can be observed between the vertebrae only below where the laminae have been removed. (B) Arterial flow from vessel in spinal cord. (C) Venous flow from vessel in spinal cord. ........................................................................................................... 145
List of Appendices

Appendix 1: Glossary ..................................................................................................................137
Appendix 2: Abbreviations ......................................................................................................139
Appendix 3: Additional Data ....................................................................................................142
1 Background & Introduction

1.1 Rationale

The central nervous system (CNS) is the integration center for signals that control bodily functions, and these signals are transmitted through the peripheral nervous system. The CNS is composed of the brain and the spinal cord, both of which are encased in bone as well as 3 semi-impermeable meninges. Additionally, blood vessels in the CNS tightly regulate molecular passage by means of the blood-brain and blood-spinal cord barrier. In traumatic spinal cord injury (SCI), some or all of these layers of protection can be compromised, leading to loss in limb, bowel, and sexual function, also making victims more susceptible to infections [1, 2]. Medical techniques are advanced enough to stabilize SCI patients immediately after injury, giving these patients high life expectancies, yet no standard clinical treatment exists to prevent the devastating loss of function. These patients permanently rely on wheelchairs or other devices for locomotion, and require ongoing medical treatment. The resulting economic impact is enormous on both the family of these patients and the government for medical care, with federal estimates at $10 billion/year in the US and $1 billion/year in Canada [3]. The National Spinal Cord Injury Statistical Center (NSCISC) estimates that there are more than 300,000 patients who have suffered and survived an SCI in the US, with more than 12,000 new cases each year [4], although a recent report suggests more than 1.2 million people live with SCI in the US [5]. The economic and physical hardships that SCI patients endure emphasize the need for developing new treatment strategies to improve the quality of life for these patients.

Current clinical treatment for SCI is intravenous injection of the steroid methylprednisolone sodium succinate (MP). However, very high doses are required to elicit these responses [6, 7], and is no longer standard practice since the limited functional benefit seen in clinical trials has been debated [6]. Many drugs and proteins are under investigation for treatment of spinal cord injury [8], and can generally be classified as either neuroprotective – those which limit neural tissue loss after acute SCI – or neuroregenerative – those which promote neural tissue regeneration after SCI. While intravenous injections are simple and the most widely used method clinically, the high doses required to reach the injury site lead to unwanted systemic side
effects. Additionally, many molecules delivered in this manner either have a short systemic half-life or cannot be transported across the blood-spinal cord barrier. To overcome these limitations, our lab first developed an injectable collagen gel for delivery to the intrathecal space – the space in which cerebrospinal fluid (CSF) cushions the spinal cord – thus circumventing the blood-spinal cord barrier [9]. Subsequently, Gupta et. al. [10] characterized a fast gelling, injectable biopolymer blend of hyaluronan and methylcellulose (HAMC) that improved some of the qualities of the collagen gel, making it more suitable as an intrathecal drug delivery system. In vivo results showed that this physical gel was biocompatible and safe for localized delivery to the injured spinal cord. The research projects described in this document were undertaken to further investigate the potential of HAMC as a delivery system for neuroprotective agents after traumatic SCI.

1.2 Hypothesis and Objectives

The hypothesis investigated in this body of work is that:

Localized delivery of neuroprotective agents with hyaluronan and methylcellulose will mitigate tissue damage after spinal cord injury.

To test this hypothesis, two proteins were investigated as neuroprotective agents – erythropoietin (EPO) and fibroblast growth factor 2 (FGF2). Three objectives were identified for this project as described below, each one followed by a short summary of the supporting work presented in this dissertation.

1. Characterize the in vitro and in vivo release of neuroprotective agents from HAMC

The release mechanism of EPO from HAMC was determined in vitro and is further described in chapter 2. Both in vitro release and in vivo tissue delivery of FGF2 from HAMC was investigated and improved by conjugation of poly(ethylene glycol) to FGF2 and is discussed in chapter 3.

2. Determine the extent of in vivo neuroprotection achieved following localized delivery of neuroprotective agents to the injured spinal cord
Neuron sparing achieved with EPO delivered locally from HAMC was compared to that of other common delivery strategies and is further discussed in chapter 2. Spinal cord blood flow and permeability in the spinal cord were assessed following localized delivery of FGF2 with HAMC and is discussed in chapter 4.

3. Develop a live animal imaging strategy to assess spinal cord hemodynamics after delivery of neuroprotective agents with HAMC

Methods for assessing functional spinal cord blood flow and permeability are limited, thus computed tomography imaging was developed as a technique to assess spinal cord blood flow and permeability following localized delivery of FGF2 with HAMC. This technique and the models employed for it are presented in chapter 4.

1.3 Anatomy and Physiology of the Human Spinal Cord

The CNS is composed of the brain and spinal cord, both of which have multiple layers of protection. The skull and the spinal column are the first layer of protection, providing a rigid encasement for the fragile tissue within. Second, the meninges are membranes that are selectively permeable to only very small molecules – cells, blood, and proteins are inhibited by this fibrous membrane that envelops the CNS. The outermost layers of the meninges consist of dense connective tissue and are known as the dura mater and the arachnoid membrane. The innermost layer closest to the spinal cord is the pia mater, which is a highly vascularized, loose connective tissue membrane. Between the pia and the arachnoid membrane is a space where cerebrospinal fluid (CSF) flows and is known as the subarachnoid space or the intrathecal (IT) space (Figure 1.1) [11]. Finally, the blood-brain and blood-spinal cord barrier (BSCB) restricts the passage of substances into the CNS, allowing tight control of the extracellular environment in the parenchyma.

The CSF cushions the brain and spinal cord from sudden movements, delivers nutrients and removes waste for the CNS. It is an acellular solution that is mainly composed of salts, and is produced in the choroid plexuses of the lateral, third and fourth ventricles of the brain. CSF flows caudally and in a rhythmic pattern contributed by respiration, blood flow, and heartbeat at
a rate varying from -2cm/s to 8cm/s for humans [12]. The fluid circulates in the IT space around the brain and the spinal cord before being reabsorbed via the arachnoid granulations in the superior sagittal sinus and sinuses in the meninges of the spinal nerve roots [13].

![Diagram of spinal cord](image.png)

**Figure 1.1: Cross section of spinal cord showing the multiple layers of protection in CNS (Copyright Michael Corrin).**

There are several different cell types that make up the CNS, each of which tightly regulates and maintains the extracellular environment. Neurons form the information conducting pathways between the brain and the rest of the body. Glial support cells – oligodendrocytes, astrocytes, and microglia – act to support neuronal function, regulate the BSCB, and protect the tissue from infection. Neurons are polarized cells where dendrites receive electrical impulses from preceding neurons, and axons transmit these electrical signals to successive neurons [11]. The electrical signals are generated by changes in ion gradients across the cell membrane, propogating down the axon to the synapse where the signal is passed to the subsequent neuron, and this cycle is repeated until the signal reaches the target site or tissue. Neurons can be up to a meter long, but must transmit these electric impulses very rapidly [11], thus can be insulated by oligodendrocytes which form myelin sheath around axons. The spinal cord is comprised of the white matter, densely populated with axons and dendrites, and gray matter, populated mainly by cell bodies and the synapses which connect neurons [14]. The white and gray matter in the
spinal cord is segregated such that the butterfly shaped gray matter is surrounded by white matter (Figure 1.1). Oligodendrocytes are found mainly in the white matter as anchorage and maintenance cells, and astrocytes regulate the extracellular chemical environment in both the white and gray matters [14]. Microglia are phagocytic cells that are normally dormant in the CNS, but are activated to macrophages after injury, infection, or disease [14]. Ependymal cells that line the central canal of the cord are considered an endogenous precursor cell population with the potential to differentiate into several cell types in the CNS.

Three main cell types regulate the BSCB, acting in tandem to dynamically regulate ion balance, facilitate nutrient transport, and prevent the entry of potentially harmful molecules. Endothelial cells, pericytes and astrocytes comprise what is known as the neurovascular unit [15]. The endothelium and abluminal pericytes that line blood vessels are surrounded by a thick basal laminae composed mainly of collagen IV, and finally astrocytic endfeet ensheath these capillaries. The endothelial cells are tightly connected by tight junction proteins which limit the passage of molecules based on size, and it is thought that the astrocytic endfeet further limit what passes into the parenchyma of CNS tissue [16].

1.4 Pathology and Prevalence of Spinal Cord Injury

The National Spinal Cord Injury Statistical Center (NSCISC) estimates that there are more than 300,000 patients who have suffered from SCI in the US and Canada, with 12,000 new cases each year [4]. A recent report by the Christopher and Dana Reeve Foundation estimates a far higher incidence where over 1.2 million people in the US report being paralyzed due to a spinal cord injury, and nearly 50% of those people reported “a lot of difficulty in movement” or “a complete inability to move” [5]. The economic impact is estimated to be $10 billion/year in the US and $1 billion/year in Canada [3]. According to NSCISC estimates of SCI, 42 % are caused by motor vehicle accidents, 27 % caused by falls, 15 % from acts of violence, 8 % from sports accidents, and 8 % from other causes. More than 81% of SCI patients are male, and the average age at injury is 40 years old [4]. Patients who survive the initial injury can have a normal lifespan, they cannot however, lead a normal life. They and their families are burdened with lifetime healthcare and living expenses of up to $2 million, yet SCI patients often have difficulty integrating into the workforce to allay these costs.
Table 1.1: Secondary injury timeline

<table>
<thead>
<tr>
<th>Acute (hours to ~2 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edema</td>
</tr>
<tr>
<td>Ischemia</td>
</tr>
<tr>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>Free Radical formation</td>
</tr>
<tr>
<td>Hemorrhage</td>
</tr>
<tr>
<td>Inflammation</td>
</tr>
<tr>
<td>Neuronal/Axonal changes</td>
</tr>
<tr>
<td>Demyelination</td>
</tr>
<tr>
<td>Intermediate (days to weeks)</td>
</tr>
<tr>
<td>Microglial responses</td>
</tr>
<tr>
<td>Astrocytic responses</td>
</tr>
<tr>
<td>Vascular responses</td>
</tr>
<tr>
<td>Late (weeks to months/years)</td>
</tr>
<tr>
<td>Wallerian degeneration</td>
</tr>
<tr>
<td>Scar formation</td>
</tr>
<tr>
<td>Cavity formation/elongation</td>
</tr>
</tbody>
</table>

Bunge et. al. [17] has provided a simple classification method for SCI based on gross findings: solid cord injury, contusion/cavitation, laceration, and massive compression. Complete cord transection is a paradigm used in experimental animal models, but is not commonly found in humans [18]. Compression of the cord can be caused by dislocation or fracture of the vertebra in the spine, and is the most common form of SCI [3]. The anatomic level and severity of injury determine the extent of the resulting loss of function. An injury at a given vertebral level impacts all levels below the injury. The cervical levels and the upper thoracic region control the upper extremities and respiration. A patient that survives an SCI in this region may require a respirator and could be a quadriplegic. If an injury is sustained in the lower thoracic region or the upper lumbar region, they may be a paraplegic without bladder or bowel function. Injuries in the lower lumbar region would also affect the posterior of the lower extremities and may necessitate the use of crutches or other devices to assist them in locomotion. Since no clinical treatment is effective at limiting the tissue degeneration or regenerating functional CNS tissue, these patients live with these limitations for the rest of their lives.

The primary insult to the spinal cord in a compression injury is the mechanical trauma that crushes the tissue. While this causes immediate cell death and vascular leakage, these impacts cause subsequent pathological events that continue to occur well after the injury, and this cascade of events is termed the secondary injury. The secondary injury has a significant impact on the outcome of the tissue, thus has serious implications on the potential for functional recovery. The timeline of events discussed here is indicative of the secondary injury process in humans (see Table 1.1). The early phase, beginning within hours of the injury and lasting for ~2 days post-injury, is characterized by edema, hemorrhage, inflammation, vascular changes, as well as neuronal and myelin changes [18]. Edema is fluid accumulation in the extracellular space that leads to swelling, and can be caused by hemorrhage and leakage of plasma fluid into the parenchyma as a result of vascular rupture. The inflammatory response is a complex process
involving cellular and chemical mediators. Neutrophils and other immune regulators begin infiltration within hours of injury, and may persist for 3-4 days. Relative to the neutrophilic response in other tissues this is quite short, but they release free radicals that contribute to tissue damage [19]. Microglia native to the CNS start to become activated macrophages early post-injury, and begin phagocytosis of cellular debris. The necrotic and apoptotic neurons from the primary and secondary injury flood the extracellular space with intracellular ions, neurotransmitters, and other cell regulators, leading to a feedback loop in which further neuronal cell death is propagated. Oligodendrocytes forming the myelin sheath swell after injury and eventually are fragmented, in turn contributing to the hostile extracellular environment. These excess neurotransmitters, reactive oxygen species, ammonia, and free radicals, among others [18] accumulate in the extracellular space, disrupting homeostasis of a much larger region than that affected by the primary injury.

The intermediate phase of the secondary injury occurs within days to weeks after the initial injury [18]. The activated macrophages continue to phagocytose cellular debris, secreting factors that are considered harmful, such as nitric oxide, glutamate and proteases, although they also release protective cytokines, such as interleukin-1β [20] and tumor necrosis factor-α [21]. Several days after the initial insult, astrocytes will undergo hypertrophy and begin to swell. These are considered activated astrocytes, and after 2-3 weeks will begin to expand and send out thick processes, ultimately forming what is termed the glial scar, or the astrocytic scar [18]. This scar was once believed to be a physical barrier for regeneration, but more recent reports have shown that by the time the scar fully forms, regenerating axons should be beyond the scar [18]. Additionally, astrocytes are involved in restoration of the extracellular ionic environment, uptake of neurotransmitters, free radical scavenging, and provision of nutrients, among others [22]. They are also sources of growth factors and neurotrophic compounds which can provide neuroprotection and promote recovery. Within a week after SCI, spontaneous angiogenesis is observed in the spinal cord [18] and restoration of the BSCB begins to occur, which are thought to be regulated by factors secreted from astrocytes [23, 24]. In this intermediate phase, it is evident that while macrophages, astrocytes, oligodendrocytes, and neurons each play an important role in restoring homeostasis within the spinal cord, each of these can also have negative impacts on the tissue. Thus, designing effective strategies for neuroprotection and neuroregeneration that can lead to functional recovery is much more complex than simple
inhibition or down-regulation of a specific cellular response, suggesting the need for a combination drug or protein therapy.

In the late stages of secondary injury, further neuronal death may occur through a process known as Wallerian degeneration. This is characterized by anterograde degeneration of axons and the myelin sheaths, a process which can occur for over a year [18]. The astrocytic scar also is stabilized and the astrocytic processes become tightly interwoven with extracellular matrix proteins such as chondroitin sulfate proteoglycans [25, 26] thereby stabilizing the early effects of the secondary injury such as hemorrhage, edema and cellular debris and leading to a fluid-filled cavity or cyst in the spinal cord. While most factors are cleared from the fluid over time, this fusiform cavity provides no scaffold or substrate for future neural regeneration. This cavity persists for the lifetime of the patient, and in some occasions a pressure filled cyst called a syrinx elongates, causing further damage to the spinal cord [18]. Syrinx formation is often associated with arachnoid or fibrous adhesions that prevent clearance of fluids, and can lead to conditions such as syringomyelia [27]. The early cell death and accumulation of toxic factors which progressively lead to further degeneration emphasizes the importance of neuroprotective strategies. Reducing tissue loss from necrosis and apoptosis may lead to better tissue and functional outcomes [28]. Neuroprotection in the early stages of secondary injury may also lead to an environment that is more conducive to regeneration.

1.5 Animal Models of Spinal Cord Injury

The timeline and the events discussed above are with respect to the pathological changes seen in the human spinal cord. Because the overall goals for drug delivery to the spinal cord are for human use, it is important to understand these conditions. However, for the in vivo testing phase of materials and drugs that will be used in drug delivery systems, animal models are very important and are commonly used. There are several animal models that can be used for testing biocompatibility and efficacy of the DDS, as well as various injury models. Rodent models are the most commonly used for initial animal studies, but studies have also been done in guinea pigs [29, 30], canine [31], feline [32], murine [33], and primate [34, 35] subjects as well. Rats
are the most common small animal model for traumatic SCI because the morphological, biochemical, and functional changes that occur after SCI are similar to those seen in humans, although differences are likely at the molecular level [36-38]. Female rats are preferred because manual bladder expression after SCI is required since this function is affected by SCI at almost all vertebral levels normally tested in vivo.

Four main injury models have been developed to study the mechanisms of SCI: hemisection [39], transection [40], compression [34, 41-43], and contusion [44, 45]. A more complete review of these models can be found in [46], and are illustrated in Figure 1.2. Hemisection models are done by either cutting a dorsoventral or mediolateral section from the cord. These are useful models since the injury site is reproducible and the contralateral or uninjured segment of the cord can be used as a control in some cases. However, the extent of tissue that is removed is difficult to control, sometimes leading to high variability in tissue and functional recovery. The complete transection model, which involves opening the dura and transecting the entire spinal cord, has advantages of certainty about the extent of injury, but the severity of the injury makes animal care more difficult [47]. These two previous models are particularly useful for regenerative strategies because there is little question between spared and regenerating axons. However, compression and contusion models are more relevant to human injuries, and are ideal for studying mechanisms of neuroprotection [35, 42]. Contusion injuries in humans occur from blunt trauma where an external force causes a quick but considerable blow to the cord. Some well established models in rats and mice for contusions are the New York University Impactor [44] and the Ohio State Accelerator [45], where a weight is dropped onto the exposed spinal cord to apply a quick and measurable force. These models have extensive variability between animals unless properly controlled by computer automation [48]. These types of injuries tend to damage the large myelinated axons and spare the smaller axons [49]. Compression injuries, the most common form in humans, are caused by prolonged pressure on the spinal cord tissue producing blockage of blood vessels and axonal conduction [49]. Two models of compression used to induce SCI are subdural balloon inflation [50] and the modified aneurysm clip [34, 41-43]. In the clip compression method, the cord is compressed for 1 min by a clip calibrated to a specific closing force, and is reasonably reproducible. With this model, mechanisms of secondary injury and the effects of neuroprotective drugs can be studied. Since compression injuries are more clinically relevant and the clip compression model has been well characterized [43, 51], all
animal studies discussed in this dissertation were done with female rats using the clip compression method for inducing traumatic SCI.

![Illustration of leading models of spinal cord injury](Figure 1.2: Illustration of leading models of spinal cord injury (Copyright Michael Corrin)).

### 1.5.1 Measurement Techniques for Spinal Cord Hemodynamics

Grossly injured vessels from the primary injury cause immediate hemorrhage and progressive edema in the parenchyma of the spinal cord; both spinal cord blood flow (SCBF) and the BSCB are compromised by these events. The accumulation of plasma constituents in the cord alters the tightly controlled chemical environment of the CNS, and ischemia and hypoxia are ongoing until blood flow is resumed. Breakdown of the BSCB is manifested by permeability to serum proteins within 3 h in areas adjacent to the injury epicenter [52], and SCBF is reduced within 2 h [53]. While hypervascularity can occur after injury to restore blood flow [54], permeability of the BSCB remains quite high even at 28 d post-injury [55]. This quantitative spatial analysis of the BSCB was performed by injecting $[^{14}\text{C}]\alpha$-aminoisobutyric acid intravenously at a time of interest post-injury followed by rapid harvesting of the spinal cord. Segments were then sectioned and optical density of autoradiograms measured densitometrically were used to assess the amount of tracer present in the spinal cord tissue. Another study showed that the BSCB is re-established within 14 d post-injury [52], assessed by injecting horseradish peroxidase into the
bloodstream and analyzing tissue sections with electron microscopy. While these studies were useful to demonstrate the dynamic changes of the BSCB, they illustrate that permeability measurements are highly dependent on the extent of the injury and the size of the molecule used to assess leakage. Recently, magnetic resonance imaging has been utilized to assess permeability, but thus far has been limited to qualitative assessments [56, 57].

SCBF has been measured using several techniques, but most are invasive or limited to select regions or time points. Kety and Schmidt first proposed gas clearance techniques for measuring SCBF, and developed models based on Fick’s principles of diffusion [58, 59]. In these techniques, an inert gas is breathed in through the nose or mouth, and allowed to equilibrate with the tissue of interest. Because most gases are quite permeable to the BSCB, the gas will equilibrate within minutes. The gas flow through the nose or mouth is then stopped, and a probe placed directly into spinal cord tissue measures gas pressures as the gas clears from the tissue or it can also be measured by continuous sampling of uncontaminated venous blood coming from the tissue. Gases that have been used for this purpose are mainly xenon [60, 61] and hydrogen [53, 62]. While this technique allows for repeated measurements while the probe is in place, the probe insertion causes trauma and data is only indicative of local measurements rather than a composite of the whole cross sectional segment of the cord. Radioactive gases or particles have also been used in combination with autoradiography to measure SCBF quantitatively. In these methods, the gas or particulate tracer is abruptly introduced into the arterial blood immediately prior to harvesting the tissue. After sectioning, autoradiographs of frozen tissue are used to measure concentrations of the gas or particulate densitometrically. Trifluoroiodomethane labeled with $^{131}$I was the first to be used with this method [63], but $^{14}$Cantipyrine developed later is more commonplace for these methods [50, 64]. These measurements allow only a single measurement from an animal since tissue harvesting is a necessity, and variability between animals can be problematic. More recently, the Laser Doppler technique has been used, but since it requires the probe to be on the surface of the spinal cord, it is limited to a relatively short timeframe after the animal has undergone the injury or a terminal time point [65]. Permeability has only been assessed quantitatively using histological and immunohistochemical techniques on sectioned tissue samples. While recent studies have attempted to utilize magnetic resonance imaging (MRI) techniques [56, 57], measurements were only qualitative.
Recent advances in MRI and computed tomography (CT) imaging techniques have produced better resolution of tissues than in previous decades. In addition, dynamic contrast enhancement (DCE) with contrast agents injected into the bloodstream has allowed measurements of blood flow and permeability in soft tissues with these techniques [66-74]. CT has been used to investigate both hemodynamic changes in growing tumors [67, 69, 71-74] and cerebral blood flow [67, 73]. These techniques are preferable since they are minimally-invasive techniques and allow repeated measurements to be taken over a relatively large tissue range in the same subject. In these methods, a relatively impermeable tracer, such as iodinated compounds or gadolinium particles are injected into a vein close to the heart while fast acquisition CT or MRI scans are being performed. Two main models are used to mathematically determine both blood flow and permeability parameters from the scans: the modified Toft’s model [75, 76] and the adiabatic tissue homogeneity model [77, 78]. Generally, these are quite similar models, but the adiabatic tissue homogeneity model requires higher signal to noise ratios to generate accurate data. One study of SCBF in humans has been reported using dynamic perfusion CT [66]; however, these have not been replicated in small animal studies. Techniques such as CT and MRI which could be performed in both humans and small animals could prove very beneficial in comparing outcomes between preclinical animal studies and clinical trials of treatment strategies.

1.5.2 Motor Function Outcome Measures

Since animal models are common for testing SCI treatments, several tests have been developed to assess functional recovery following SCI. Because complete high cervical injuries require extensive animal care and often have higher mortality rates, forelimb testing is mainly used for some hemisection models. For complete SCI models which are mainly done in thoracic and lumbar regions, such as the compression model, hindlimb testing is most common. The inclined plane test [79], narrow beam walk [80], grid walk test [81, 82], footprint analysis [83], and the Basso, Beattie, and Bresnahan (BBB) open field locomotor test [84] are primarily used, several of which were tested in both a hemisection and contusion injury model in Metz et. al. [85]. In any SCI model, a variety of tests are often performed, however, the BBB test is most prolific and is often used as a baseline of comparison between studies performed in different laboratories. It is a 0-21 point scale based on anatomical markers and specific locomotion behaviors which are
observed for rats allowed to freely walk in an open field environment. These are summarized in Table 1.2 and further described below.

**Table 1.2: Basso, Beattie and Bresnahan (BBB) locomotor score rankings.**

<table>
<thead>
<tr>
<th>Score</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No observable hindlimb (HL) movements</td>
</tr>
<tr>
<td>1</td>
<td>Slight (≤ 50 %) movement of one or two HL joints</td>
</tr>
<tr>
<td>2</td>
<td>Extensive (&gt;50 %) movement of one HL joint and possible slight movement of one other joint</td>
</tr>
<tr>
<td>3</td>
<td>Extensive movement of two HL joints</td>
</tr>
<tr>
<td>4</td>
<td>Slight movement of all three HL joints</td>
</tr>
<tr>
<td>5</td>
<td>Slight movement of two HL joints and extensive movement of third HL joint</td>
</tr>
<tr>
<td>6</td>
<td>Extensive movement of two HL joints and slight movement of third HL joint</td>
</tr>
<tr>
<td>7</td>
<td>Extensive movement of all three HL joints</td>
</tr>
<tr>
<td>8</td>
<td>Sweeping (rhythmic extension of three HL joints) with no weight support OR Plantar placement with no weight support</td>
</tr>
<tr>
<td>9</td>
<td>Plantar placement with weight support in stance only OR Occasional, frequent or consistent weight supported dorsal stepping and no plantar stepping</td>
</tr>
<tr>
<td>10</td>
<td>Occasional (&gt;5 % and ≤50 %) weight supported steps with no forelimb-hindlimb (FL-HL) coordination (one HL step per FL step, alternating HL steps)</td>
</tr>
<tr>
<td>11</td>
<td>Frequent (51-94 %) to consistent (95-100 %) weight supported steps and no FL-HL coordination</td>
</tr>
<tr>
<td>12</td>
<td>Frequent to consistent weight supported steps and occasional FL-HL coordination</td>
</tr>
<tr>
<td>13</td>
<td>Frequent to consistent weight supported steps and frequent FL-HL coordination</td>
</tr>
<tr>
<td>14</td>
<td>Consistent coordinated plantar stepping, predominant paw position is rotated at initial contact and liftoff; Frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping</td>
</tr>
<tr>
<td>15</td>
<td>Consistent coordinated plantar stepping, no or occasional toe clearance during forward limb advancement, predominant paw position as parallel to body at initial contact</td>
</tr>
<tr>
<td>16</td>
<td>Consistent coordinated plantar stepping, frequent toe clearance, and predominant paw position is parallel to body at initial contact and rotated at liftoff</td>
</tr>
<tr>
<td>17</td>
<td>Consistent coordinated plantar stepping, frequent toe clearance, predominant paw position is parallel to body at initial contact and liftoff</td>
</tr>
<tr>
<td>18</td>
<td>Consistent coordinated plantar stepping, consistent toe clearance, predominant paw position is parallel to body at initial contact and liftoff</td>
</tr>
<tr>
<td>19</td>
<td>Consistent coordinated plantar stepping, consistent toe clearance, predominant paw position is parallel at initial contact and liftoff, tail is down part or all of the time</td>
</tr>
<tr>
<td>20</td>
<td>Consistent coordinated plantar stepping, consistent toe clearance, predominant paw position is parallel to body at initial contact and liftoff, tail is consistently up, and trunk instability</td>
</tr>
<tr>
<td>21</td>
<td>Consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and liftoff, tail consistently up, and consistent trunk stability</td>
</tr>
</tbody>
</table>
Various regions of the scale reflect the severity of injury and the degree of recovery [86]. Generally, the scores from 0-8 focus on simple limb movements that are displayed in the initial stages of recovery, and animals that have an extensive injury, such as transection or massive compression of the spinal cord, often never recover beyond this range. The 9-14 range describes weight support and coordination behavior seen in the intermediate stage of recovery. Animals with moderate compression or contusion injuries often plateau in this range 2-4 weeks post-injury. The 15-20 range encompasses more subtle movements like coordination, foot placement, and tail and trunk posture in walking behavior. This range is useful for assessing locomotor changes after very mild injuries or for assessing the safety of a treatment in uninjured animals. Methods such as the grid walk test are used to examine differences in more fine motor skills, such as coordination in stepping and foot placement. In this type of test, animals are trained to walk across a grid and footsteps and footfalls through the grid indicate improvement in fine motor skills where fewer footfalls suggests better motor control.

1.6 Treatment Strategies for Spinal Cord Injury Repair

Currently, there are no effective strategies for repairing the spinal cord after traumatic injury, but many neuroprotective and neuroregenerative strategies are under development. Some of these involve drug delivery systems [10, 87], cellular delivery systems [88-90], modification of drugs to cross the blood-brain barrier for intravenous delivery [91, 92], scaffolds for promoting axonal outgrowth [93, 94], entubulation of the spinal cord to induce tissue bridge formation [90, 95], and transplantation of adult or fetal neural tissue [96]. The discussion here will focus on clinically tested treatments for SCI and potential neuroprotectants that can be delivered locally with HAMC.

1.6.1 Clinical Trials and Treatments

Immediately following SCI, the priority is stabilizing the vital signs of a patient. The location, degree, and type of injury dictate the type of treatment a patient receives. Strong experimental evidence as well as class II and III clinical data suggest that surgery should be done to remove bone fragments and decompress the spinal cord for acute, non-penetrating SCI [97, 98]. However, this is performed at the discretion of the physician, and recent recommendations
suggest this should be done 8-24 h post-injury [99]. The only drug treatment used is intravenous administration of MP, as previously discussed. The mechanistic rationale for use of this corticosteroid is to reduce edema and ensuing swelling that occurs after primary injury. MP also has a strong antagonistic effect on lipid peroxidation, which occurs extensively after injury [100]. In parallel, MP has been shown to inhibit ischemia, support aerobic energy metabolism, reduce intracellular overload of calcium, and attenuate calpain-mediated neurofilament loss [28]. However, clinical trials of MP showed that high doses were required to benefit patients, yet the functional impact was minimal [101-104]. Concerns surround the small sample size as well as the non-standardized surgical protocols [6, 7], and currently is not used in many Canadian and European countries, although it is still widely used in the United States.

Several other drug treatments using systemic delivery have undergone clinical trials, such as tirilizad mesylate [105], thyrotropin releasing hormone [106], monosialotetrahexosylganglioside-1 (GM-1) [107, 108], naloxone [103, 104], gacyclidine [109], and nimodipine [110]. GM-1 showed faster achievement of peak functional recovery, but did not show an increase in functional improvement. In animal studies, Naloxone demonstrated improved spinal conduction and a reduction of edema, but in clinical trials failed to show therapeutic effects [103, 111]. Tirilizad mesylate possesses antioxidant properties and stabilizes cell membranes by limiting lipid peroxidation [112, 113]. However, it did not show functional benefit over placebo controls in Phase III trials, and is not used as a clinical treatment [105]. Molecules that were tested in small clinical trials were gacyclidine [109], thyrotropin releasing hormone [106], and nimodipine [110], yet none showed significant beneficial results, thus were not further pursued. Recent trials are underway for Cethrin, riluzole, and anti-Nogo A, however, these results are not yet available [114]. The US Food and Drug Administration did not approve the delivery of Anti-Nogo A with minipumps since they can cause scarring and compression of the cord [115], so these studies are currently being done in Europe and Canada only [114]. No clinical trial has shown impairment due to any drug treatment, however, the lack of effective treatment resulting from so many clinical trials underscores the need for more research to develop effective treatment strategies with the goals of improving functional outcome for SCI patients.
1.6.2 Experimental Neuroprotective Agents

Neuroprotective agents are those molecules which have an impact on neuronal survival, directly or indirectly, following an injury or biochemical insult. After traumatic SCI, most cells damaged from the primary injury cannot be spared; however, manipulation of the biochemical events which occur in the secondary injury may allow for greater survival and tissue sparing in the penumbra surrounding the injury. A plethora of literature exists for neuroprotective agents that have been tested in vivo for treatment of SCI, as reviewed in [8, 116]. Table 1.3 summarizes some of these neuroprotective agents, identifying the benefits and limitations of each. This table however, should not be considered an exhaustive list, but only a guide to the molecules that have been published to date. Each molecule listed is classified based on the secondary injury pathway affected, although some may have a variety of actions. Delivery with HAMC was expected to result in the greatest benefit by delivering proteins that possessed several molecular targets and a wide range of beneficial actions in vivo. For this reason both erythropoietin (EPO) and fibroblast growth factor 2 (FGF2) were selected as good candidates to deliver with HAMC; the actions of each are further described in subsequent sections. Localized delivery of EPO with HAMC was investigated in comparison to conventional drug delivery strategies since it is a neuroprotective molecule that crosses the blood-spinal cord barrier. Since FGF2 does not cross the blood-spinal cord barrier, it was investigated with respect to intrathecal controls only. Both proteins provided a unique perspective on the performance of HAMC as an intrathecal drug delivery system.
Table 1.3: Drugs for SCI treatment in clinical or experimental studies indicating potential benefits and limitations

<table>
<thead>
<tr>
<th>Class of drug</th>
<th>Drug</th>
<th>Potential/Perceived Benefit</th>
<th>Potential Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroids</td>
<td>Methylprednisolone Sodium Succinate (MP) [101, 103, 105]</td>
<td>Phase III clinical trials indicate minimal functional improvement; Prevents lipid peroxidation</td>
<td>Functional benefit is limited; High doses increase wound infections, pneumonia, and respiratory problems</td>
</tr>
<tr>
<td></td>
<td>Tirilizad mesylate (TM) [105]</td>
<td>Does not activate glucocorticoid receptors, so may not have negative side effects of MP</td>
<td>Showed no improvement in functional recovery during clinical trials</td>
</tr>
<tr>
<td>Gangliosides</td>
<td>GM-1 [107, 108]</td>
<td>Prevents lipid peroxidation and may enhance neuronal survival</td>
<td>Only improved recovery for lower extremities, but effect was not significant for higher SCI</td>
</tr>
<tr>
<td>Opiate blockers</td>
<td>Naloxone [103, 104, 117]</td>
<td>Improves spinal cord conduction and reduces edema</td>
<td>Failed in clinical trials to show therapeutic benefit and functional recovery</td>
</tr>
<tr>
<td>Glutamate receptor antagonists</td>
<td>NBQX [118]</td>
<td>Attenuates neurotoxicity, and has been shown to be safe in vivo</td>
<td>Large doses required to provide neuroprotection can globally affect nervous system</td>
</tr>
<tr>
<td></td>
<td>Gacyclidine [109, 119, 120]</td>
<td>Improved functional, histological and electrophysiological status after contusion</td>
<td>Long term functional benefit was lacking in clinical trials; Global affect on nervous system</td>
</tr>
<tr>
<td></td>
<td>Taurine [121]</td>
<td>Modulates intracellular calcium homeostasis; prevents neurotoxicity in vitro</td>
<td>Globally affects glutamate receptors in the nervous system</td>
</tr>
<tr>
<td></td>
<td>mGluR antagonists [122]</td>
<td>Decreases excitotoxicity; Improved locomotor function and spares gray matter</td>
<td>Require spinal delivery for beneficial affect</td>
</tr>
<tr>
<td>Ca$$^{2+}$$ channel blockers</td>
<td>Nimodipine [110]</td>
<td>Prevents excitotoxicity and vasospasm induced ischemia</td>
<td>Clinical trials failed to show neurological benefit</td>
</tr>
<tr>
<td>Sodium channel blockers</td>
<td>Tetrodotoxin [123]</td>
<td>Reduces excitotoxicity from ion influx; Axonal loss attenuated after contusion in rodents</td>
<td>Global affects on nervous system for intravenous administration</td>
</tr>
<tr>
<td></td>
<td>Riluzole [124, 125]</td>
<td>Reduces excitotoxicity from ion influx; White and gray matter sparing seen after contusion in rodents; approved by FDA for ALS therapy</td>
<td>Global affects on nervous system for intravenous administration</td>
</tr>
<tr>
<td>Calpain antagonists</td>
<td>CEP-4143 [126, 127]</td>
<td>Prevents destabilization of cytoskeleton and myelin protein catabolism; In vivo tests show decreased neurofilament breakdown and improved locomotion</td>
<td>Requires invasive subdural delivery to show benefit</td>
</tr>
<tr>
<td>Immune blockers</td>
<td>Cyclosporin-A [128]</td>
<td>Suppresses the immune system, prevents lipid peroxidation better than MP; reduces demyelination and neuronal cell death and improves motor outcomes</td>
<td>Globally inhibits immune system and could cause increased infections and complications</td>
</tr>
<tr>
<td></td>
<td>Anti-Integrin mAbs [129, 130]</td>
<td>Prevents leukocyte and neutrophil tethering and infiltration; Enhanced neurological function after intravenous administration</td>
<td>Globally inhibits immune system and could cause increased infections and complications</td>
</tr>
<tr>
<td></td>
<td>Minocycline [131, 132]</td>
<td>Antibiotic could decrease inflammation; Decreases oligodendrocyte apoptosis and microglia activation, reduce lesion size and improve neurologic deficit</td>
<td>Reduced microglia activation may not be beneficial because macrophages phagocytose debris and secrete growth promoting factors</td>
</tr>
<tr>
<td>Polymers</td>
<td>Polyethylene glycol (PEG) [133, 134]</td>
<td>Dehydrates membranes and prevents lipid peroxidation; Has shown functional benefit with severe SCI models</td>
<td>Inappropriate neuronal connections may form since PEG is a fusogen</td>
</tr>
</tbody>
</table>
### Proteins and peptides

<table>
<thead>
<tr>
<th><strong>Proteins and peptides</strong></th>
<th><strong>Anti-NOGO-A [135-137]</strong></th>
<th><strong>Targets myelin growth inhibitors; Prevents demyelination and promotes axonal survival</strong></th>
<th><strong>Very specific to oligodendrocytes, so would be best in combination with other neuroprotective agents</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Basic Fibroblast Growth Factor (bFGF/FGF-2) [87, 138, 139]</strong></td>
<td><strong>Angiogenic factor; Has been co-delivered with EGF to stimulate proliferation of ependymal cells</strong></td>
<td><strong>Has not been extensively studied in spinal drug delivery; may cause tumor formation</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Brain Derived Neurotrophic Factor (BDNF) [140]</strong></td>
<td><strong>Promotes survival of sensory and motor neuron populations; Promotes axonal regeneration</strong></td>
<td><strong>Controversial because other labs report no neuro-protective or regenerative affects</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Epidermal Growth Factor (EGF) [87, 141]</strong></td>
<td><strong>Stimulates ependymal cell proliferation; Co-delivery with bFGF may stimulate differentiation</strong></td>
<td><strong>Used mainly for stimulating differentiation of neural stem cells</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Erythropoietin (EPO) [49, 142]</strong></td>
<td><strong>Reduces lipid peroxidation, inflammation and apoptosis; Increased functional benefit and reduced lipid peroxidation in vivo</strong></td>
<td><strong>May increase hematocrit when administered systemically which can amplify SCI</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Neurotrophin-3 (NT-3) [143, 144]</strong></td>
<td><strong>Mediate apoptosis in development; Promotes regeneration of axons after SCI</strong></td>
<td><strong>Optimal affects seen with long term delivery; cannot cross BSCB</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Thyrotropin releasing hormone (TRH) [106, 145]</strong></td>
<td><strong>Antagonizing effect on endogenous opioids, platelet activating factor, and excitatory amino acids; Promising results seen for clinical trials</strong></td>
<td><strong>Clinical trials showed improved recovery, but had small patient population and lacked long term data</strong></td>
</tr>
<tr>
<td></td>
<td><strong>z-VAD-fmk Tripeptide caspase inhibitor [146, 147]</strong></td>
<td><strong>Caspase-3 inhibition prevents apoptosis when administered topically on dura following SCI; May show increased benefit with co-delivery of calpain inhibitors</strong></td>
<td><strong>Some studies showed benefit only with pre-treatment of drug, others showed no benefit after SCI</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Rho pathway antagonists Y-27632 [148, 149]</strong></td>
<td><strong>Rho inhibition promotes axon sprouting; demonstrated axonal sprouting on inhibitory substrates when present</strong></td>
<td><strong>Regenerative effects are greater for C3 transferase</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Cethrin (C3 transferase with transport sequence) [150]</strong></td>
<td><strong>Promotes axon sprouting, improves blood flow to ischemic regions; Phase I clinical trial for extradural administration was completed</strong></td>
<td><strong>Delivered epidurally from fibrin, limiting amount diffusing into spinal cord</strong></td>
</tr>
</tbody>
</table>

### 1.6.2.1 Erythropoietin

Erythropoietin is a highly glycosylated 30kDa hormone that acts as the primary regulator of red blood cell production in mammals [151]. The peptide moiety is 165 amino acids with 4 glycosylation sites; the average carbohydrate content is ~40%, which plays an important role in its stability, solubility and biological activity [152]. In addition to its hematopoietic properties, it also plays a role in central nervous system development [153-155]. Recent reports show that EPO and its receptors are upregulated in the brain and spinal cord within minutes to hours after an ischemic injury [156, 157]. The exact neuroprotective mechanisms of EPO are unclear; most studies focus on its role in limiting ischemia in the CNS, but some studies show that it has neurotrophic properties as well [158, 159]. In cultured rat cortical neurons, EPO has been shown...
to reduce hypoxic [160] and glutamate [155] induced cell death. Tissue loss is decreased when EPO is administered following ischemic damage [161], and it is thought to reduce inflammation [162, 163] and spare white matter tracts in the spinal cord [49, 164]. EPO is thought to have a proliferative effect on both oligodendrocytes and astrocytes, which potentially have an indirect effect on sparing neurons after ischemic injury [156, 159, 165, 166]. The proliferative and antiapoptotive effects of EPO are thought to act through the JAK2/STAT3 and the PI3K/AKT activation pathways[158]. Positive functional recovery has been observed in stroke models following intravenous EPO administration, which is attributed to the neuroprotective effects of the glycoprotein which has been shown in hypoxic and ischemic neonatal and adult rat brains [167-170]. In ischemic spinal cord injury, EPO delivered systemically reduced apoptosis of motor neurons[161]. In a compressive SCI model, increased motor score and reduced ischemic injury was observed [171], supported by another study with contusive SCI [49], where significant functional recovery was observed. These studies showed evidence that localized delivery of EPO would limit tissue damage and achieve significant neuroprotection after SCI.

Since EPO can cross the BSCB [172], it was an ideal neuroprotective molecule to test with the HAMC delivery system to determine if localized delivery would be more beneficial than systemic. Additionally, previous studies in the spinal cord showed that multiple systemic injections of EPO was not better than a single immediate injection, suggesting that the neuroprotective actions occurred very early in the secondary injury phase [49], which was an optimal time frame for delivery from HAMC.

1.6.2.2 Fibroblast Growth Factor 2

Fibroblast growth factor 2 (FGF2) is a protein that exists in several forms of 18, 22, and 24 kDa, all of which are highly regulated in vivo [173]. The 18 kDa FGF2 is the most ubiquitous, and sequence homology is very high (>90%) across a wide range of species [174]. It contains a large number of basic residues (pI=9.6) and 4 cysteine residues, none of which participate in disulfide bonding, unlike most other proteins [175, 176]. It has 2 binding sites for FGF receptors (which bind to other FGF family proteins as well) and also has a high binding affinity for heparin and heparin sulfate proteoglycans [177]. Heparin acts to stabilize the tertiary structure of FGF2 and enhances its bioactivity [178, 179]. FGF2 is best known for its trophic properties in promoting
angiogenesis [180-182], as well as the capacity for promoting differentiation of stem cells [183]. It is expressed in the CNS, and following brain injury [184] and SCI [185, 186], it is upregulated although still tightly regulated [173]. FGF2 has also been shown to be neuroprotective [187, 188] and produce functional recovery in experimental models of SCI [138, 139]. It stimulates endogenous ependymal cells to proliferate when co-delivered with EGF into either the ventricles [189] or into the intrathecal space [87]. Ex vivo experiments show that it can modulate the number of blood vessels in the CNS, and induce expression of tight junction proteins, suggesting that it might reduce permeability of the blood-brain barrier in CNS lesions [190, 191]. FGF2 has also been used to limit ischemic CNS injuries [187, 192], and has been tested clinically for the treatment of stroke [193]. Because this molecule is so ubiquitous and has diverse effects, a single mechanism of action is unlikely. It is thought to promote angiogenesis primarily by inducing the proliferation of endothelial cells, and also by upregulating the expression of vascular endothelial growth factor (VEGF) [194]. FGF2 acts synergistically with platelet derived growth factor to induce vessel maturation [195]. It is thought to reduce permeability of the blood-brain barrier by upregulating tight junction proteins that limit passage of molecules across the endothelium in the brain [190, 191].

These studies suggest that FGF2 will provide neuroprotection, stimulate endogenous stem cells in the ependyma, and promote angiogenesis, perhaps limiting the detrimental effects of secondary injury. Notwithstanding these obvious benefits, FGF2 does not cross the intact blood-brain barrier [196], and is known to be mitogenic and upregulated in cancer [182], reflecting the limitations of systemic administration [197]. Thus FGF2 was an excellent choice for local delivery using HAMC after SCI.

1.6.3 Routes of Drug Administration

Traditional routes of drug delivery include intravenous bolus injections and oral delivery. Oral delivery is disadvantageous for SCI since it must first be absorbed through the gastrointestinal tract into the bloodstream, thus extending the time before treatment can reach the injury site. Intravenous delivery is a simple method (Figure 1.3), but has several major limitations. Large doses are required so that an adequate dosage will reach the target tissue, potentially causing negative systemic side effects or toxicity to other tissues. Many drugs have short half-lives in
blood, and thus must be infused continuously. Additionally, the BSCB is a selective barrier that normally keeps drugs and proteins out of the CNS, and although it is compromised at the site of injury, several studies have shown that blood flow is severely decreased SCI, as previously discussed. Systemic delivery is therefore not optimal for SCI drug treatments.

Localized spinal drug delivery has obvious benefits, but does not lack limitations. Epidural delivery, into the soft tissue between the vertebral organs and the dura surrounding the spinal cord, can be administered much closer to the site of injury. Toxicity and side effects are reduced, but transportation across the meninges and through the pulsating CSF is still required to reach the tissue. Intrathecal and intramedullary methods are much closer to the site of injury and do not require passage across the BSCB, but are more invasive (Figure 1.3). Intramedullary methods involve injections directly into the cord, which could cause more damage to the injured cord [198]. Osmotic mini-pumps have been used for intrathecal delivery into the CSF, and have shown some promising results for delivery of analgesics (Figure 1.3) [199]. Unfortunately, the implanted catheters can compress the spinal cord and are prone to infection since the dura may not seal around the catheter insertion site. Additionally, the catheters can cause scar formation in the intrathecal space, leading to catheter blockage, thus impeding drug flow [115]. Both intrathecal and intramedullary delivery are invasive since the dura must be breached, potentially resulting in long term leakage of CSF which causes massive headaches and pain. However, intrathecal delivery may be an optimal route for delivery of some neuroprotective agents since they can be delivered as close to the injury as possible without impinging on the injury site itself and have the least systemic side effects. For acute SCI patients undergoing decompressive surgery, the dura is exposed at the site of injury, providing a unique opportunity for treatment in this manner. Intrathecal delivery provides direct contact with the spinal cord tissue, and only requires the drugs to traverse the pia mater, which is more permeable than both the dura and arachnoid mater [200].
1.7 Polymers for Drug Delivery

Polymers, composed of identical repeating units, have emerged as useful materials for drug delivery because their properties can be tailored for biological applications. They can act as reservoirs for biomolecules and release can be controlled with specific material modifications. Polymers can also be used to stabilize proteins and prolong the half-life of molecules or enhance their distribution through tissue. Because polymers can have very different properties, such as stiffness, porosity, and water content, drug delivery systems can vary widely from solid reservoir systems [115], to sol-gel systems [203], films or wafer systems [204-206], or injectable flow systems [9, 10, 87]. Although polymers are not without limitations, such as material-tissue interactions or immunogenic responses, they are optimal biomaterials for drug delivery.

Drug release from polymer systems can be achieved through many approaches. Bulk drug release that is diffusion mediated is the simplest method. Degradation mediated release can be achieved by either entrapping a drug in a polymer with a mesh size smaller than the drug [207], or by incorporating the drug or protein of interest into the backbone of the material or as a graft.
Degradation controlled systems generally have potential for longer delivery than diffusion mediated systems, where drug can diffuse through the polymer network; however, degradation in vivo can be too slow and degradation products can have toxic side effects [19]. Micro- and nano-particles have been widely developed for drug delivery to slow the rate of release for long time periods [19]. Particles can also be incorporated into polymer networks to achieve extended delivery of molecules [209], and mechanisms for release include diffusion, matrix or bulk degradation, and surface erosion [210].

Polymer systems can be crosslinked systems, physically interacting systems, or interpenetrating networks. Hydrogels are crosslinked hydrophilic polymer networks that contain high water content, and are formed by physical or chemical crosslinks. Physically-crosslinked hydrogels are less stable than covalently crosslinked hydrogels, and generally have faster degradation rates. Physical interactions include hydrogen bonding, hydrophobic junctions, and electrostatic charge interactions [19], making these hydrogels ideal for minimally invasive injection techniques [10, 211] or environmentally responsive to temperature or pH changes [19, 212]. Chemically-crosslinked hydrogels can be formed in situ through chemical techniques such as Michael-type addition reactions [213], or through UV photopolymerization [214] at or just prior to the time of implantation. However, chemical reactions such as these can cause proteins to be crosslinked into the gel structure, and crosslinking and initiating components can also have toxic affects [215]. Since hydrogel properties are easily controlled, they are excellent space-filling materials for irregularly shaped gaps or cavities while minimizing the invasiveness of implantation.

Natural polymers, which include proteins and polysaccharides, are those that have biologic origin and have the advantage of being similar to the natural biologic environment. Because of this, they are often degraded by naturally present enzymes, and degradation products are metabolized through physiological mechanisms [19]. However, since they are source specific, they can produce a significant immunogenic response, although this can be modulated by identification and modification of antigenic determinants [19]. Synthetic polymers can be less immunogenic than natural polymers and are generally easier to chemically manipulate for specific applications. The most common biomedical hydrogels include some derived from natural sources, such as chitosan, collagen, hyaluronan, agarose, alginate, dextran, and fibrin; as well as synthetic polymers, such as polyesters, poly(acrylic acid), and their derivatives like poly(ethylene glycol) (PEG), poly(2-hydroethyl methacrylate) (PHEMA) and poly(N-isopropyl
acrylamide) (pNiPAAm). Poly(lactic acid) and polyglycolide are synthetical polyesters with unique biomedical properties since lactic acid is produced within the body and therefore has no immunogenicity [216]. PEG is a material that has been approved by the FDA for several medical applications due to its biocompatibility and is used as coatings for nanoparticles or in micelles to minimize immune detection. It has also been used to modify proteins to increase the systemic half-life or biodistribution into tissues [217]. Clearly, the biomedical application for which a material is needed will influence the choice of a natural or synthetic polymer. The following sections describe in more detail the three polymers that were used for various aspects of this research.

1.7.1 Hyaluronan

Hyaluronan (HA) is a naturally-derived polysaccharide composed of the repeating disaccharide D-glucouronic acid β(1-3) N-acetyl-D-glucosamine. It is found in the extracellular matrix of many tissues, including the CNS, and is a main constituent of synovial fluid [218]. The linear chains range from 10-15,000 disaccharide units, and form random coils that are highly hydrated and able to form entangled molecular networks [219]. At low strain frequencies, the molecules are capable of aligning parallel to the direction of stress and flow, dissipating energy as viscous flow and heat. At high strain frequencies, the molecules cannot adjust configurationally, and act as elastic solids, which is why they are widely used as treatments for osteoarthritis and tissue augmentation [220]. These shear thinning properties that allow the molecular chains to align under force make HA an optimal material for an injectable gel because the molecular weight of the polymer does not break down from the injection force.

Although concern surrounds natural polymers because of immunogenic affects, HA promotes wound-healing by reducing inflammation and minimizing tissue adhesion and scar formation. HA has differing properties dependent on its molecular weight where high and low molecular weight references are generally thresholded at 3-6 x10^6 Da [220]. Low molecular weight HA can stimulate angiogenesis [221], but can also increase the macrophage proliferation rate [222]. High molecular weight HA at concentrations greater than 1mg/ml decreases proliferation of and increases apoptosis of macrophage in vitro [222]. Hyaluronan has found widespread use because of these non-immunogenic and biocompatible properties [218].
Hyaluronan is degraded enzymatically by hyaluronidase, and more quickly in an inflammatory environment [223]. Degradation can also be mediated by chain diffusion since there is little interaction between the long MW chains. Chemical modification of HA can occur at the carboxyl or hydroxyl groups, or with the amino group once the N-acetyl group is removed (Figure 1.4) [220]. Chemical modification of HA can be accomplished using relatively simple methods, and depending on the modification, can be used to modulate the properties of the material. Unlike many other natural polymers, HA alone does not form a gel, however, this facilitates blending it with other polymers. The shear thinning property of HA makes it optimal for injectable applications, while the ease and diversity of modification sites allow drug delivery properties to be tailored.

![Chemical structure of hyaluronan (A), methylcellulose (B), and poly(ethylene glycol) (C)](image)

**Figure 1.4: Chemical structure of hyaluronan (A), methylcellulose (B), and poly(ethylene glycol) (C)**

### 1.7.2 Methylcellulose

Methylcellulose (MC) is a carbohydrate that is derived from cellulose (Figure 1.4). When degree of methylation is between 1.4 and 1.9, methylcellulose has inverse thermal gelling properties, causing it to gel upon a temperature increase [224]. Water molecules form cagelike structures around the hydrophobic moieties of MC at low temperatures. Upon a temperature increase, these cagelike structures break, exposing the hydrophobic methyl groups which then form
entropically favored hydrophobic junctions (Figure 1.5) [225]. In water, MC can form a weak gel at physiological temperatures (37°C), but salt solutions decrease the gelation temperature, thus increasing the stability of the gel at physiological temperatures [226]. This phenomenon occurs because water molecules associate with salt molecules more readily than the hydrophobic sugar units of MC, thus reducing its solubility. Although MC is not enzymatically degraded, MC chains dissolve over time, thereby breaking the gel. Cellulose based membranes have been shown to activate the complement pathway in the immune system [227-229], although this response is lowered for modified cellulose structures [230, 231]. Despite the concern of activating the immune system, MC was used as a scaffold for experimental traumatic brain injury in vivo, and showed good biocompatibility over a two week time span [232, 233]. It has also been used for peripheral nerve scaffolds and did not show adverse pathological reactions over 8 weeks [232, 233]. These data support the use of MC in biomedical applications, particularly for nervous tissue.

![Figure 1.5: Gelation of MC caused by heating via formation of hydrophobic junctions](image)

Figure 1.5: Gelation of MC caused by heating via formation of hydrophobic junctions
Blending HA and MC forms a viscous material at room temperature that forms a physically crosslinked hydrogel when heated. When prepared in the salts that comprise the CSF, a 2% HA and 7% MC formulation is a hydrogel at physiological temperatures [10]. The HA acts as a thickener for the gel yet maintains its molecular weight upon injection since it is shear thinning. These properties of HA make the solution more viscous, thus retaining the solution at the site of injury during the gelation process. HA also has a “salting out” effect on MC, causing gelation to occur more rapidly since the hydrophilic hydroxyls of HA draw water molecules away from the hydrophobic MC chains. The MC component forms the physical crosslinks that produce the hydrogel structure. These properties make this blend of HAMC is injectable through a small needle (30 G) for use as a minimally invasive intrathecal delivery system for local administration of drugs or proteins.

1.7.3 Poly(ethylene glycol)

Poly(ethylene glycol) is a hydrophilic, nonionic polyether with a narrow polydispersity. PEG is unique in that it is capable of dissolving in both organic and aqueous solvents, and it can bind 2-3 water molecules per ethylene oxide unit [234]. The binding of water and the flexibility of the backbone chain have been postulated as reasons for the unique biomedical properties of PEG. The process by which PEG is covalently conjugated to peptides or proteins is known as PEGylation. This process has been used to improve molecular stability by increasing resistance to proteolysis [235], and reducing immunogenicity by shielding immunogenic binding sites [236]. PEG can decrease tissue clearance of proteins [237], thus enhancing its penetration into tissue. It has been shown to increase the biologic half-life of several molecules and also enhance distribution in tissues [238]. The distribution of PEGylated proteins in tissue depends on both the physico-chemical factors of the PEG-protein conjugate and the physiological/anatomical factors of the host tissue. Since PEG can be functionalized with a variety of reactive groups, it can be attached to proteins using various chemistries, which are reviewed in [234]. In this work, PEG was used to improve the diffusion of FGF2 into injured spinal cord tissue [239], as discussed in detail in chapter 3.
1.8 Historical Context and Motivation for this Research

Injectable polymers are excellent materials to utilize for localized drug delivery after SCI. The intrathecal space is irregularly shaped, but is an optimal site for drug delivery since this route circumvents the BSCB without causing damage to the cord. Additionally, a very small insertion site is highly desirable to minimize a breach in the dura and leakage of CSF. Since the dura is exposed after decompressive surgery, this offers a unique clinical window for this type of treatment after SCI. Figure 1.6 illustrates an injectable system for intrathecal drug delivery, which was first developed by Jimenez Hamann et. al. [9], consisting of a viscous collagen solution that was injected into the intrathecal space. This collagen gel was used to co-deliver recombinant human epidermal growth factor and FGF2 [87]. Compared to bolus injections, the epidermal growth factor penetrated deep into the injured spinal cord and was found to be present for up to 6 h within the cord. FGF2 was only detected on the outer periphery of the tissue, likely because it is tightly regulated in vivo through extracellular matrix binding, thus inhibiting its diffusion into the tissue. Nonetheless, these experiments verified that an injectable polymeric drug delivery system could achieve the goals of localized delivery, and resulted in better tissue penetration than a transient injection into the CSF of diffusible proteins. However, the collagen gel was not entirely biocompatible for this purpose since it is a cell adhesive material, which led to cellular build-up around the material.

Subsequently, Gupta et. al. [10] developed an injectable blend of hyaluronan and methylcellulose for this purpose, improving upon the initial design of the collagen gel. The design criteria for this material was: (1) Injectable through a 30 gauge needle, (2) Fast gelling to facilitate localized drug delivery, (3) Biodegradable to avoid the need for removal, (4) Minimally swelling to not further compress the injured cord, (5) Non-cell adhesive to prevent scar formation, and (6) Biocompatible. In vitro work showed that HAMC met all of the first 5 design criteria [10], and an in vivo study was done to test the biocompatibility. This biocompatibility/safety study was done in both uninjured and injured rats to determine any potential impact this would have on tissue recovery and motor function behavior. The in vivo results using the clip compression SCI showed that compared to a control injection of artificial CSF intrathecally, HAMC injected intrathecally did not significantly increase the lesion volume or the number of apoptotic cells, and in fact, showed a reduction in the inflammatory response following injury [10]. Behavioral testing with the BBB locomotor scale showed animals that received HAMC performed better
than aCSF controls 1 week following injury and performed similarly from 2-4 weeks after injury. Uninjured animals that received either aCSF or HAMC intrathecally did not show any loss of function over the same period. These results suggested that HAMC was a safe and biocompatible intrathecal drug delivery system.

The research described herein describes the research performed to further characterize HAMC as a minimally invasive, intrathecal drug delivery system, as well as build on the existing framework of HAMC to improve delivery of therapeutic agents. Initially, we studied the \textit{in vivo} degradation properties of HAMC to determine the longest period of delivery that could be achieved with this promising drug delivery system. The neuroprotective molecule EPO, which can cross the blood-brain barrier, was delivered with HAMC and compared to traditional routes of delivery, specifically, an intraperitoneal (systemic) injection and intrathecal bolus injection. This study was done to determine if localized delivery can improve upon these delivery routes with respect to tissue and functional recovery, as well as to assess the potential of EPO as a neuroprotective agent. Chapter 2 describes this work in detail, and has been published in Tissue Engineering [240]. Following this work, PEG was conjugated to FGF2 to improve its diffusion into spinal cord tissue, since previous work with the collagen gel showed that FGF2 did not diffuse into tissue. Both FGF2 and PEG-FGF2 were delivered with HAMC in the intrathecal space, and their diffusion pattern into injured spinal cord tissue was elucidated using ELISA detection of serially digested tissue samples. This study was done to improve the delivery of FGF2 to the injured spinal cord and also understand the diffusion characteristics of proteins delivered from HAMC in the intrathecal space. Chapter 3 describes the results of this work, and has been accepted for publication in the Journal of Controlled Release [239]. Finally, the therapeutic benefit of FGF2 delivered with HAMC was studied with respect to the hemodynamic changes that occur following SCI. Since the methods used to study hemodynamic properties require large animal numbers, a new method utilizing dynamic CT was developed for this purpose. Both this new method and the impacts of FGF2 delivered from HAMC are discussed in a manuscript that is in review for submission to the Journal of Neurotrauma [241], and is described in Chapter 4. The entirety of this dissertation describes the use of HAMC as an intrathecal drug delivery system for neuroprotective agents after acute SCI.
Figure 1.6: Injectable polymeric drug delivery system for intrathecal delivery (copyright Micheal Corrin).
2 Localized Delivery of Erythropoietin with the Hyaluronan-Methylcellulose Blend

A New Paradigm for Local and Sustained Release of Therapeutic Molecules to the Injured Spinal Cord for Neuroprotection and Tissue Repair*

*This chapter has been peer-reviewed and published in Tissue Engineering with the following authors: Catherine Kang, Peter Poon, Charles Tator, and Molly Shoichet [240]

2.1 Abstract (Local Delivery of EPO)

After spinal cord injury (SCI), a complex cascade of events leads to tissue degeneration and a penumbra of cell death. Neuroprotective molecules to limit tissue loss are promising; however intravenous delivery is limited by the blood-spinal cord barrier and short systemic half-life. Current local delivery strategies are flawed: bolus injection results in drug dispersion throughout the intrathecal space and catheters / pumps are invasive and open to infection. Our laboratory previously developed a hydrogel of hyaluronan (HA) and methylcellulose (MC) (HAMC) that, when injected into the intrathecal space was safe and remarkably, had some therapeutic benefit on its own. In order to test this new paradigm of local and sustained delivery, relative to conventional delivery strategies, we tested for the first time the in vivo efficacy of HAMC as an intrathecal drug delivery system by delivering a known neuroprotective molecule, erythropoietin (EPO). In vitro studies showed that EPO was released from HAMC within 16 h, with 80% bioactivity maintained. When the material alone was injected in vivo, individual fluorescent labels on HA and MC showed that HA dissolved from the gel within 24 h, whereas the hydrophobically associated MC persisted in the intrathecal space for 4-7 days. Using a clip compression injury model of moderate severity, HAMC with EPO was injected in the intrathecal space and, in order to better understand the potential of this delivery system, compared to the therapeutic effect of both common delivery strategies – intrathecal EPO and intraperitoneal EPO – and a control of intrathecal HAMC alone. Intrathecal delivery of EPO from HAMC resulted in both reduced cavitation after SCI and a greater number of neurons relative to the other delivery strategies. These data suggest that the localized and sustained release of EPO at the tissue site by HAMC delivery enhances neuroprotection. This new system of intrathecal delivery holds great promise for the safe, efficacious and local delivery of therapeutic molecules directly to the spinal cord.
2.2 Introduction (Local Delivery of EPO)

Treatment for the devastating condition of spinal cord injury (SCI) is currently limited, in part due to the complexity of the pathophysiology after trauma and the lack of effective therapies [242]. After an initial traumatic injury to the spinal cord, a cascade of events causes further tissue damage. This process is termed the secondary injury and is characterized by ischemia, hemorrhage, inflammation, and edema within the cord. These secondary events traumatize a large area of tissue that was previously unaffected or sublethally affected by the primary insult, and is thought to be responsible for the significant further loss of function that occurs after SCI [28]. Recent research has focused on administering neuroprotective agents to interrupt this cascade, thereby minimizing tissue degeneration.

High doses of therapeutic agents are required to cross the blood spinal cord barrier (BSCB) and reach the site of injury when delivered by the traditional systemic route, often leading to widespread side effects. Often only a brief period of delivery is achieved due to renal clearance and the short half-life of molecules in blood. These limitations suggest that localized delivery of these agents would result in greater neuroprotection and tissue sparing. Bolus delivery of therapeutic agents to the intrathecal space has been investigated; however, the therapeutic window is short due to drug clearance by cerebrospinal fluid (CSF) flow and absorption [243, 244]. Osmotic minipumps became popular in the last decade for localized and sustained experimental delivery of drugs to the intrathecal space, and implanted pumps have been used in humans for the delivery of analgesics and other agents [199]. However, widespread utilization of pumps has been slow due to catheter blockage, infections, and formation of proliferative lesions around the insertion site [115]. Our laboratory has pioneered an injectable, polymeric drug delivery system for intrathecal drug administration whereby therapeutic agents dispersed throughout a fast-gelling polymer can be released directly at the site of injury [9, 87]. A new material blend of 2% hyaluronan (HA) and 7% methylcellulose (MC) was developed and shown to be fast gelling within the intrathecal (IT) space, minimally invasive by injection through a 30G needle, non-cell adhesive, biodegradable, and biocompatible in vivo [10]. The goal herein was to test the potential of this new paradigm of intrathecal HAMC delivery to conventional intrathecal bolus and intraperitoneal delivery strategies.
Methylprednisolone, a steroid administered systemically, is one of the few agents that can cross the blood-spinal cord barrier (BSCB) and shown to have a positive effect on recovery in clinical trials, although there was only a mild benefit [245] and the trials have been widely criticized [6, 7]. Many other therapeutic agents are being investigated which show reduced apoptosis in several cell types [161, 246], reduced demyelination [247, 248], reduced inflammation [249, 250], among others. These affects may directly prevent or indirectly lead to sparing of neurons which can result in greater remodeling of the sensory and motor spinal tracts to improve functional recovery post-injury. Recent reports show that the hematopoietic protein erythropoietin (EPO) and EPO receptors, which play a role in central nervous system development, are upregulated in the brain and spinal cord within minutes to hours after injury [156, 157]. EPO has shown very positive functional recovery in stroke models, and the neuroprotective benefit of this glycoprotein has been proven in hypoxic and ischemic neonatal and adult rat brains [167-170]. Various groups have also shown a neuroprotective benefit of EPO in spinal cord injury, both in ischemic and compressive/contusive models [49, 142, 157, 161]. The exact neuroprotective mechanism of EPO after SCI is still unclear, but it is postulated that mechanisms may include reduction of ischemic damage [161], reduction of inflammation [162, 163], and sparing of white matter tracts in the spinal cord [49, 164]. EPO may also have more direct actions on neurons, oligodendrocytes and astrocytes, all of which express receptors for this molecule [156, 159, 165, 166]. Since EPO can cross the BSCB [172], it was an ideal molecule to test in terms of delivery strategy in the context of neuroprotection after SCI. Since HAMC forms a loose polymer network, fast drug release from this material was expected; however Gorio et. al. [49] demonstrated that a single intraperitoneal (IP) injection of EPO provided similar neuroprotective effects after SCI to repeated IP injections for several days, further emphasizing EPO as a choice to test in intrathecal HAMC delivery.

The objective of this study was to investigate the benefit of intrathecal HAMC delivery relative to conventional delivery strategies intrathecally and intraperitoneally. EPO was chosen as the molecule to test in this new delivery paradigm because of its demonstrated neuroprotective effects. We first studied the in vitro release of EPO from HAMC to better understand the time course of release in vivo. The in vivo degradation of HAMC was also determined to ensure a sufficient time window for in vivo EPO delivery. Finally, the in vivo tissue benefit of EPO delivered in HAMC was compared to the conventional delivery strategies of intrathecal EPO.
alone and intraperitoneal (IP) EPO alone, using intrathecal HAMC alone as a control for the delivery vehicle. We hypothesized that the localized and sustained release achieved with intrathecal delivery of EPO in HAMC would result in greater neuroprotection and tissue sparing than the controls.

2.3 Materials & Methods (Local Delivery of EPO)

Media and cells were purchased from ATCC (American Type Culture Collection, Rockville, MD) and reagents were sterile filtered before use. Unless otherwise indicated all chemicals were purchased from Sigma Aldrich Chemical Co. (Mississauga, ON) and used as received.

2.3.1 Material preparation

Sodium hyaluronate was purchased from Novamatrix (1.5x10^6 Da, Drammen, Norway) and sterilized prior to use. To sterilize HA, a 0.1% HA solution in Millipore deionized water (dH2O) was filtered through a 0.22 µm PES filter (Nalgene, Rochester, NY). The solution was then lyophilized under sterile conditions by covering 50 ml tubes with nylon 0.22 µm filters (Millipore, Billerica, MA), producing sterile HA powder. Methylcellulose was sterilized by autoclaving for 20 min at 120°C. Artificial cerebrospinal fluid (aCSF) was prepared in dH2O with 148 mM NaCl, 3 mM KCl, 0.8 mM MgCl2, 1.4 mM CaCl2, 1.5 mM Na2HPO4, and 0.2 mM NaH2PO4 [9].

Sterile HAMC was produced by mixing polymer solutions in a laminar flow hood. The MC powder was added to half of the appropriate amount of sterile filtered aCSF at 90°C and vortexed until all polymer particles were wetted. The remaining amount of aCSF was added cold at 4°C and the solution was shaken on an ice bath for 30 min. HA powder was then added to the MC solution, vortexed, and allowed to dissolve overnight at 4°C. This resulted in a 2% HA and 7% MC solution.

2.3.2 In vitro release of EPO from HAMC

The EPO/HAMC blend was similarly prepared to that of HAMC, with EPO dissolved in cold aCSF and added to MC that was dispersed in hot aCSF prior to the addition of HA. This order of addition was important to achieve a well-mixed suspension of EPO in HAMC. The solution was then loaded into a Hamilton syringe and 10 µl injected into the bottom of eppendorf tubes.
containing 100 µl of aCSF, thereby approximating the large ratio of CSF to HAMC that is expected in vivo by injection into the intrathecal space. These samples were incubated at 37°C on an orbital shaker and aCSF was fully removed and replaced with fresh aCSF at t=0, 10 min, 20 min, 40 min, 1, 2, 4, 8, 16, 32, 64, and 128 h. An ELISA assay (R&D Systems, Quantikine IVD EPO Kit) performed in triplicate was used to determine the concentration of EPO in the aCSF that was removed at each time point.

2.3.3 Bioactivity of released EPO

A cellular assay was used to determine the activity of the released EPO on the basis of TF-1 cell proliferation in response to EPO. Cells were maintained in RPMI-1640 media with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2ng/ml of granulocyte macrophage-colony stimulating factor (GM-CSF). A 10 µl aliquot of HAMC containing EPO was injected into 90 µl of culture media lacking GM-CSF. These samples were incubated for t=10 min, 20 min, 40 min, 1, 2, 4, 8, and 16 h, and the media was fully transferred to a fresh plate. A TF-1 cell suspension was added to each sample to achieve a final cell density of 1x10⁴ cells/ml in each well. A standard curve was obtained using samples of EPO dissolved in media at known concentrations, and kept in similar conditions to those with EPO in HAMC. All cells were incubated for 2 d at 37°C and 5% CO₂, and cell density then assayed with Cell Titer 96, a substrate metabolized into a colored product only by live cells. The absorbance was measured at 490 nm with a VERSAmax tunable microplate reader and cell density was calculated. Based on the standard curve correlating expected cell density to known concentrations of EPO, the concentration of bioactive EPO released from HAMC was determined for samples collected at each time point.

2.3.4 Degradation of HAMC in vitro and in vivo

Using carbodiimide chemistry HA was conjugated to a BODIPY-Fluorescein (BODIPY-FL) hydrazide and MC was conjugated to Texas Red hydrazide for visualization within the intrathecal space. For HA conjugation, a 1% HA in dH₂O solution was adjusted to pH 4.6 and 1-ethyl-3-(3-diamethylaminopropyl) carbodiimide hydrochloride (EDC) was added at a 2:1 molar ratio to HA. N-hydroxysuccinimide (NHS) was added in a 1:1 molar ratio to EDC to stabilize the intermediate and BODIPY-FL hydrazide was then added in a 1:4 molar ratio to HA [251]. This was allowed to react at room temperature for 24 h, and was then dialyzed overnight (12,000 MWCO, Spectrum laboratories Inc., Los Angeles, CA) and sterilized as described above.
for unmodified HA. For MC, the hydroxyl groups were first modified to carboxyl groups with bromoacetic acid using a protocol obtained from Hermanson [252]. Methylcellulose was added to 1 M bromoacetic acid in 3 M NaOH and reacted at room temperature for 1.5 h. The reaction was stopped by adding solid monobasic sodium phosphate and neutralizing with 6 N HCl and fluorescent modification of MC was then performed as described above for HA, with Texas Red hydrazide used in place of BODIPY-FL hydrazide. MC was then dialyzed overnight and sterilized in the same way as unmodified MC. In the fluorescent HAMC solution, fluorescent HA accounted for 0.5% of the total HA in the blend, and fluorescent MC accounted for 1% of total MC in the blend, which was prepared by the same procedure as their unmodified counterparts.

To determine if degradation of the labeled material matched that of the unlabeled HAMC, the mass loss of each was determined over time in vitro. To maintain the ratio of HAMC to CSF expected in vivo, 100 μl of unlabeled HAMC or fluorescent HAMC was injected into 1 ml of aCSF which was equilibrated to 37 °C. At t=0, 1, 2, 4, 8 h and 1, 2, 4, 7, and 14 d, aCSF was fully removed from the samples and the remaining material was lyophilized and weighed. Mass at each time was compared to that at t=0 to determine material loss over time.

Animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals developed by the Canadian Council on Animal Care and approved by the Animal Care Committee at the Research Institute of the University Health Network. Fifteen Sprague-Dawley rats (200-250 g; Charles River, Montreal, QC) were anesthetized by inhalation of halothane, and a laminectomy performed at the T1-2 vertebral level. Fluorescently labeled HAMC was injected intrathecally as described in Jimenez-Hamann et. al. [87]. Following injection, the overlying muscles and fascia were sutured closed, and the rats were ventilated with pure oxygen and placed under a heat lamp for recovery. Buprenorphine was administered every 12 h for 3 d post-surgery for pain management.

At t=0 (immediately after injection), 1, 2, 4, and 7 d, animals were administered a lethal dose of sodium pentobarbital, and a 2 cm section of the spinal cord was removed at the T1-2 level. Tissue was removed fresh to prevent the gel from dislodging during the fixation process. Cords were sectioned parasagittally and imaged on a Leica DMRB inverted microscope with Stereo Investigator Software, version 6. For each component of HAMC, the fluorescent area within a
given intensity range was calculated for each image. Fluorescence loss observed over time was indicative of bulk hydrogel degradation, which was calculated according to equation (1) where $A_o$ is the initial fluorescent area (at $t=0$) within intensity limits and $A_t$ is the fluorescent area at time $t$ within the same intensity limits:

$$100\% - \left( \frac{A_o - A_t}{A_o} \times scaling\ factor \times 100\% \right) = \%\ Fluorescence\ Remaining \quad (1)$$

The loss of fluorescence was scaled according to the % of HA or MC that was labeled. For HA a scaling factor of 4 was used because 1/4 of the total HA was fluorescent-HA; for MC a scaling factor of 7 was used because 1/7 of the total MC was fluorescent-MC.

### 2.3.5 EPO Efficacy Study

The operative procedure for 47 Sprague-Dawley rats (220-300g; Charles River, Montreal, QC) was performed as described in the degradation study, except that rats sustained an SCI before injection. SCI was performed with the aid of an operating microscope by placing a modified aneurysm clip calibrated to a closing force of 35 g on the spinal cord. The cord was acutely compressed with this clip for 60 sec, as previously described [43]. Immediately following the injury, one of four injections (10 µl) was performed: 1) injection of HAMC containing EPO into the intrathecal space ($n=10$); 2) HAMC without EPO injection into the intrathecal space ($n=13$); 3) bolus injection of EPO into the intrathecal space ($n=12$); 4) bolus injection of EPO into the intraperitoneal cavity ($n=12$). An injection volume of 10 µl was constant amongst all groups, and all EPO solutions were prepared at 100 IU/µl. Animal weights varied from 250-300 g, resulting in a dosage of $3500\pm200$ IU/kg. This dosage was used based on previous studies with EPO in SCI where an intraperitoneal dosage of 5000 IU/kg produced the greatest functional benefit [49]. Previous work in our lab showed that intrathecal injection of aCSF was comparable to HAMC injection, and therefore the aCSF group was excluded from this study [10]. Postsurgical care of rats was identical to that in the degradation study except that rat bladders were manually expressed 3 times daily.
2.3.6 Functional Assessment

To determine the efficacy of EPO in enhancing motor behavior after injury, open field motor function was assessed using the Basso, Beattie, and Bresnahan (BBB) scoring method [84] daily for 1 week and then weekly thereafter for 6 weeks. Each hindlimb was ranked by two blinded observers and concurrently videotaped. BBB scores range from 0 (no hindlimb movement) to 21 (normal gait behavior) and are used to assess functional improvement after injury and treatment.

2.3.7 Histology & Immunohistochemistry

Animals for the degradation study were sacrificed at t=0 (immediately following injury), 1, 2, 3, and 5 d following intraperitoneal injection of an overdose of sodium pentobarbital. Fresh tissue was harvested to prevent dislodging HAMC from the intrathecal space, then cryoprocessed and stored at -80°C until cut into 20 µm parasagittal sections. Images were obtained at 10x with a Leica DMRB inverted microscope and Stereo Investigator Software (version 6). The area of HA and MC at each timepoint was determined by pixel intensity using Image J software.

Animals for the EPO efficacy study were sacrificed 42 d after surgery and perfused intracardially with 4% paraformaldehyde under deep anesthesia. A 2 cm segment of spinal cord encompassing the injury site was harvested from each animal and cryoprocessed. Cords from three to four animals from each group were sectioned parasagittally at 20 µm thickness, and every sixth section was stained with either 1) Luxol Fast Blue/Hematoxylin and eosin (LFB/H&E) for general morphology and to assess cavity area (volume and area measurements were equivalent for the 35g clip injury model that we have utilized here [51]), 2) ED-1 (activated macrophage stain), or 3) glial fibrillary acidic protein (GFAP – activated astrocyte stain). Images were taken at 5x (LFB/H&E) or 10x (ED-1, GFAP) with a Leica DMRB inverted microscope using Stereo Investigator Software (version 6), and sections in the maximal cavity area were analyzed for area of macrophages and astrocytic scar formation based on pixel intensity using Image J software.

Cords from three other animals in each group were cross-sectioned at 20 µm for 1 cm caudal to the injury, and every fourth section stained with NeuN (Neural cell body stain) or CC-1 (Oligodendrocyte stain) to assess sparing of these cell types caudal to the injury. Images were obtained at 20x with a Olympus BX61 Microscope with Image Pro Plus Software (version 5.1). Neurons were counted manually and oligodendrocytes were counted in Image Pro Plus, which counted only objects within limits on intensity and object size.
2.3.8 Statistical Analysis

All statistics were performed using Microsoft Excel. One way ANOVA followed by the Tukey’s post hoc t-test was used to compare BBB scores of all groups. A student’s t-test was used to compare the maximal cavity area and neuron counts. Differences were accepted to be statistically significant at $p < 0.05$. All errors are given as standard deviations.

2.4 Results (Local Delivery of EPO)

2.4.1 In vitro release of EPO from HAMC

Prior to studying the therapeutic and tissue benefit of intrathecal EPO delivered in HAMC relative to conventional delivery techniques, we investigated the *in vitro* release profile where the volume ratio of EPO/HAMC injected into aCSF mimicked that of the animal model. The release profile of EPO was investigated over 128 h using an ELISA assay, from which it was determined that 99% of EPO was released from HAMC during the first 16 h (Figure 2.1A). Peppas and others [253] have previously established models showing that the amount of drug release is proportional to $t^n$ where $n$ is indicative of the transport mechanism from polymeric drug delivery systems. In unidimensional diffusion mediated release from a slab geometry, $n$ is equal to 0.5 and a linear relationship exists between drug release and $t^{0.5}$ for ~60-80% of release [253]. This is a good approximation for the HAMC hydrogel geometry both *in vitro* in a test tube where the gel is a flat slab and *in vivo* in the intrathecal space where the polymer blend is bound by the spinal cord and the dura. In Figure 2.1B, cumulative release *in vitro* is plotted versus $t^{0.5}$ and a linear relationship is maintained for the first ~75% of release ($R^2 = 0.992$), providing evidence that EPO release from HAMC is diffusion mediated. Deviation from the model was expected for >80% release due to depletion of drug within the hydrogel, resulting in $n > 1$ [254].

A TF-1 cellular assay demonstrated that 80% of the released EPO remained bioactive over the 16 h release time (Figure 2.1C). The ratio of bioactive EPO released to cumulative EPO released over time remained constant, showing that EPO bioactivity did not decrease with time. The 20% loss of activity was likely a result of the increased temperature needed to dissolve MC and obtain a good dispersion of EPO within HAMC. With most of the EPO released being bioactive, the therapeutic benefit of local intrathecal delivery was investigated.
Figure 2.1: (A) EPO release from HAMC over 128 h shows fast release within 16 h. (B) Cumulative release of EPO vs. the square root of time demonstrates a diffusion-mediated release profile for the first 75% of EPO released. (C) EPO released from HAMC is 80% bioactive over time relative to cumulative EPO released. (■=Cumulative EPO released; ○=Bioactive portion of EPO released)
2.4.2 Degradation of HAMC *in vitro* and *in vivo*

Having established *in vitro* EPO release and bioactivity profiles from HAMC, we were interested in understanding the HAMC degradation profile *in vivo*. An *in vitro* degradation profile, shown in Figure 2.2A, demonstrated that fluorescently labeled HAMC had similar mass loss over time as that of the unlabeled material. Figure 2.2B shows longitudinal spinal cord sections where each of the HA and MC are labeled with a different fluorescent molecule. The profile of HAMC loss was followed over time by monitoring the change in area of fluorescence within the intrathecal space. HA degraded quickly, exhibiting a ~95% loss in fluorescent area after 24 h. In contrast, MC showed an initial degradation of ~65% after 24 h and then continued to persist within the intrathecal space for at least 4 days. After 7 days, traces of neither HA nor MC could be detected (Figure 2.2C).

Figure 2.2 (see next page): (A) *In vitro* mass degradation is the same for labeled and unlabeled HAMC. (B) Brightfield image of a parasagittal spinal cord section with corresponding fluorescent images of HA (Green) and MC (Red) in the IT space immediately after injection. (C) Loss in the area of fluorescence shows that HA degrades faster than MC in the IT space. Scale bars =1mm. (n=3, mean ± standard deviation plotted) (■=mass loss of labeled HAMC; ●=mass loss of unlabeled HAMC) ◆=fluorescence loss of HA; ▲=fluorescence loss of MC)
2.4.3 *In vivo* delivery of EPO with HAMC

To determine the efficacy of intrathecal HAMC delivery relative to conventional intrathecal bolus and intraperitoneal delivery, EPO was chosen as the therapeutic molecule due to its known neuroprotective effects and ability to cross the BSCB, and then further compared to a control of intrathecal HAMC alone. After SCI, secondary events that lead to inflammation and extensive cell death result in fluid accumulation or edema within the spinal cord [255]. Subsequently, a cyst or a cavity forms within the cord, filled with cellular debris and toxic factors [3]. The ultimate size of this fluid-filled cavity is proportional to the severity of the injury that is sustained, but the size may be reduced due to tissue sparing. To quantify any change in the size of the cavity due to HAMC or EPO, the maximal cavity area was measured in parasagittal histological sections stained with LFB/H&E (Figure 2.3A). A reduction in the cavity area suggests tissue sparing, which is important for both neuroprotective and regenerative strategies. Figure 2.3B shows that the maximal cavity area for EPO delivered intrathecally from HAMC was significantly lower than that of the HAMC control (p<0.05), demonstrating the benefit of intrathecal HAMC delivery of EPO over HAMC alone. While not significantly different, intrathecal HAMC delivery of EPO had less tissue loss than either intrathecal EPO or intraperitoneal EPO delivery. Thus, of the three delivery strategies, intrathecal HAMC delivery of EPO resulted in the greatest tissue sparing, demonstrating the benefit of local and sustained release.
Figure 2.3: (A) Parasaggital section of a representative spinal cord of an animal that received IT administration of EPO-loaded HAMC at maximal cavitation stained with LFB/H&E. (B) Comparison of maximal cavity area measured from parasaggital spinal cord sections. (n=4, mean ± standard deviation; horizontal bars show statistical significant based on a t-test, *p<0.05)

The general morphology of the tissue suggested that some cells may have been spared due to local delivery of EPO to the spinal cord. To determine which cells were spared, both neurons and oligodendrocytes were counted. Significantly higher numbers of neurons were counted in animals that received intrathecal HAMC delivery of EPO than animals that received EPO by either conventional route of delivery – intrathecal bolus or intraperitoneal (Figure 2.4) – thereby showing an added tissue benefit of localized and sustained delivery with HAMC. Interestingly,
bolus intrathecal injection shows similar neuron counts to intraperitoneal injection, substantiating studies that show EPO can cross the BSCB, particularly after an injury to the CNS [172]. Intrathecal HAMC alone resulted in highly variable neuron counts, demonstrating that its previously reported benefit [10] alone is insufficient to achieve neuroprotection.

![Neuron counts observed in the ventral horns caudal to the injury site based on NeuN staining.](image)

**Figure 2.4:** Neuron counts observed in the ventral horns caudal to the injury site based on NeuN staining. (n=3, mean + standard deviation; horizontal bars show statistical significance based on t-test, *p<0.05)**

Based on previous reports that describe white matter sparing with EPO delivery [49], we postulated that there may also be a reduction in demyelination within the spinal cord, translating into a greater number of oligodendrocytes. However, oligodendrocyte counts showed no significant differences between any groups (data not shown). While it is generally known that reactive glial cells secrete factors that can lead to cell death, this action is more commonly associated with astrocytes than oligodendrocytes. Since no significant increase in oligodendrocytes or decrease in glial scar formation from reactive astrocytes (GFAP immunoreactivity, data not shown) was observed, it is unlikely that either of these indirect mechanisms of neuroprotection occurred. Combined with the neural sparing observed, these results suggest a more direct action of EPO binding to EPO receptors on neurons.

EPO has also been shown to decrease inflammation [163], and for this reason ED-1 immunoreactivity was investigated (Figure 2.5). ED-1 stains activated macrophages and
microglia within the spinal cord and the area over which these cells reside can be measured by pixel intensity of the images obtained. At the maximal cavity area, the total area of inflammation was measured, but EPO delivery did not appear to reduce the inflammatory area. Since it is possible for EPO delivered intraperitoneally to cross the BSCB, this small amount could decrease the macrophage response similar to EPO delivered intrathecally. Also, because inflammation was measured 6 weeks post-injury, the difference in the inflammatory response may have abated by this stage of recovery. Previous work with HAMC alone showed a significant decrease in inflammation relative to injection of aCSF without any therapeutic agents [10], which was attributed to the wound healing property of HA [219].

![Figure 2.5: Comparison of maximal inflammation area measured with ED-1 immunostaining of macrophages and microglia from parasagittal spinal cord sections. (n=3, mean ± standard deviation).](image)

The functional benefit of EPO delivered with HAMC was tested by locomotor functional analysis using Basso, Beattie Bresnahan scoring. Interestingly, at 7 days after EPO injection, animals that received intrathecal HAMC delivery of EPO behaved significantly better than those that received intrathecal bolus EPO (p<0.05); however, in subsequent weeks, these animals behaved similarly to all other animals. Although Gorio et al. [17] showed a functional benefit with intraperitoneal EPO injections, this study was later found to be irreproducible [256]; the present study also showed no functional benefit from EPO delivery, either intrathecal or intraperitoneal except at 7 days from HAMC (Figure 2.6). The BBB scores were very similar for
all groups, including those that received the control injection of HAMC alone. Within 3 weeks of injury, most animals had reached a plateau in functional recovery, with all animals exhibiting weight support of hindquarters.

**Figure 2.6**: Open field motor scores as assessed by the BBB scale over six weeks. (*shows statistical significance measured by ANOVA; n=9 to 11, data shown as mean ± standard deviation) (◆=IT administration of EPO-loaded HAMC; ■=IT administration of HAMC alone; ▲=IT administration of EPO; ○=IP administration of EPO)

### 2.5 Discussion (Local Delivery of EPO)

The diffusional release observed *in vitro* can be interpreted using a scaling argument where drug release for a characteristic time (τ) follows equation (2):

\[
\tau \sim \frac{L^2}{D_{\text{drug}}} \quad (2)
\]

where L is the characteristic length and \(D_{\text{drug}}\) is the diffusion coefficient of the drug [257]. The thickness of the gel was estimated at 2 mm based on the volume of gel and dimensions of the reservoir. The diffusion coefficient of the 30 kDa EPO molecule was estimated to be on the order of \(1 \times 10^{-10} \text{ m}^2/\text{s}\) [258]. Thus, the characteristic diffusion time for EPO from HAMC is on the order of 10 h, consistent with the 16 h release observed experimentally. While this time scale is relatively short in the scheme of tissue remodeling that can occur for weeks or months after SCI,
intrathecal HAMC delivery of EPO likely has longer lasting effects on the tissue because the apparent concentration of EPO at the site of injury remains more than 100 times longer after injury when delivered with HAMC than either intrathecal bolus or intraperitoneal delivery of EPO, based on the average velocity of CSF flow [12].

Upon injection in vivo, the temperature of HAMC increases from room temperature to 37°C, resulting in the formation of hydrophobic junctions of MC. These junctions act as physical crosslinks within the hydrogel, preventing fast dissolution of MC. However, an initial loss of MC is observed, with a slower degradation observed over a period of days. The initial 65% loss of MC in the first 24 h after injection may occur due to either quick dissolution prior to full gelation of MC or polydispersity of the MC chains, where lower molecular weight chains erode more quickly than higher molecular weight chains. Furthermore, non-homogeneous distribution of methyl groups on MC could result in chains with only a few hydrophobic domains dissolving away more quickly, leaving only chains stabilized by several hydrophobic junctions.

The degradation of HAMC observed in vivo was faster than that observed in vitro, where degradation occurred over a period of 10-15 days. The difference may be due to continual CSF flow in the intrathecal space, which amplifies the hydrogel dissolution rate. Also, the spatial constraints on the gel in vivo are greater than those in vitro; the spinal cord and dura limit gel layer thickness in vivo and thinner slabs degrade more quickly due to increased disentanglement of polymer chains [254]. Importantly, the degradation of the fluorescently labeled HAMC was not statistically different from that of the unlabeled HAMC (Figure 2.2A), validating the in vivo degradation results.

The rapid loss of HA from the hydrogel blend can be explained either by dissolution or degradation. Since HA forms crosslinks with neither MC nor itself, it is expected to dissolve away from the gel, assisted by CSF flow in the intrathecal space. The natural enzyme hyaluronidase has a high specific activity for cleaving the N-acetyl-D-glucosamine in HA, and at concentrations found in serum [259], it would degrade all of HA in HAMC within minutes of injection. Because this rate would cause complete loss of HA before tissue harvesting at initial timepoints and hyaluronan is present in the CSF [260], it is unlikely that enzymatic degradation contributed significantly to the loss of HA in the intrathecal space. This suggests that dissolution is likely the dominant mechanism for HA loss after injection into the intrathecal space.
The concentration of EPO at the injury site is likely lowest for EPO delivered intraperitoneally due to dilution in the blood, presence of the BSCB and CSF flow. Moreover, EPO causes red blood cell production [261] and thus systemic administration can lead to thickening of the blood and increase the time needed to clear the hemorrhage after injury. These may account for the lack of neuroprotection after intraperitoneal relative to intrathecal HAMC delivery of EPO. Similarly, intrathecal bolus delivery of EPO resulted in lower neuroprotection relative to intrathecal HAMC delivery of EPO, likely due to the fast clearance by CSF flow. The improvement in neuron counts may be attributed to longer residence time and bioavailability of EPO released from HAMC at the site of injury combined with the wound healing properties of HA. Spatial limitations of the hydrogel-spinal cord interface also cause a constant supply of EPO on the surface of the spinal cord, and EPO diffusing from HAMC into the cord in this manner may provide enhanced neuronal protection due to drug-receptor binding on the surface of neurons where EPO receptors are present [155, 159]. Cultured primary neurons treated with EPO have been protected from excitotoxic cell death [155], and experiments with EPO in stroke and traumatic brain injury models [170] have also shown this neuroprotective effect in hypoxic and ischemic environments [169]. While EPO delivery in HAMC showed greater neuroprotection than HAMC alone, the difference was not statistically significant; the high standard deviation in the HAMC group suggests that HAMC may play some role in neuroprotection, but not strong enough to reproducibly achieve neuron sparing. Together these data suggest a synergistic effect between the drug delivery system and the delivered molecule.

We have shown here that EPO delivered locally with HAMC can increase neuronal sparing as compared to the traditional routes of drug delivery. The enhanced tissue and neuron sparing achieved demonstrate the importance of our localized release strategy to limit degeneration and ultimately as a means to enhance functional recovery.

Behavioral recovery was significantly greater at 1 week post-injury for animals that received intrathecal HAMC delivery of EPO relative to animals that received intrathecal delivery of EPO alone; however, animals that received the bolus intrathecal delivery of EPO alone had acute respiratory complications during surgery. Within 15-30 s of injection, animals that received an intrathecal bolus injection of EPO alone began gasping deeply for breath, and after 3-5 gasps became apnoeic. Of the 16 rats undergoing surgery in this group, 12 exhibited this behavior, 4 of which could not be resuscitated and died. The resuscitative efforts consisted of terminating
anesthesia and applying external compression of the thoracic region until a pulse was identified and respiration restored. A recent report shows that slowed breathing is observed when EPO is administered in the central nervous system [262]; however cessation of breathing was not listed as a side effect. This unexpected respiratory complication may have contributed to the lack of early functional recovery in this group. This was not observed in animals that received intraperitoneal EPO or intrathecal HAMC alone, but was observed for intrathecal HAMC delivery of EPO in 2 of the 10 animals, both of which were successfully resuscitated. Interestingly, when the dose of the intrathecal bolus EPO was reduced to 80%, to adjust for the bioactivity of EPO in HAMC, gasping was observed, but animals did not cease to breathe, suggesting that this effect is dose-dependent. Although the enhanced locomotor functional benefit of EPO delivered via HAMC was only apparent at 7 days post-injury, delivery with HAMC allowed slower release of EPO and fewer respiratory complications relative to intrathecal infusion of EPO alone. The functional benefit observed at 7 days may have occurred due to other secondary injury events such as reduced inflammation or decreased apoptosis at early time points; EPO has been shown to reduce the inflammatory response following traumatic brain injury [163, 263, 264] and decrease apoptosis [163, 264]. The early improvement suggests a localized delivery strategy with a longer release profile would sustain recovery further. This illustrates an added benefit of HAMC in terms of improved safety for intrathecal drug administration and the potential for even greater functional recovery with prolonged delivery times.

The rate of dissolution of HAMC in vivo and the release rate of EPO in vitro suggests that this is a useful strategy for the localized release of neuroprotective molecules with an early therapeutic window. This work showed that intrathecal HAMC delivery of EPO provided the greatest neuroprotective benefit in terms of tissue sparing after SCI. This was exemplified by decreased cavitation over HAMC alone and a greater number of spared neurons as compared to the intrathecal bolus of EPO and intraperitoneal EPO. Additionally, respiratory complications associated with EPO administration were minimized when delivered with HAMC. In ongoing studies, the intrathecal HAMC delivery system is being further refined for prolonged degradation and release of other therapeutically relevant molecules with a focus on improving neuroprotection and regeneration after spinal cord injury.
3 Enhancing Localized Delivery of Fibroblast Growth Factor 2

Poly(ethylene glycol) Modification Enhances Penetration of Fibroblast Growth Factor 2 in Injured Spinal Cord Tissue from an Intrathecal Delivery System*

*This chapter has been peer-reviewed and is in press in the Journal of Controlled Release with the following authors: Catherine Kang, Charles Tator, and Molly Shoichet [239].

3.1 Abstract (Penetration of FGF2)

Spinal cord injury is a condition for which there is no effective treatment and clinical drug delivery techniques for central nervous system conditions are limited by the blood-spinal cord barrier. Our lab has developed an injectable drug delivery system consisting of a biopolymer blend of hyaluronan and methylcellulose (HAMC) that can sustain drug release for up to 24 h in the intrathecal space. Fibroblast growth factor 2 (FGF2) has great potential for treatment of spinal cord injury due to its angiogenic and trophic effects, but previous studies showed no penetration into spinal cord tissue when delivered locally. Conjugation to poly(ethylene glycol) (PEG) is known to improve penetration of proteins into tissue by reducing clearance and providing immunogenic shielding. We investigated conjugation of PEG to FGF2 and compared its distribution relative to unmodified FGF2 in injured spinal cord tissue when delivered intrathecally from HAMC. Importantly, PEG conjugation nearly doubled the concentration of FGF2 in the injured spinal cord when delivered locally and, contrary to previous reports, we show that some FGF2 penetrated into the injured spinal cord using a more sensitive detection technique. Our results suggest that PEGylation of FGF2 enhanced tissue penetration by reducing its rate of elimination.

3.2 Introduction (Penetration of FGF2)

Spinal cord injury is a serious condition affecting nearly 300,000 people in the US. Most frequently, a spinal cord injury is caused by broken fragments of the vertebral column compressing the spinal cord causing immediate and delayed cell death and blood vessel rupture. The primary injury causes limited tissue damage, but the series of events in the hours to days after injury include hemorrhage, ischemia, hypoxia, inflammation, and edema, all of which
contribute to the characteristic tissue degeneration and major loss of function [265]. The current clinical treatment for this condition includes an intravenous injection of high dose methylprednisolone; however, the clinical benefit of this treatment has been highly debated [6, 7] and at present there are no other proven treatments [242].

Drug delivery to the central nervous system (CNS) is particularly difficult due to the blood-spinal cord barrier and the dura and arachnoid membranes that surround the cord, both of which are effective in preventing the passage of most drug and protein therapeutics delivered systemically. This emphasizes the need for other methods than the traditional intravenous and oral delivery strategies, which often require high doses for penetration and can lead to significant and undesirable side effects. Intrathecal drug delivery strategies to circumvent these barriers have been developed, such as bolus delivery, implantable catheters, or sustained delivery from minipumps. However, these strategies are invasive and prone to infections [115] and bolus intrathecal delivery is evanescent. To achieve sustained intrathecal delivery, our lab has developed a minimally invasive, injectable drug delivery system consisting of a physical blend of hyaluronan and methylcellulose (HAMC) which has proven safe for in vivo use [10]. A therapeutic agent can be dispersed either alone or in polymeric nanospheres within this hydrogel [209], and the hydrogel alone has shown promise in vivo to provide short-term, localized delivery of therapeutic molecules when injected into the intrathecal cavity following spinal cord injury [240]. To relieve pressure on the spinal cord, decompressive surgery is often required to remove vertebral fragments. During this procedure, HAMC could be injected into the intrathecal space directly over the injury site (Figure 3.1A) to achieve maximal therapeutic benefit from localized release.

Fibroblast growth factor 2 (FGF2) is a protein with multiple modes of action in the CNS and has been tested both in experimental models of spinal cord injury [138, 139] and clinically for the treatment of stroke [193]. Aside from its well known trophic properties in promoting angiogenesis [180-182], FGF2 has also been shown to be neuroprotective [187, 188]. It stimulates endogenous ependymal cells to proliferate when co-delivered with EGF [87] and can reduce permeability of the blood-brain barrier [190, 191]. Additionally, FGF2 has enhanced functional recovery in rats after spinal cord injury when delivered locally from an osmotic minipump [138, 139]. However, because FGF2 does not cross the blood-spinal cord barrier, a localized delivery system is necessary and this need is underscored by FGF2 being mitogenic.
and upregulated in cancer [182] reflecting the limitations of systemic administration [197]. Notwithstanding the interesting functional benefits ascribed to FGF2, its penetration into the spinal cord when delivered locally beneath the dura and arachnoid mater has only been studied once. This study was based on immunohistochemistry and demonstrated that FGF2 delivered intrathecally from a collagen hydrogel did not penetrate into spinal cord tissue [87]. To enhance tissue penetration and increase local concentrations, we conjugated poly(ethylene glycol) (PEG) to FGF2, using a methodology similar to PEG modification of other proteins [266, 267]. We then investigated the penetration and distribution of PEG-FGF2 in the spinal cord relative to unmodified FGF2.

PEG modification has been used to improve molecular stability [234], as well as reduce immunogenicity [236] and decrease tissue clearance [237], thus enhancing its penetration into tissue. It has been shown to increase the biologic half-life of several molecules and also enhance distribution in tissues [238]. The distribution of PEGylated proteins in tissue depends on both the physico-chemical factors of the PEG-protein conjugate and the physiological/anatomical factors of the host tissue. To better understand FGF2 biodistribution in spinal cord tissue, we developed a tissue Enzyme-Linked Immunosorbent Assay (ELISA) technique to study the penetration of FGF2 vs. PEG-FGF2 delivered locally from the injectable HAMC hydrogel. This technique was more sensitive than the previously used immunohistochemical method and demonstrated tissue penetration of both FGF2 and PEG-FGF2. Our results suggest that the greater tissue penetration observed for PEG-FGF2 than FGF2 is ascribed to reduced elimination.

3.3 Materials & Methods (Penetration of FGF2)

All chemicals were purchased from Sigma-Aldrich Chemical Co (Mississauga, ON) and used as received unless otherwise noted. Media and cells were purchased from ATCC (American Type Culture Collection, Rockville, MD) and reagents were sterile-filtered in a 0.22 µm Stericup filter before use.

3.3.1 Synthesis of PEG-FGF2

Poly(ethylene glycol) was modified with a maleimide functional group prior to conjugation with FGF2. PEG monomethyl ether (PEG, 5000 Da) was dissolved in dichloromethane and reacted
with a 3x molar ratio of succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Pierce, Chicago, IL) in the presence of 1.5x molar ratio of triethylamine under nitrogen. After 24 h PEG-maleimide (PEG-mal) was precipitated dropwise in cold ether and filtered through a 0.42 µm glass microfiber filter. PEG-mal was dried under vacuum overnight to remove excess ether and stored at -20°C. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS, Voyager Elite Mass Spectrometer, Toronto Integrated Proteomics Lab) was performed to characterize PEG (CH₃(OCH₂CH₂)₁₁OH (M⁺) 5,000 Da, found 5,088 Da) and PEG-mal (CH₃(OCH₂CH₂)₁₁OC₁₁H₁₄NO₄ (M⁺) 5,236 Da, found 5,223 Da).

Recombinant human fibroblast growth factor 2 (FGF2, Biovision, Mountain View, CA) was dissolved in 5mM Tris buffer to a final concentration of 1 mg/ml. PEG-mal was added in a 5x molar ratio to 300 µl of FGF2 and was reacted at room temperature on a shaker at 500 rpm for 3 h. A heparin affinity binding column (GE Healthcare Biosciences AB, Uppsala, Sweden) was used to remove free PEG from the reaction mixture. Purified PEG-FGF2 was dialyzed (10,000 MWCO) overnight at 4°C and subsequently lyophilized in conical tubes with sterile nylon 0.2 µm filter caps prior to use.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed with a 12% acrylamide resolving gel and 5% stacking gel (Biorad, Constant Voltage = 120V) to confirm the PEG-FGF2 product had formed and that free PEG was fully removed. A Benchmark pre-stained protein ladder (Invitrogen, Burlington, ON) was used for molecular weight classification. Proteins were stained with Simply Blue™ Safestain (Invitrogen, Burlington, ON) and PEG was stained for 20 min with sequential submersion in 2.5% barium chloride and 1.3% iodine/2% potassium iodide using a modified protocol from [268].

### 3.3.2 Bioactivity of PEGylated FGF2

Dose dependent proliferation of a Balb/3T3 fibroblast cell line (ATCC, CCL-163) was used to investigate the bioactivity of FGF2 and PEG-FGF2 products. Cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 1% Penicillin/Streptomycin and 10% calf bovine serum. To test the activity of PEG-FGF2 relative to that of FGF2, Balb/3T3 cells were cultured in serum free media with either FGF2 or PEG-FGF2 mitogens at 10, 20, and 40 ng/ml with a 20:1 weight ratio of Heparin:FGF2. These cells were incubated for 24 h at 37°C and 5% CO₂, and cell density then assayed with Cell Titer 96, a substrate metabolized into a
colored product only by live cells. The absorbance was measured at 490 nm with a VERSAmax tunable microplate reader and cell density was calculated relative to a standard curve of known cell density.

3.3.3 **In vitro** release of FGF2 vs. PEG-FGF2 from HAMC

Sodium hyaluronate (Novamatrix, 1.5x10^6 Da, Drammen, Norway) was sterilized by filtering a 0.1% solution through a 0.2 μm filter and lyophilizing prior to use. Methylcellulose (13x10^3 Da), was sterilized similarly. Following lyophilization in conical tubes with sterile nylon 0.2 μm filter caps, sterile HAMC was produced by mixing polymer solutions in a laminar flow hood. Artificial cerebrospinal fluid (aCSF) was prepared in dH₂O with 148 mM NaCl, 3 mM KCl, 0.8 mM MgCl₂, 1.4 mM CaCl₂, 1.5 mM Na₂HPO₄, and 0.2 mM NaH₂PO₄ [9]. The MC and HA powders were sequentially dissolved in a solution of 100 μg/ml of either FGF2 or PEG-FGF2 in aCSF at 4°C. This resulted in 100 μg/ml FGF2 or PEG-FGF2 loaded into a 2% HA and 7% MC solution. HAMC with no protein was similarly prepared as a control to ensure HAMC did not interfere with the ELISA assays. A 100 μl aliquot of this solution was injected into the bottom of centrifuge tubes containing 900 μl of aCSF at 37°C, approximating the ratio of HAMC to CSF that is expected *in vivo* by injection into the intrathecal space of a rat. These samples were incubated at 37°C on an orbital shaker and aCSF was fully removed and replaced with fresh aCSF at t=1, 3, 6, 12, 24, and 48 h. A sandwich ELISA assay (R&D Systems, Human FGF basic Duoset) was used to determine the concentration of FGF2 or PEG-FGF2 in the aCSF that was removed at each time point (n = 4).

3.3.4 **In vivo** distribution of FGF2 in spinal cord tissue

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals developed by the Canadian Council on Animal Care and approved by the Animal Care Committee at the Research Institute of the University Health Network. Fifty two adult Sprague-Dawley rats (200-250 g; Charles River, Montreal, QC) were anesthetized by inhalation of halothane, and a laminectomy performed at the T1-2 vertebral level. A modified aneurysm clip calibrated to a closing force of 26 g was applied to the spinal cord at T1 for 1 min to simulate a spinal cord injury as previously described [43]. A durotomy was performed immediately caudal to the injury site with a 30 G bent beveled needle and then a 30 G blunt, bent tipped needle was inserted into the intrathecal space (see Figure 3.1A) for injection of HAMC.
To allow for HAMC gelation, the needle was held in the intrathecal space for 1 min before removal, at which time a small plug of HAMC would form at the durotomy site and prevent leakage of CSF [10]. Animals received a 10 µl injection in the intrathecal cavity of HAMC loaded with either 10 µg/ml FGF2 (n=5/timepoint) or 10 µg/ml PEG-FGF2 (n=5/timepoint). To control for cross-reactivity in the ELISA of endogenous rat FGF2 with the recombinant human FGF2 injected, animals were injured but not injected (n=3/timepoint). Following injection, the overlying muscles and fascia were sutured closed; rats were ventilated with pure oxygen and placed under a heat lamp for recovery. Buprenorphine was administered post-surgery for pain management.

At t=0 (immediately after injection), 3, 6, and 24 h, animals were administered a lethal dose of sodium pentobarbital, and a 2 cm section of the spinal cord was removed at the T1-2 level. Removal of fresh tissue prevented loss of HAMC in the intrathecal space and FGF2 from tissue during the fixation process. Tissue was flash frozen in 2-methylbutane on ice and subsequently cut parasagittally on a tissue chopper (McIlwain, Redding, CA) into 1 mm thick slices. The most medial two slices were then cut into 300 µm longitudinal sections dorsoventrally so that FGF2 could be detected spatially through the depth of the spinal cord (See Figure 3.1B). Homogenate buffer was prepared in distilled H2O with 20 mM HEPES, 10 mM KCl, 1.5mM MgCl2, 1mM ethylenediamine tetraacetic acid (EDTA), 1mM ethylene glycol tetraacetic acid, 1mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride. Each 1mm x 300 µm x 2 cm segment of spinal cord was digested in homogenate buffer using a Pellet Pestle (Kontes, USA). Samples were centrifuged for 15 min at 15,000 RPM at 4°C and the supernatant collected. A sandwich ELISA assay (R&D Systems, Human FGF basic Duoset) performed in duplicate on supernatant samples was used to determine the concentration of FGF2 and PEG-FGF2 in each tissue segment. Low concentrations of rat FGF2 cross reacted with human FGF2 antibodies; these concentrations were then subtracted from concentrations detected in the cords with rhFGF2 and PEG-rhFGF2 that was delivered locally for each timepoint, respectively.
Figure 3.1: (A) Diagram of injection paradigm shows hydrogel spreading following intrathecal injection. Arrows indicate potential directions for protein flux from HAMC into the CSF or into spinal cord tissue. (B) Tissue sampling paradigm for observing depth of dorsoventral penetration. Spinal cord segments of 2 cm spanning the entire rostro-caudal spread of the hydrogel were removed and then sectioned sagittally into 1mm thick sections. The most medial two sections were then sliced longitudinally into 300 μm.
sections. These 1mm x 300µm x 2cm sections were used to measure the dorsoventral penetration of FGF2 or PEG-FGF2 from the dorsal aspect to the center of the spinal cord.

3.3.5 Statistical Analysis

One way analysis of variance (ANOVA) followed by Tukey’s post-hoc t-test was used to compare the depth of dorsoventral penetration concentrations of FGF2 and PEG-FGF2. Differences were considered statistically significant at p<0.05. All error bars shown represent standard deviations.

3.4 Results (Penetration of FGF2)

3.4.1 PEGylation of FGF2

A maleimide functionalized PEG (Figure 3.2) was conjugated to free FGF2-cysteine thiols. The crystal structure of FGF2 reveals four free cysteine residues that do not participate in disulfide bonding in the active FGF2 structure or in the well known binding sites for heparin sulfate proteoglycans [178]. These free cysteines also do not interfere with the binding sites for the FGF receptors 1 and 2 [269].

![Figure 3.2: FGF2 with free cysteine groups reacts with PEG-mal to produce single PEGylated FGF2 and double PEGylated FGF2 (with some unreacted, non-PEGylated FGF2).](image)

Gel electrophoresis showed 3 bands in the purified PEG-FGF2 mixture (Figure 3.3, lane 3): ~17kDa, ~25 kDa, and ~35 kDa. Although there are 4 free cysteines on FGF2, Cys 96 and Cys 78 are more readily available on the surface of the protein for conjugation [176]. Cys 34 and Cys 101 face the internal portion
of the protein when the active conformation is maintained, making the conjugation of PEG-maleimide difficult on these residues [176]. For this reason, the PEG/FGF2 reaction mixture delivers 3 distinct bands: FGF2, mono-PEG-FGF2, and di-PEG-FGF2. The molecular weights expected were ~22kDa and ~27 kDa for single and double PEGylation respectively; however, bands were observed shifted higher at ~25 and ~35kDa. This is likely due to the complexation of SDS with PEG, which is known to slow the movement of PEGylated proteins in SDS-PAGE [270].

When the reaction was allowed to proceed for several hours longer, 5 bands were observed (data not shown), also shifted higher than the expected molecular weights, with the higher molecular weights corresponding to three and four PEG molecules bound to FGF2. Because these products with 3 and 4 PEGs would likely exhibit reduced bioactivity due to the conformational change necessary to expose the internal Cys residues, the protocol which produced only single, double, and unPEGylated FGF2 was used, and this mixed product is henceforth referred to as PEG-FGF2.

Lane 3 in Figure 3.3 was loaded with products previously separated on a heparin affinity binding column and shows no PEG band at low molecular weights. Lane 4 shows the flow through of the reaction solution, with a dark band of PEG observed near ~6 kDa. These results show that free PEG was fully removed from the PEG-FGF2 solution prior to dialysis. Densitometry of the bands showed that of the products, 18% was non-PEGylated FGF2, 44% was mono-PEG-FGF2, and 38% di-PEG-FGF2. Thus 82% of the FGF2 was PEGylated.

Figure 3.3: Representative gel electrophoresis shows molecular weight ladder, control FGF2, products of PEG- 
FGF2 reaction isolated from heparin binding column, and flow through PEG from heparin binding column.
3.4.2 PEG-FGF2 Bioactivity

Dose dependent proliferation of Balb/3T3 cells was used to ensure that the PEG-FGF2 maintained bioactivity after PEG modification and processing steps. The cell density increased similarly with increasing concentrations of either FGF2 or PEG-FGF2 (Figure 3.4), indicating that PEG-FGF2 maintained dose dependent activity following the PEGylation and purification steps.

![Graph](image.png)

Figure 3.4: Dose dependent activity of FGF2 (white bars) and PEG-FGF2 (black bars) show that bioactivity based on cell density is maintained after PEGylation and subsequent purification. (n=5, mean ± standard deviation are shown).

3.4.3 FGF2 Release from HAMC *In vitro*

The *in vitro* release profiles of PEG-FGF2 and FGF2 from HAMC were compared to understand whether PEG modification would influence diffusion of FGF2 and to establish the duration of release. Since the HAMC hydrogel is comprised of 9% solids and 91% buffer and is highly porous, a diffusive mechanism of release was expected based on previous results with other proteins of comparable size [240]. The release profiles of PEG-FGF2 and FGF2 were similar and release was complete after 24 h (Figure 3.5).
Figure 3.5: *In vitro* release of FGF2 (□) and PEG-FGF2 (●) from HAMC drug delivery system shows diffusive release within 24 h in almost identical concentrations. (n=5, mean ± standard deviation are shown).

3.4.4 *In vivo* Distribution of FGF2 and PEG-FGF2

Previously, our laboratory found that FGF2 did not penetrate into spinal cord tissue based on detection by immunohistochemistry where the limit of detection was 100 µg/ml [87]. This restricted delivery of FGF2 motivated the present study to enhance the diffusive penetration into the spinal cord of FGF2 by PEG modification. To overcome the limited sensitivity of immunohistochemistry, we used the more sensitive tissue ELISA technique that increased the sensitivity by four orders of magnitude to 10 ng/ml. With ELISA, both FGF2 and PEG-FGF2 were detected in spinal cord tissue when delivered locally (Figure 3.6). The spinal cord was removed and frozen with the dura intact to enable measurement of the concentration of FGF2 and PEG-FGF2 in both the drug delivery system and the tissue. At 3 h, protein release from HAMC was constant since the concentration in the gel did not change from t = 0 h. At this time, a significantly greater concentration of PEG-FGF2 than FGF2 was detected in the spinal cord tissue (Figure 3.6B). There was generally a greater concentration of PEG-FGF2 than FGF2 at all depths of penetration from the dorsal surface of the spinal cord, with significant differences at 0.6 and 1.75 mm from the dorsal surface (Figure 3.6B). Concentrations within the spinal cord
were highest near the dorsal surface in close contact with HAMC; however, because HAMC spread around the spinal cord tissue, diffusion of PEG-FGF2 and FGF2 was likely from both dorsal and ventral sides, thereby accounting for the increase in protein concentration in the center of the cord. Additionally, cerebrospinal fluid transport of the protein through the central canal may have contributed to these elevated protein concentrations. At 6 h, the concentration in the gel had dropped to ~1 µg/ml and release was no longer constant due to drug depletion. Concentration profiles at 6 h show that concentrations of PEG-FGF2 tended to be higher than FGF2 throughout the cord, but not statistically significant at this time (Figure 3.6C). At 24 h, no FGF2 or PEG-FGF2 could be detected in the gel, and only small amounts (10-15 ng/ml) were detected in the spinal cord (data not shown). We used Equation 1, based on concentrations detected in tissue, to better understand FGF2 and PEG-FGF2 diffusion in vivo [271, 272]:

\[ C = C_o \exp \left[ -\varphi \left( \frac{x}{a} - 1 \right) \right] \]  \hspace{1cm} (1)

where \( C \) is the concentration in the tissue, \( C_o \) is the initial concentration in the polymer delivery system, \( x \) is the distance of the protein from the polymer and \( a \) is the characteristic thickness of the polymer gel that is bound by the spinal cord and the dura. The \( \varphi \) is termed the diffusion/elimination modulus [271, 273] and is defined as:

\[ \varphi = a \sqrt{\frac{k}{D}} \]  \hspace{1cm} (2)

where \( k \) is a lumped elimination parameter accounting for cellular interactions and \( D \) is the diffusion coefficient. Because this equation is applicable when drug delivery is at steady state, estimated concentration profiles were obtained for \( t = 3 \) h only, where drug release was constant. In vivo steady state model predictions using equation 1 above, as has previously been described [273, 274] for similar systems, were fit to the data for up to 1.2 mm into the cord. These predicted concentration profiles of FGF2 and PEG-FGF2 are shown in Figure 3.6B, from which the \( \varphi \) values were calculated using the least fit squares method. This model fits those points closest to the surface of the cord up to ~1.2 mm into the tissue, where release is most similar to a point source. Deviation occurs beyond this depth likely due to concentric protein flux from other surfaces of the cord (see Figure 3.1B, inset) as well as from CSF transport of protein through the
central canal. Importantly, this analysis provides useful insights into mechanism that accounts for the greater tissue penetration of PEG-FGF2 vs. FGF2 by providing an understanding of the relationship between the elimination constant (k) and diffusion coefficient (D). The φ values, based on in vivo concentration values of $\phi_{\text{FGF2}}$ and $\phi_{\text{PEG-FGF2}}$, were 1.2 and 0.7, respectively. Because $a$ is constant, the φ values indicate the relationship between elimination and diffusivity of FGF2 and PEG-FGF2, where $\phi > 1$ indicates greater elimination than diffusivity.

Figure 3.6: Cumulative comparison shows a greater amount of PEG-FGF2 (●) in the injured spinal cord than FGF2 (□) when delivered from the intrathecal HAMC drug delivery system. B At 3 h post-intrathecal injection, higher concentrations of PEG-FGF2 (●) than FGF2 (□) have diffused into the cord. Lines indicate the predicted concentration profiles of PEG-FGF2 (solid) and FGF2 (dotted). C At 6 h post-intrathecal injection, drug depletion from the hydrogel results in a plateau of drug in the tissue, but elevated concentrations of PEG-FGF2 (●) are still observed in the spinal cord. Asterisks indicate statistical significance at $p<0.05$. (n=5, mean ± standard deviation are shown).
3.5 Discussion (Penetration of FGF2)

Intrathecal injection of HAMC containing either FGF2 or PEG-FGF2 allows local release of the protein at the site of spinal cord injury. The HAMC hydrogel forms a gel spatially bound by the dura and arachnoid membrane and the spinal cord from which the protein can diffuse laterally into the CSF, radially into the spinal cord tissue, as well as across the dura and arachnoid membrane into the epidural space (Figure 3.1A). Since the dura and arachnoid membrane contributes to the blood-spinal cord barrier and is highly impermeable to proteins, minimal diffusion is expected in this direction [275]. The spinal cord-hydrogel interface and the CSF-hydrogel interface are the main sites for drug release from the hydrogel. The gel spreads over the surface of the spinal cord and fills much of the intrathecal space at the site of injection, thereby resulting in a significant spinal cord-hydrogel interface; however, protein flux into the CSF is also expected. Penetrability of proteins into the spinal cord tissue is affected by diffusion of the molecule in the extracellular space (which is bounded by cell membranes), as well as by the elimination rate dictated by surface receptor binding or internalization of the protein, degradation, and blood-spinal cord barrier clearance. Penetrability increases with dispersivity and decreases with elimination, as is described in equations 1 and 2 above.

To further probe how PEG affected penetrability, we used the \( \phi \) values calculated from in vivo concentration values of FGF2 and PEG-FGF2, (1.2 and 0.7, respectively), which showed an overall decrease in \( \phi \) due to PEGylation. While the absolute values of \( k \) and \( D \) cannot be determined for FGF2 and PEG-FGF2 within tissue, the ratios of each indicates relative differences between the two proteins, which can be used to better understand why greater concentrations of PEG-FGF2 are observed in the spinal cord. This ratio is shown as:

\[
\frac{\phi_{\text{FGF2}}}{\phi_{\text{PEG-FGF2}}} = \sqrt{\frac{k_{\text{FGF2}}}{k_{\text{PEG-FGF2}}} \frac{D_{\text{PEG-FGF2}}}{D_{\text{FGF2}}}}
\]  

(3)

The ratio of \( \phi_{\text{FGF2}}/\phi_{\text{PEG-FGF2}} \) obtained from the in vivo concentration profiles yields a ratio of 1.7. Given that FGF2 has a higher diffusion/elimination modulus, we then investigated how the relative differences of the elimination and diffusion constants, which define \( \phi \), were affected by PEGylation.
Absolute diffusion coefficients for FGF2 and PEG-FGF2 in tissue cannot be calculated due to the complexities of protein-tissue interaction, but the relative difference of diffusion coefficients between FGF2 and PEG-FGF2 can be estimated based on the ratio of molecular weight, where

\[
D \propto \frac{1}{M^{1/3}} \quad [276].
\]

The weighted average MW of PEG-FGF2 based on the relative amounts of the reaction products was 23.2 kDa. The ratio of diffusion coefficients,

\[
\frac{D_{FGF2}}{D_{PEG-FGF2}} = 1.1,
\]

indicated that diffusion should decrease due to PEGylation. Because \( D \) is inversely proportional to \( \varphi \), if penetration of each molecule was only affected by diffusion then \( \varphi \) would be expected to increase as a result of PEGylation, and result in

\[
\frac{\varphi_{FGF2}}{\varphi_{PEG-FGF2}} < 1.
\]

However, \textit{in vivo} measurements yield \( \frac{\varphi_{FGF2}}{\varphi_{PEG-FGF2}} = 1.7 \), suggesting that PEGylation impacted the elimination parameter. The relation of \( k \) to \( \varphi \) (Equation 2) suggests that elimination would have to decrease significantly after PEGylation, with more than a three-fold change needed to produce the nearly two-fold change in \( \varphi \) that was observed, where

\[
\frac{k_{FGF2}}{k_{PEG-FGF2}} = 3.2.
\]

Since \( k \) is a lumped elimination parameter, it can be impacted by several biological factors. Elimination can occur through intra- or extra-cellular degradation of the protein, phagocytosis by cellular components, surface receptor binding, and clearance via the bloodstream. PEG is known to enhance molecular stability of proteins [234], and conjugation to FGF2 could decrease elimination by reducing extracellular degradation of the protein. In addition, PEG conjugation is known to mask proteins via preventing phagocytosis by the reticulo-endothelial system, which has been most commonly studied in renal clearance of intravenously delivered PEG conjugates [217]. In the CNS, the reticulo-endothelial system is represented by specialized immune cells called microglia, which become activated macrophages following an injury. These cells phagocytose foreign material and cellular debris at the injury site. If exogenously delivered FGF2 is detected as foreign, it will be cleared from the extracellular space as it diffuses into the tissue. In Figure 3.6B, concentrations of FGF2 are highest near the surface of the spinal cord where the drug loaded HAMC is releasing protein into the tissue. As FGF2 diffuses into the tissue, macrophages that accumulate at the injury site may internalize the foreign recombinant human FGF2. The PEG-FGF2 is thought to evade phagocytosis due to the PEG chains that shield the exogenous FGF2, thus allowing greater
concentrations to reach the center of the cord. The analysis described here suggests that PEGylation of FGF2 reduces elimination, further supporting previous reports of PEGylation leading to enhanced molecular stability and biological evasion of macrophage phagocytosis.

3.6 Conclusions (Penetration of FGF2)

Using a reproducible method for PEGylation of FGF2, the penetration of FGF2 and PEG-FGF2 were compared: FGF2 and PEG-FGF2 were detected at ng/ml concentrations within the injured spinal cord. These results demonstrate that exogenous FGF2 penetrates into the spinal cord when delivered locally, and importantly, that higher concentrations of PEG-FGF2 vs. FGF2 can be achieved. Analysis of the diffusion/elimination modulus suggests that elimination of FGF2 decreased with PEGylation, supporting biological models of PEG-mediated penetration of proteins \textit{in vivo}. These results support the concept that therapeutic proteins can be locally delivered to the injured spinal cord with a minimally invasive injection of HAMC into the intrathecal space.
4 Live Animal Imaging of Post-Injury Spinal Cord Blood Flow

Spinal Cord Blood Flow and Permeability Measured by Dynamic Computed Tomography Imaging in Live Animals After Localized Delivery of Fibroblast Growth Factor 2*

*This chapter is in preparation for submission to the Journal of Neurotrauma (2010) with the following authors: Catherine Kang, Richard Clarkson, Charles Tator, Ivan Yeung, and Molly Shoichet.

4.1 Abstract (Hemodynamics with FGF2)

Following spinal cord injury, profound vascular changes lead to ischemia and hypoxia of spinal cord tissue. Since fibroblast growth factor 2 (FGF2) has angiogenic effects, its delivery to the injured spinal cord may attenuate the tissue damage associated with ischemia. To limit systemic mitogenic effects, FGF2 was delivered to the spinal cord from a gel of hyaluronan and methylcellulose (HAMC) injected in the intrathecal space and compared to controls of HAMC alone and artificial cerebrospinal fluid alone. Dynamic perfusion computed tomography (CT) was employed for the first time in small animals to serially measure blood flow and permeability in the injured and uninjured spinal cord. Spinal cord blood flow (SCBF) and permeability-surface area \((PS)\) measurements were obtained near the injury epicenter and at two regions rostral to the epicenter. As predicted, SCBF measurements decreased and \(PS\) increased after injury. FGF2 delivered from HAMC after injury restored SCBF towards pre-injury values in all regions, and increased blood flow rates at 7 d post-injury compared to pre-injury measurements. \(PS\) was stabilized at regions rostral to the epicenter of injury when FGF2 was delivered with HAMC, with significantly lower values than aCSF controls at 7 d in the region farthest from the epicenter. These data demonstrate that both localized delivery of FGF2 improves spinal cord hemodynamics following injury and perfusion CT is an important technique to serially measure these parameters in small animal models of spinal cord injury.
4.2 Introduction (Hemodynamics with FGF2)

Traumatic spinal cord injury (SCI) is a serious condition that often causes paralysis and severe loss of function. A recent study shows that more than 1.2 million people in the US report being paralyzed due to SCI, and nearly 50% of those people reported “a lot of difficulty in movement” or “a complete inability to move” [5]. Pharmacotherapy for acute SCI is currently limited to intravenous administration of the steroid methylprednisolone; however its efficacy in improving functional recovery has been questioned [6]. The early secondary events following the initial spinal cord trauma lead to rapid and profound tissue changes, including hemorrhage, inflammation, edema, and ischemia, all of which have significant impacts on tissue and functional recovery. Several drug treatments using systemic delivery are designed to target and limit these secondary injury mechanisms and have undergone clinical trials, as reviewed in [114, 242]; however, none has shown sufficient improvement in human functional recovery to be established as a standard clinical practice. Re-establishing blood flow to the injured tissue could have a neuroprotective effect by limiting degeneration resulting from ischemia.

Fibroblast growth factor 2 (FGF2) has been shown to both promote angiogenesis [180-182] and be neuroprotective [187, 188]. Ex vivo studies suggest that FGF2 reduces permeability of the damaged blood-brain barrier [190, 191]. In vivo studies demonstrate that FGF2 enhances functional recovery in spinal cord injured rats when delivered locally from an osmotic minipump [138, 139]. Thus, we hypothesized that FGF2 can improve spinal cord blood flow (SCBF) and reduce permeability following injury, yet requires a local delivery strategy because it does not cross the blood-spinal cord barrier (BSCB) [182]. While systemic drug delivery is the most common practice clinically, it requires that molecules cross the BSCB, and high doses are necessary to reach therapeutic levels at the site of injury, often leading to undesirable side effects. Drug delivery systems have been designed to circumvent these limitations, such as bolus intrathecal delivery and implantable catheters/minipump devices. However, these systems are either transient or can cause complications, such as infection [115]. To overcome these limitations, our lab has developed a minimally invasive, intrathecal drug delivery system that is safe for local delivery of therapeutic agents to the site of injury [10, 240]. This localized delivery system, composed of a biopolymer blend of hyaluronan and methylcellulose (HAMC), is injected into the intrathecal space and locally releases FGF2 into the injured spinal cord for at least 6 h [239].
In order to evaluate the potential benefit of local FGF2 delivery from HAMC to the injured spinal cord, a dynamic method to serially study hemodynamics in the same animal over time was desired. Early work in cerebral blood flow using gas clearance techniques were well characterized and modeled by Kety and Schmidt based on Fick’s principles of diffusion [58, 59]. Utilizing inert gases to which the BSCB is completely permeable, the intravascular space, $i$, and the extravascular space, $e$, are considered to be a single compartment and rate constants for gas movement are equal between the extravascular and the intravascular spaces ($k_{ei}$ and $k_{ie}$ in Figure 1A). In this method, an inhalable and inert gas is first allowed to permeate the tissue until saturation, and the clearance of the gas is then measured by electrodes placed within the tissue as a function of time to determine the flow rate. SCBF is then calculated as shown in equation (1) [59]:

$$SCBF = \frac{\frac{dQ_T}{dt}}{\frac{W}{t_0}(c_A - c_V)dt}$$  \hspace{1cm} (1)$$

where $Q_T(t)$ is the quantity of gas in tissue $T$ at time $t$, $W$ is the weight of the tissue, and the denominator is the difference between the arterial and venous curves for gas concentration over time. Although very useful for dynamically measuring blood flow, tissue damage occurs from the probes that are inserted in the tissue for measurement. Other methods for measuring SCBF, such as autoradiography, require a timed infusion of a radioisotope and subsequent sectioning of the tissue [277]. The radioisotope is detected in tissue sections and quantified using densitometry to assess regional flow into grey and white matter in the spinal cord. Indicator fractionation techniques are similar, but instead use labeled microparticles in place of a radioisotope [277]. More recently, the Laser Doppler technique has been used, but since it requires the probe to be on the surface of the spinal cord, it is limited to either a relatively short timeframe after the animal has undergone the injury or a terminal time point [278].

Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) now have improved resolution, and when combined with dynamic contrast enhancement blood flow and permeability can be measured after contrast agents are injected into the bloodstream [66-74]. These techniques build on the models used previously, but employ different assumptions and indicator techniques. With MRI and CT, gadolinium-chelates and iodinated contrast agents, respectively,
can be introduced into the bloodstream and the perfusion rate measured as they pass through the tissues. Because these contrast agents or tracers do not diffuse freely in the tissue as gases would, the intravascular and extravascular spaces within the tissue can be viewed separately (see Figure 1B), unlike models developed by Kety and Schmidt. A consensus paper by Tofts et. al. [75] describes this model in detail, known as the Modified Tofts Model, including the limitations under different conditions and the association of fitting parameters to physiological parameters. In this two-compartment model, the tissue is composed of the intravascular and extravascular components, and a permeable tracer moves from the intravascular space to the extravascular space at a volume rate constant of $K_{\text{trans}}$. Because the tracer is sequestered in the plasma of blood, the rate constant $k_{ep}$ is defined as the rate from the extravascular space to the plasma portion of blood. The unidirectional transport of tracer is dependent on the permeability and the surface area of the endothelial layer that separates the intravascular and extravascular spaces in the selected region. Thus, this method calculates the product of permeability and surface area of endothelium in selected regions, known as the permeability-surface area product, or $PS$. The generalized kinetic model for two compartments as previously described by Tofts et. al. [75] is shown in equation (2a):

$$C_T(t) = K_{\text{trans}} \int C_p(\tau) e^{-k_{ep}(1-t)} d\tau$$  \hspace{1cm}(2a)$$

and with the initial conditions that $C_p = C_T = 0$ at $t=0$, this equation simplifies to:

$$\frac{dC_T}{dt} = K_{\text{trans}} (C_p - C_e)$$  \hspace{1cm}(2b)$$

where $C_p$ is the concentration in the plasma (defined as $C_a/(1-Hct)$ where Hct is the blood hematocrit), and $C_e$ is the concentration in the extravascular space (defined as $C_T/v_e$, where $v_e$ is the fractional volume of the extravascular space). This model can be used for any tissue, but the parameters will vary based on the assumptions applied to the system. In a flow-limited situation where a tracer is very permeable across the endothelial layer (e.g., where $PS >> F$), the equation further simplifies to the Kety model shown in equation (3) where contrast enhancement is related to flow ($F$) in the following manner:
\[ \frac{dC_T}{dt} = F \rho (C_A - C_V) = F \rho (1 - Hct)(C_p - C_e) \]  

(3)

where \( C_A = C_p(1-Hct) \) and \( C_V = (1-Hct)C_e \) (tissue density \( \rho \) is required since \( F \) is per unit mass of tissue). SCBF measurements made with the models developed by Kety have customarily been reported as ml/min/100g of tissue rather than per g of tissue, and this convention is maintained here to allow for comparison with previous work. In a permeability-limited situation, where the tracer is not very permeable to the endothelial layer (e.g., where \( F >> PS \)) then the situation simplifies to equation (4):

\[ \frac{dC_T}{dt} = PSF (C_p - C_e) \]  

(4)

where the units of \( PS \) are ml/min/g. Thus, in a flow limited situation, \( K^{\text{trans}} = F \rho (1-Hct) (PS >> F) \), and in a permeability limited situation, \( K^{\text{trans}} = PS \rho (F >> PS) \). In the central nervous system, the BSCB is mostly impermeable to many compounds, thus \( F >> PS \) and the \( K^{\text{trans}} \) value can be used to estimate \( PS \). Thus, Equation 4 was utilized in these studies to calculate \( PS \) values in the uninjured and injured spinal cord.

A simpler method for measuring \( F \) has been estimated from the first pass of an iodinated compound through the tissue, and flow is only dependent on the maximum amount flowing into the tissue shown in equation (5) [68, 279, 280]:

\[ F \rho = \frac{(dC_T/dt)_{\text{max}}}{C_A_{\text{max}}} \]  

(5)

where flow is equivalent to the ratio of the maximal slope of the tissue intensity curve and the peak height of the arterial curve. In this case, the perfusion rate through tissue is only depicting the perfusion of the intravascular component, ignoring the extravascular component. The underlying assumption is that in the short time used to measure peak tissue enhancement (usually done in the first 10 sec), an insignificant amount of the tracer has permeated into the tissue and thus can be ignored. This equation is only valid for cases where the tracer is not extremely permeable to the membrane, and thus is measuring tracer uptake into the tissue rather than tracer
washout. Therefore, equation 5 was used to determine SCBF in the uninjured and injured spinal cord from the first pass of contrast through the tissue.

The dynamic perfusion CT method has been used in several applications in both animal and human studies. Initially, the method was used to investigate renal blood flow [68, 279, 280], but further technical developments allowed measurements of tumor blood flow in cerebral tumors [67, 73], lung cancers [72, 74], as well as renal and hepatic metastases [68, 70, 71]. A recent report discusses the first use of CT perfusion for SCBF in humans [66], but this technique has not yet been reported for small animals. In the present paper we evaluate dynamic CT imaging for repeated measurements of SCBF and PS in rats after SCI, and with this technique assess the effect of local delivery of FGF2 in HAMC on SCBF and permeability of the injured spinal cord.

![Figure 4.1: (A) One-compartment and (B) two-compartment model of blood flow.](image)

#### 4.3 Materials & Methods (Hemodynamics with FGF2)

All chemicals were purchased from Sigma-Aldrich Chemical Co (Mississauga, ON) and used as received unless otherwise noted.
4.3.1 HAMC preparation

2 wt% HA and 7 wt% MC was prepared as previously described [239]. Briefly, sterile methylcellulose (13x10³ Da) and sodium hyaluronate (Novamatrix, 1.5x10⁶ Da, Drammen, Norway) powders were sequentially dissolved into artificial cerebrospinal fluid (aCSF) in a laminar flow hood. The aCSF was prepared in dH₂O with 148 mM NaCl, 3 mM KCl, 0.8 mM MgCl₂, 1.4 mM CaCl₂, 1.5 mM Na₂HPO₄, and 0.2 mM NaH₂PO₄ [9]. To distribute recombinant human FGF2 (Biovision, Mountain View, CA) in HAMC, a solution of 10 µg/ml of FGF2 dissolved in aCSF was used in place of aCSF alone in the procedure above. This resulted in 10 µg/ml FGF2 loaded into a 2% HA and 7% MC solution.

4.3.2 In vivo Surgical Procedures

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals developed by the Canadian Council on Animal Care and approved by the Animal Care Committee at the Research Institute of University Health Network. Twenty-four adult Sprague-Dawley rats (200-250 g; Charles River, Montreal, QC) purchased with implanted jugular vein catheters were anesthetized by inhalation of isoflurane, and a laminectomy performed at the T1-2 vertebral level. Animals were imaged with dynamic perfusion CT immediately following the laminectomy to obtain normal, pre-injury measurements. A modified aneurysm clip calibrated to a closing force of 26 g was applied to the spinal cord at T2 for 1 min to produce SCI as previously described [43]. A durotomy was performed immediately caudal to the injury site with a 30 g bent beveled needle and then a 30 g blunt, bent tipped needle was inserted through the durotomy into the intrathecal space. Animals received either 10 µl of aCSF (n=8), 10 µl of HAMC (n=8), or 10 µl of FGF2 loaded HAMC (10 µg/ml FGF2, n=8) through the needle that was then maintained in the intrathecal space for 1 min before removal to allow HAMC gelation in the intrathecal space [10]. Following injection, the overlying muscles and fascia were sutured, rats were ventilated with pure oxygen, and then placed under a heat lamp for recovery. Buprenorphine was administered post-surgery for pain management. CT imaging was performed at this time to obtain immediate post-injury measurements, and serial imaging was performed at 1 d post-injury and 7 d post-injury, in the same animals.
4.3.3 Computed Tomography Imaging Acquisition with Dynamic Contrast Enhancement

Animals were kept under isoflurane anaesthesia for the duration of the CT scans, which were obtained with a MicroCT scanner (Locus Ultra MicroCT, GE Medical Systems; Milwaukee, WI) in the Spatio-Temporal Targeting and Amplification of Radiation Response Facility (Toronto, ON). A scout x-ray was performed to define the level of interest corresponding to approximately C5 through T4. A perfusion scan was then performed over the selected region, at an in-plane resolution of 0.15 mm x 0.15 mm with a slice thickness of 0.15 mm covering a range of $z = 13.5$ mm at an energy of 60 kV and 90 mA. The iodinated contrast agent Visipaque™ (270 mg I/mL, GE Healthcare, Milwaukee, WI) was injected as the scan started by means of a foot-pedal controlled injection pump (NE-1000 Single Syringe Programmable Pump, New Era Pump Systems, Inc., Farmingdale, NY). The contrast was warmed to 37°C to reduce viscosity and delivered in the first 10 s of the scan at a rate of 124.2 mL/hr (total 354 µL delivered) through the implanted jugular catheter. A 3 minute multiphase scan protocol was used, acquiring a volume every second for the first 30 s, then every 10 s for the remaining 150 s.

4.3.4 Image Analysis

Post-processing was done using the open source ClearCanvas Software (Toronto, ON) with a Dynamic Contrast Enhancement (DCE) tool developed at the University Health Network in Toronto. A region of interest in the ascending aorta superior to the heart (at T3) was defined and the time-intensity curve produced for the arterial input function. A tissue region of interest for the spinal cord was then defined using average spinal cord measurements for long and short axes of the spinal cord at each vertebral level [281] and using the remaining portions of the vertebrae as boundaries (see Figure 2). Tissue-intensity curves were produced for 3 regions: 1) a region immediately adjacent to the injury epicenter between T1 and T2 which is immediately rostral to T2; 2) a region centered at the vertebral body of T1; and 3) a region between T1 and C7. Each region corresponded to a 150 µm thick segment of tissue. An average hematocrit of 0.4 was used for all measurements [281]. The onset time at which contrast was seen to enter each region was manually determined and the tissue-intensity data was fit to a curve using the DCE tool.
Values for $K_{trans}$, initial slope in the first 10 s, and the peaks of the arterial and tissue curves were calculated using the Modified Tofts Model, as described above. This procedure was performed for each animal, at each time that a CT scan was obtained ($t =$ pre-injury, 1 h post-injury, 1 day post-injury, and 7 d post-injury).

4.3.5 Statistical analysis

All statistics were performed using two factorial ANOVA followed by paired Student’s $t$-test to compare SCBF and $PS$ values. Differences were accepted to be statistically significant at $p < 0.05$. All errors are given as standard error of the mean.

Figure 4.2 (See next page): (A) Representative arterial region of interest at T3 where a cross section of the entire rat is shown. The box outlines the inset on right showing anatomical landmarks, including the contrast filled catheter inside the jugular vein, the aorta (used for determining the arterial input function), the trachea, and the intact T3 vertebra. The corresponding arterial input function is shown below the inset. (B) A representative region of interest near the injury epicenter (T1/T2), and the corresponding tissue uptake curve below. (C) A representative region of interest 0.75-1 mm rostral to epicenter (T1) where a laminectomy was performed and the corresponding tissue uptake curve below. (D) A representative region of interest 1.8-2 mm rostral to the epicenter (T1/C7) and the corresponding tissue uptake curve below.
4.4 Results (Hemodynamics with FGF2)

4.4.1 SCBF and PS Measurements in the Uninjured Spinal Cord

Initial pre-injury measurements were taken at three distinct regions in the spinal cord as a baseline comparison for all post-injury measurements. These regions were selected to investigate the hemodynamics at the epicenter of injury and two rostral regions since the initial mechanical impact is known to have significant biochemical and hemodynamic effects for a considerable rostro-caudal distance. Since the lungs are directly ventral to the spinal cord in regions caudal to the injury, respiratory movement prevented accurate measurements in these regions. All pre-injury SCBF and PS data were averaged from all animals since treatment was not administered until post-injury. At the region nearest to the injury epicenter (T1/T2), SCBF of all groups pre-injury was measured at 49.7 ± 1.6 ml/min/100g. For the areas centered at T1 and T1/C7, the pre-injury means and standard error of the means were, respectively, 45.7 ± 1.6 and 45.7 ± 1.7 ml/min/100g. These SCBF measurements for pre-injury are comparable to previously reported values with the hydrogen clearance technique, where composite SCBF was observed to be ~ 55-65 ml/min/100g [53].

In calculating the \( PS \) value, the assumption of the model is that \( K_{\text{trans}} = PS \) only when \( PS \ll F \). However, immediately after the trauma of spinal cord compression, there is extensive hemorrhage into the extravascular space of the parenchyma, and therefore, this assumption does not apply at 1 h post-injury. Thus, only pre-injury, 1 d post-injury, and 7 d post-injury \( PS \) data are reported for each of the three regions studied. The injury epicenter at T1/T2 and the region at T1 had identical pre-injury \( PS \) values of 0.32 ± 0.03 ml/min/g. At T1/C7, the pre-injury \( PS \) value was calculated at 0.25 ± 0.03 ml/min/g.

4.4.2 Epicenter of Injury (T1/T2)

At 1 h post-injury and post-injection of treatment, there was a significant decrease in SCBF at the epicenter to approximately 40 ml/min/100g for all groups (Figure 3A). At 1 d post-injury, SCBF remained statistically lower than pre-injury values for animals that had received aCSF or HAMC, with values of 38.0 ± 3.8 and 39.1 ± 3.5 ml/min/100g, respectively. This persistence of decreased SCBF 1 d post-injury has been shown previously [282], and could be due to persistent
leaky vasculature or destruction of vasculature at the epicenter. However, animals that received FGF2 with HAMC had a SCBF value of $47.3 \pm 2.6 \text{ ml/min/100g}$, which was not significantly different from pre-injury measurements. This suggests some therapeutic benefit of local release of FGF2 in HAMC (vs. HAMC alone and aCSF alone) where blood flow has returned to near pre-injury values at 1 d. At 7 d post-injury, all groups had elevated SCBF values compared to pre-injury measurements; however, these were only statistically higher in those animals that received FGF2 with HAMC and not those that received either aCSF or HAMC control injections.

At 1 d post-injury at the epicenter, $PS$ had increased significantly from pre-injury measurements for all groups, and values for all groups were comparable (Figure 3B). By 7 d, $PS$ values had declined such that none of the groups was significantly higher than pre-injury values, but animals that received FGF2 from HAMC had slightly lower $PS$ values than controls. This impact on permeability may be a reflection of improved SCBF observed in the FGF2 group at this time. The process of angiogenesis creates leaky vasculature initially, but FGF2 is known to promote maturation of newly formed vessels [283]. Since permeability did not decrease at 1 d for FGF2 treated animals, but SCBF did increase, it is possible that FGF2 stimulated the formation of small vessels. At 7 d, these new vessels promoted by FGF2 at 1 d post-injury may have matured, thus contributing to the reduced $PS$ observed and the significant increase in SCBF.
Figure 4.3: (A) Spinal cord blood flow (SCBF) and (B) permeability-surface (PS) area products nearest to injury epicenter at T1/T2 for: ▲ = FGF2/HAMC; ◊ = aCSF; □ = HAMC (n=8, mean ± standard error of the mean are shown; letters indicate statistical differences as compared to pre-injury values where a = aCSF, b = HAMC, c = FGF2/HAMC).
4.4.3 0.75-1 mm Rostral to Injury Epicenter (T1)

At 1 h post-injury and post-injection, SCBF decreased rostrally for aCSF and HAMC to 38.2 ± 3.1 and 35.1 ± 4.0 ml/min/100g, respectively (Figure 4A). Animals that received FGF2 locally with HAMC had a SCBF of 44.1 ± 2.5 ml/min/100g at 1 h post-injury, only 1.6 ml/min/100g lower than the pre-injury value. At 1 d post-injury, all groups showed some improvement in SCBF, although FGF2 treated animals had values most similar to pre-injury measurements. As was observed at the epicenter, higher SCBF was observed 7 d post-injury for the FGF2/HAMC treated group, as compared to pre-injury measurements. A trend toward higher SCBF was also observed for HAMC alone in this region. The aCSF group, however, only returned to pre-injury values and did not show higher reperfusion rates at T1.

PS values rostral to the injury increased moderately at 1 d post-injury relative to pre-injury measurements (Figure 4B), with smaller changes than those observed at the epicenter. Animals that received FGF2 with HAMC showed a marginal increase of only 0.02 ml/min/g as compared to the aCSF and HAMC groups, which increased by 0.11 and 0.12 ml/min/g, respectively. At 7 d, aCSF treated animals remained higher than pre-injury values, and animals that received HAMC alone showed a reduction in PS to nearly that at pre-injury. In those animals that received FGF2 from HAMC, the PS value at 7 d was less than that prior to injury.
Figure 4.4: (A) SCBF and (B) PS at 0.75-1 mm rostral to epicenter at T1 for: ▲ = FGF2/HAMC; □ = aCSF; ▣ = HAMC (n=8, mean ± standard error of the mean are shown; letters indicate statistical differences as compared to pre-injury values where a = aCSF, b = HAMC, c = FGF2/HAMC).
4.4.4 1.8-2 mm Rostral to Injury Epicenter (T1/C7)

As far as 2 mm away from the injury epicenter, at T1/C7, SCBF decreased significantly from the pre-injury measurement of 45.7 ± 1.7 ml/min/100g for all groups 1 h post-injury, to values of 38.2 ± 3.4, 36.3 ± 3.1 and 36.5 ± 1.6 ml/min/100g for aCSF, HAMC, and FGF2/HAMC, respectively. At 1 d, all groups increased to values that were lower, but not significantly different from pre-injury values. At 7 d, higher reperfusion rates were observed for all groups, similar to the findings at the epicenter and T1 regions. The aCSF and HAMC treated groups increased to 49.3 ± 2.4 and 53.5 ± 5.1 ml/min/100g, respectively. In this region at 7 d, SCBF in animals that received FGF2 treatment was 60.9 ± 3.5 ml/min/100g, which is significantly higher than both pre-injury values and aCSF controls.

At 1 d post-injury, the $PS$ value for the FGF2/HAMC-treated group was 0.28 ± 0.10 ml/min/g, which was similar to the pre-injury value of 0.25 ± 0.03 ml/min/g whereas the $PS$ values for both aCSF and HAMC were higher, with values of 0.42 ± 0.08 and 0.37 ± 0.10 ml/min/g, respectively. At 7 d, $PS$ values of all groups decreased: FGF2/HAMC-treated animals showed the greatest decrease in $PS$ values to 0.18 ± 0.05 ml/min/g (lower than pre-injury values), followed by HAMC-treated animals where the $PS$ of 0.28 ± 0.07 ml/min/g was similar to pre-injury values, and then aCSF-treated animals had $PS$ values of 0.36 ± 0.03 ml/min/g. The FGF2/HAMC treatment showed significantly lower $PS$ values than aCSF-treated animals at this time. The reduction in $PS$ and increase in SCBF observed with FGF2 delivery further supports the hypothesis that early delivery of FGF2 has prolonged effects on tissue recovery. The decrease observed for delivery of HAMC alone suggests a therapeutic benefit of HAMC, which is consistent with previous reports [10].
Figure 4.5: (A) Spinal cord blood flow and (B) permeability-surface area products at 1.8-2 mm rostral to epicenter at T1/C7: ▲ = FGF2/HAMC; ◊ = aCSF; □ = HAMC (n= 8, mean ± standard error of the mean are shown; letters indicate statistical differences as compared to pre-injury values where a = aCSF, b = HAMC, c = FGF2/HAMC; * indicates statistical significance between aCSF and FGF2/HAMC groups at that time).
4.5 Discussion (Hemodynamics with FGF2)

Dynamic perfusion CT was utilized, for the first time, to serially measure SCBF and $PS$ of the uninjured and injured rat spinal cord. Controls showed the characteristic drop in SCBF that has been observed with other methods [53, 282] and the spontaneous recovery that occurs within 7 d, a period in which spontaneous angiogenesis is thought to occur [284]. $PS$ increased following injury as has previously been shown [52, 55], and to some extent was attenuated over time, although it remained higher than pre-injury values at regions nearest to the site of injury. Notwithstanding the variability in $PS$ measurements, the results shown here suggest that dynamic CT is an effective tool with which to study SCBF and permeability, and has the advantages of being minimally-invasive and repeatable in the same animal.

Immediately following SCI several vascular changes occur at the site of injury. Grossly injured vessels leak blood and fluid into the parenchyma of the tissue, leading to edema. This leads to a disruption of the BSCB since cells and proteins normally absent in the spinal cord consequently accumulate in the tissue. To seal burst blood vessels and limit hemorrhage, widespread vasoconstriction occurs, thus lowering blood flow to the area. However, this global vasoconstriction leads to ischemia in the surrounding tissue penumbra. Systemic FGF2 infusion causes vasodilation in vivo, by opening ATP-sensitive potassium channels and enhancing release of nitric oxide [285]. In the experiments performed here, localized delivery of FGF2 from HAMC raised SCBF to near pre-injury values at the injury epicenter and at two rostral sites for the first 24 h post-injury. The immediate delivery of FGF2 with HAMC in the intrathecal space may cause vasodilation of the large peripheral vessels on the surface of the cord, potentially leading to this stabilization of blood flow very early in the secondary injury process. In vivo angiogenesis requires persistent vasodilation, which allows existing vessels to expand into poorly vascularized tissue. Previously we showed that FGF2 delivered locally from HAMC was observed to diffuse into the spinal cord for up to 6 h [239]; this localized delivery may have contributed to the higher perfusion rates observed at 7 d post-injury at all three tissue sites, where FGF2 concentrations sustained over several hours within the cord could have persistently dilated small vessels, thereby stimulating angiogenic pathways and increasing blood flow relative to controls. Moreover, FGF2 may directly stimulate angiogenesis by inducing endothelial proliferation, or stimulating expression of VEGF [194] or nitric oxide [286] in endothelial cells and astrocytes, respectively, to regulate angiogenesis. While the mechanism is not well-
understood, it is clear that notwithstanding the brief period of FGF2 delivery following SCI, long lasting benefits were observed for SCBF after its localized delivery from HAMC.

The control injections of HAMC and aCSF showed a characteristic decrease in SCBF within the first hour, and remained lower than pre-injury values at 24 h. Blood flow at 7 d for these controls showed SCBF returned to pre-injury measurements, although HAMC showed a non-statistical trend toward greater reperfusion. HA is known to scavenge free radicals [287], which cause tissue damage and are present after SCI. HA has also been shown to promote angiogenesis in wound healing processes by upregulating collagen production in endothelial cells [288, 289]. HA has been observed to diffuse from out of the HAMC gel within 24 h after injection [240], although a significant improvement in locomotor function was observed at 7 d post-injury for rats injected with HAMC relative to aCSF controls [10]. The limited benefit of HAMC alone observed for SCBF rostral to the epicenter of injury may have contributed to the improved functional behavior previously reported. Taken together, these results suggest that HAMC itself may have an angiogenic impact on the tissue, which can be enhanced by the addition of FGF2.

Disruption of the BSCB occurs rapidly at the site of injury due to mechanical insult and the downstream biochemical pathways lead to similar disturbances in the areas surrounding the injury. The tight junctions between endothelial cells that line blood vessels and normally effectively block passive transport of large molecules across the BSCB into the parenchyma [290], are disrupted by injury allowing indiscriminate extravasation of plasma proteins [52]. The BSCB remains permeable to very small molecules for at least 28 d after SCI [55], although for larger molecules, the barrier is restored at 14 days [52]. Increased permeability of the BSCB has been observed several vertebral segments away from the epicenter of injury [52, 55], suggesting a widespread biochemical mechanism. FGF2 has been shown to play a role in the BSCB, where tight junction proteins are preserved in organotypic cortical slices when exposed to FGF2 in the surrounding media [190]. Moreover, the tight junction proteins occludin and zona occludens-1 were reduced in FGF2 knockout mice [191], suggesting a role for FGF2 in the regulation of these proteins. FGF2 signaling has also been shown to mediate signaling between astrocytes and endothelial cells in angiogenesis [16], and these interactions are also necessary for functioning of the BSCB [23, 291]. HAMC alone showed a trend toward reducing $PS$ values at regions rostral to the epicenter of injury, and localized delivery of FGF2 with HAMC restored $PS$ near to pre-injury values in these same regions 1 d post-injury. At 7 d post-injury, the $PS$ values were
lower than pre-injury values and significantly lower in animals treated with FGF2/HAMC than those treated with aCSF in the region most rostral to the epicenter at T1/C7. These data also support the hypothesis that short-term delivery of FGF2 with HAMC can have a long-term impact on tissue recovery.

Here we have shown that SCBF measurements at three distinct regions of the spinal cord show characteristic patterns of recovery using dynamic perfusion CT imaging. Localized delivery of FGF2 from HAMC stabilized SCBF nearest to pre-injury values, also causing increased perfusion rates at 7 d post-injury as compared to pre-injury measurements. PS measurements indicate a characteristic increase in permeability 1 d following injury. Delivery of FGF2 with HAMC stabilized PS at regions rostral to the epicenter of injury, with significantly lower PS values than aCSF controls at 7 d in the region most rostral to the epicenter at T1/C7. HAMC alone also showed a moderate improvement in PS at regions distal to the epicenter, supporting previous data that the delivery vehicle itself may have therapeutic benefit. Importantly, we report here that dynamic perfusion CT is a valid method for serially measuring SCBF and permeability after SCI in small animals, and that FGF2 delivered locally with HAMC can improve SCBF and permeability following injury.
5 Discussion

5.1 Localized Drug Delivery

Spinal cord drug delivery is particularly difficult due to the layers of protection surrounding the CNS, which effectively limits molecular diffusion into healthy tissue. After SCI the BSCB is compromised due to grossly injured vessels from the mechanical impact, which allows blood and any systemically administered drugs passage into the parenchyma. Intravenous delivery relies on this BSCB disruption to achieve a therapeutic impact; however, the high doses required to accomplish this can cause significant systemic side effects. Thus, localized delivery strategies have been developed to improve therapeutic concentrations while minimizing systemic impacts. Intrathecal injections can be done, but provide only transient delivery at the site of injury, and may be followed by leakage of CSF after injection. Minipumps can constantly deliver drugs in the epidural or intrathecal spaces for long periods of time when implanted locally, allowing high local concentrations near the site of injury. Although minipumps and catheters provide direct access, they also can cause scarring and compression of the spinal cord, which is a barrier for use post-SCI where further damage of the cord may only worsen the potential for functional recovery. Polymeric delivery systems have also been used for localized delivery. Fibrin glues have also been applied extradurally to deliver drugs [148]; however, since the dura/arachnoid mater generally only allow passage of small molecules (<400 Da) [200], proteins or larger molecules require transport moieties to gain access to the tissue. Additionally, molecules must also traverse the intrathecal space where CSF flow can disperse the drug before reaching the target tissue area. Combining the concepts of a minipump, which could sustain drug release, and an intrathecal bolus injection, where a small needle could be inserted and removed immediately after delivery, an injectable collagen matrix was developed for localized and sustained intrathecal delivery [9, 87]. A blend of 2 wt% HA and 7 wt% MC was subsequently developed, which was non-cell adhesive, unlike collagen, and also appeared to seal the dura from CSF leakage [10]. These systems were shown to be safe for intrathecal drug delivery, without the scarring and compression that resulted from catheters and minipumps. The following discussion focuses on work done to better understand the capacity of HAMC as an injectable intrathecal drug delivery system.
5.1.1 In vitro Release of Therapeutic Molecules from HAMC

Numerous molecules have been shown to provide therapeutic benefit in animal models of SCI, thus in vitro release of several molecules from HAMC was investigated. EPO is a 30 kDa and highly glycosylated protein which has shown promise for improving functional recovery after SCI. FGF2 is an 18 kDa protein which can have a spectrum of actions after SCI, including neuroprotection and angiogenesis. In addition to these proteins, two small molecules were also investigated: 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzoquinoxaline-2,3-dione (NBQX) and nimodipine. NBQX is a glutamate antagonist that may provide neuroprotection from glutamate excitotoxicity following SCI [118]. It is a small molecule of 336 Da which is quite hydrophobic, but the sodium salt form is readily soluble in aqueous medium. Nimodipine is a calcium channel blocker of 418 Da, generally used for treating vasospasm, and has been tested clinically for SCI [110]. It is highly hydrophobic and is only sparingly soluble in aqueous solutions, where the reported solubility is 2 – 4 µg/ml [292]. The release of these 4 molecules from HAMC was studied to better understand the potential of HAMC as a drug delivery system for neuroprotective molecules to be delivered early in the secondary injury process.

Both EPO and FGF2 showed similar release profiles from HAMC, although FGF2 is not glycosylated. Nearly 99% release of both molecules from HAMC occurred within 16 h in vitro, when the volume of HAMC to aCSF was similar to in vivo ratios. To understand how molecules would be released from HAMC in vivo, the mechanism for drug release was investigated in vitro where some variables such as polymer geometry could be controlled. Models that define the rate of drug release from drug delivery systems are based on Fick’s laws of diffusion, where the rate of change of the concentration, $C$, over time, $t$, is related to the rate of concentration change over its position, $x$, within the drug delivery system at that time, given by:

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2}$$

where at $t = 0$, $-\frac{1}{2} < x < \frac{1}{2}$, $C = C_i$ and at $t > 0$, $x = \pm \frac{1}{2}$, $C = C_0$; $D$ is the diffusion coefficient of a given molecule. This assumes perfect sink conditions whereby drug diffuses from a thin polymer slab at an initial uniform concentration, $C_i$, and the surrounding media into
which the drug is diffusing has zero concentration, or \( C_0 \). While numerical solutions can be obtained for this complex partial differential, simplified empirical models have been established by Peppas [253] where rate of drug release, \( M_t/M_\infty \), is related to time and distance through:

\[
\frac{M_t}{M_\infty} = 4 \left( \frac{Dt}{\pi l^2} \right)^{1/2}
\]

where \( l \) is the thickness of the polymer that the molecule must diffuse through. This is valid for the first 60-80% of Fickian release (\( M_t/M_\infty \leq 0.60 \-- 0.80 \)), and assumes a simple slab geometry from which drug can be released from planar surfaces of the slab. This can be generalized to:

\[
\frac{M_t}{M_\infty} = kt^n
\]

Where \( k \) is a constant that takes account of the \( 4(D/\pi l^2)^{1/2} \) term in the previous equation and \( n \) is the diffusional exponent indicative of the transport mechanism. Still utilizing the same assumptions, an \( n = 0.5 \) indicates Fickian diffusion and \( n > 0.5 \) indicates non-Fickian diffusion, which is the case in swelling or degradation controlled release systems. However, it is important to note that deviations from one-dimensional release and simple polymer geometries without sink conditions could impact the interpretation of both \( k \) and \( n \), thus the method by which the release mechanism is studied should be as close as possible to a polymer slab with a surface exposed to media not containing drug for release. \textit{In vitro} release of both EPO and FGF2 were obtained with similar geometries and controlled sink conditions by refreshing the media in which the HAMC containing drug was maintained. Figure 2.1B shows that cumulative EPO release increases linearly with \( t^{0.5} \) for up to 75% of release, indicating that its release is diffusion controlled. Similarly, release of FGF2 and PEG-FGF2 from HAMC showed the same pattern of linearity with \( t^{0.5} \) (Appendix 3, Figure 0.1), suggesting that protein release from the physically interacting gel network of HAMC is diffusion controlled. This would likely hold true for proteins of approximately the same molar mass that do not interact with the hydrogel polymers. Doing these experiments in a controlled, \textit{in vitro} experiment allowed us to understand better how HAMC would deliver drugs in the more complex \textit{in vivo} environment, where geometry is not simple, and ideal conditions cannot necessarily be maintained.
The release of any molecule from a drug delivery system depends on both the delivery vehicle and the diffusion rate of the drug molecule, which is encompassed in the $k$ term above. Diffusivity depends upon several factors, including size, charge, and solubility. The different slopes, indicative of the $k$ term above, observed between EPO and FGF2 demonstrate that they have different diffusivities in HAMC. Diffusive release was also observed for the small, water soluble NBQX, where complete release was observed within 1 h \textit{in vitro} and $>85\%$ of cumulative release was linearly related to $t^{0.5}$ (see Appendix 3, Figure 0.2). Additionally, a soluble formulation of nimodipine in HAMC containing 2% v/v ethanol followed a similar trend to NBQX and protein release, where complete release occurred within 8 h in a diffusion-mediated manner (Appendix 3, Figure 0.2). However, for the sparingly water-soluble nimodipine, both dissolution of the drug and its diffusion through the matrix determined the rate of drug release [293, 294]. Solid particles of nimodipine were produced with varying degrees of sonication to achieve crystalline particles of approximately 900 nm and 380 nm, and these were incorporated into HAMC. The solid particles of nimodipine followed a biphasic release pattern with a high burst release within hours and a slow, sustained release for several days (Appendix 3, Figure 0.3). The high initial burst release in 4 h for 380 nm particles was 80% and 60% for the 900 nm particles. The 380 nm particles had a secondary release phase for 2 d, and the 900 nm particles for 3d. This biphasic release suggested that HAMC somehow impacted the solubility of nimodipine. The presence of hydrophobic moieties on MC was thought to increase the intrinsic solubility of solid drug particles in aqueous systems, since polymeric excipients similar to MC, such as hydroxypropyl methylcellulose or poly(vinylpyrrolidone), have been incorporated into drug particles to increase their solubility [295-299]. This was verified by mixing nimodipine particles in HA and MC separately, revealing that MC, but not HA, increased the solubility by an order of magnitude from 2-4 µg/ml to 30-40 µg/ml (Appendix 3, Figure 0.4). Ultimately, these studies showed that HAMC accelerated the release of a hydrophobic, poorly soluble drug, also yielding highly tunable release profiles dependent on the size of solid particles. These studies with nimodipine are described more fully in [300], where numerical predictions of release were matched to this empirical data showing that drug release was dissolution controlled rather than diffusion controlled.

\textit{In vitro} data shows that proteins and water soluble small molecules are released from HAMC in a diffusion-controlled manner. Release of nimodipine, a sparingly soluble drug showed that
longer delivery could be achieved through a drug dissolution controlled mechanism. The diffusive mechanism of release for proteins suggested that in vivo delivery of these molecules with HAMC would likely be limited to 12-16 h post-injection. Hence, neuroprotection rather than neuroregeneration was targeted for events that occurred in the immediate secondary injury phase, specifically, the ischemic insult that results from vascular damage, hemorrhage, and edema. Notwithstanding this short delivery period, intrathecal HAMC delivery has potential for longer lasting effects since the apparent protein concentrations on the surface of tissue can remain more than 100 times longer after injection when delivered with HAMC than conventional methods such as intrathecal bolus or intraperitoneal delivery, based on the average velocity of CSF flow [12].

5.1.2 In vivo Delivery from HAMC

5.1.2.1 Delivery Period and Degradation of HAMC

In vitro data showed that the HAMC gel degraded within 15 d [10] but little was known about how long the gel lasted in vivo where degradation could be affected by changes in pH, gel geometry, and the presence of enzymes. Each component of HAMC was thus fluorescently labeled to observe the presence of it in the intrathecal space at the site of injection over time. Figure 2.2C shows the in vivo degradation of each component, showing that 95% of HA disappears from the gel within 24 h, and nearly 60% of the MC component is lost within that time as well. The structure of HA contains hydroxyl, carboxyl and amino groups, which are hydrophilic. MC contains some hydrophilic hydroxyls as well as the hydrophobic methyl groups. At low temperatures these polymers are simply entangled together, but after injection into the intrathecal space, the temperature increase leads to associations between MC molecules when the cagelike water structures around the hydrophobic methyl groups are broken. The blend of HAMC thus becomes a physically crosslinked network, in which MC molecules form a network in which the HA is entangled. The blend of HAMC in its crosslinked state is thus a semi-interpenetrating network, whereby MC cannot dissolve out without breaking bonds, but the disentanglement of HA from the gel does not require any bond disruption. The snake-like motion of HA out of MC is termed repatation, in which the macromolecule changes configuration as it withdraws from the gel phase. HA may be removed through this process, or
may undergo enzymatic degradation by hyaluronidases secreted locally following an injury [301] or entering the cord from hemorrhaging blood. In addition, HA can also be non-specifically degraded by reactive oxygen species, particularly after injury [302, 303]. This early loss of HA from the gel structure likely leads to a more porous network of MC, where CSF replaces the void space left by the dissolving HA. By making the gel more porous, diffusive drug release may be expedited since the diffusion path of drug is less tortuous through larger pores. While early loss of HA may lead to faster drug release, nearly 30% of MC continued to persist in the intrathecal space for up to 4 d after injection, with complete degradation by 7 d. These results indicate that HAMC permits a maximum release window of 4-7 d in vivo, if drug release could be extended beyond diffusion mediated release. Importantly, these results indicated that the gel was still present in the intrathecal space during the time window of in vitro release, suggesting that localized delivery in vivo could be achieved.

In vitro release of EPO from HAMC was found to be diffusion controlled, which suggested that HAMC was capable of releasing EPO for up to 16 h. These experiments were supported by the estimation of 10 h based on gel thickness and diffusion coefficient of EPO. Thus, the residence time of EPO on the surface of the spinal cord in contact with the gel could be estimated at 10 h. An intrathecal bolus of EPO, however, was expected to be washed away more quickly from the site of injection by the flowing CSF. The residence time at the site of injection for EPO delivered IT was estimated to be on the order of 5 min. This estimation was obtained using the same volume of injection as for EPO delivered from HAMC (10µl), but the CSF flow rate was used rather than the diffusion coefficient. This was then used to show the ratio of residence times for EPO delivered from HAMC and as a bolus IT: 

\[
\frac{\tau_{EPO/HAMC}}{\tau_{EPO IT}} = \frac{10 \text{ h}}{0.083 \text{ h}}
\]

This ratio shows that EPO delivered with HAMC could result in more than a 100 fold increase in the residence time of EPO delivered as an IT bolus, when only the site of injection is considered. These estimations suggested that EPO delivered with HAMC would provide better diffusion into the injured tissue at the site of injection.

In vitro release of both FGF2 and PEG-FGF2 from HAMC was diffusion controlled, so experiments were performed to validate that proteins diffusing out of HAMC actually penetrated into the injured spinal cord rather than simply diffusing into the CSF. This was done by
sectioning and digesting spinal cords serially after injecting HAMC/FGF or HAMC/PEG-FGF2 into the intrathecal space. Using ELISA to detect the protein in the tissue digests, FGF2 and PEG-FGF2 were confirmed to diffuse into the spinal cord (Figure 3.6, B and C). Concentrations of 5-50 ng/ml were achieved within the tissue, where the highest concentrations were observed in the outer 600 µm of tissue, nearest to HAMC. These are concentrations that are similar to culture experiments where 25-50 ng/ml of FGF2 in ex vivo brain slices showed reduction in permeability of the blood-brain barrier [190], 5-40 ng/ml is used in differentiation/proliferation media for stem cells [304], and neuronal cultures could be spared from glutamatergic excitotoxicity with 20 ng/ml [188]. These results verified that relevant tissue concentrations could be achieved using HAMC as an intrathecal delivery system.

While relevant concentrations were achieved within the tissue, the cumulative amount of protein that was delivered to the tissue relative to the amount loaded into HAMC was quite low, where only 0.5-1% of FGF2 loaded into HAMC penetrated into the tissue. One possibility is that much of the FGF2 diffused from HAMC into the CSF very quickly. This could account for the higher concentrations observed in the center of the cord, where FGF2 released from HAMC into the CSF may have circulated through the central canal of the spinal cord. However, based on a diffusion mechanism it is unlikely that 99% of the FGF2 diffused from the gel so quickly, since the CSF-polymer interface is small compared to the spinal cord-polymer interface. Additionally, if so much of the FGF2 had recirculated through the central canal, higher concentrations would have been expected in the center of the cord. FGF2 is highly regulated in vivo by its binding to heparin in the extracellular matrix, where the FGF2-heparin Kd = 39 nM [305]. The rate of association for these molecules is quite fast, whereas the rate of dissociation is quite slow. Although soluble heparin was delivered with FGF2 in HAMC to limit its sequestration in the extracellular matrix, the FGF2-heparin complex can also bind to FGF Receptors 1-4 (FGFR1-4), with FGFR1 being the most highly expressed receptor. The FGF2-FGFR1 complex has a Kd = 62 nM [305], and similar to FGF2-heparin complexation, this has a fast rate of association and a slow dissociation rate [306]. FGF2 detected using an ELISA would only detect FGF2 that was not bound to receptors on the tissue, thus, the low concentrations observed in the spinal cord would only reflect diffusing FGF2. This suggests that tissue concentrations may have been higher if FGF2 bound to receptors or to heparin in the extracellular matrix could have been detected with the ELISA.
While FGF2 and PEG-FGF2 penetrated into the spinal cord at relevant concentrations, it was only detected in the spinal cord for up to 6 h, and was not observed in the tissue 24 h after HAMC injection. The exponential decrease in concentration from the polymer surface observed at 3 h (Figure 3.6B) was beginning to plateau at 6 h (Figure 3.6C), suggesting drug depletion in HAMC. Since an intrathecal bolus injection would be transient at the tissue surface due to CSF circulation, HAMC effectively sustains the delivery of proteins to the injured spinal cord for at least several hours. Although this time period is short with respect to the secondary injury, it may be an effective time period to limit tissue damage that occurs in this timeframe. This early delivery is sufficient to have some therapeutic benefit, which is further discussed in sections 5.2.1 and 5.2.2 of this chapter, where neuroprotection was observed with EPO delivery, and increased blood flow in tissue was observed for FGF2 delivery even at times when diffusing FGF2 was no longer detected. However, as evidenced from EPO administration, the functional benefit that results from early delivery may be limited, thus a longer delivery window may be beneficial and necessary to provide greater neuroprotection after some of the early secondary injury events have subsided.

5.1.2.2 Potential Biological Role of HA In vivo

HAMC injected into the intrathecal space interfaces with the dura/arachnoid mater, spinal cord tissue, and CSF. Polymer dissolution was expected to occur mainly into the CSF since the long chains would face the lowest diffusive barrier in a fluid. The spatial limitations imposed by the dura/arachnoid and the spinal cord allowed only for rostro-caudal polymer dissolution. In assessing the safety of HAMC, significantly better functional recovery was observed in animals that received HAMC over the aCSF injection [10], several days after HA had been cleared from the intrathecal space. Also, the hemodynamic characteristics of HAMC treated groups showed trends toward improved SCBF in sites rostral to the epicenter [241]. These results suggest that HAMC plays a biological role following injury.

HA comprises a significant amount of the extracellular matrix in the CNS, and in healthy tissue is of a high molecular weight form (>3-6 x10^6 Da). However, after an injury, HA is believed to play a central role in the wound healing process, whereby the high molecular weight form that is normally found in the extracellular space is broken down locally by secreted enzymes. These
lower molecular weight products then have multiple actions through each phase of the wound healing process (reviewed in [307]). Initially, HA enhances immune cell infiltration and upregulates inflammatory cytokines [308, 309]. In addition to these early actions on cells, HA scavenges free radicals and has antioxidant properties [287, 310]. As the high molecular weight form in the tissue is cleaved, HA fragments of <2x10^5 Da have been observed to induce inflammatory or proliferative genes [311], which is thought to contribute to cell migration for tissue remodeling. These lower molecular weight forms are also thought to enhance the angiogenic processes in the later phases by upregulating collagen production in endothelial cells [221, 288, 289]. While it is unlikely that the high molecular weight, exogenously administered HA in the intrathecal space diffuses rapidly into spinal cord tissue to increase the cellular migration into the injured zone following SCI, it is possible that HA scavenges free radicals which diffuse into the intrathecal space from the injured tissue, and that secreted enzymes also lead to its degradation. The lower molecular weight fragments of HA that result from oxidation or enzymatic degradation may diffuse into the tissue or recirculate in the CSF through the central canal. Potentially, this increase in low molecular weight HA could lead to enhanced angiogenesis as the acute inflammatory response begins to subside, thus accounting for the enhanced blood flow response [241], greater neuron counts [240], and motor function [10] observed in the HAMC alone groups.

5.1.2.3 HAMC Delivery Compared to Conventional Delivery Strategies

HAMC was compared to intraperitoneal delivery and intrathecal bolus delivery to investigate the potential benefits that could be achieved with localized and sustained delivery. Intrathecal HAMC delivery was calculated to sustain release at the site of injury more than 100 times intrathecal bolus or intraperitoneal delivery, and systemic delivery via intraperitoneal injection was expected to achieve the lowest dose of EPO at the site of injury since it was diluted through the entire bloodstream. As expected, this route of delivery did result in the lowest amount of neuroprotection relative to intrathecal HAMC delivery of EPO (Figure 2.4). Similarly, the intrathecal bolus resulted in fewer surviving motor neurons relative to intrathecal HAMC delivery of EPO, likely due to the fast clearance by CSF flow. This improvement in neuron counts may be attributed to longer residence time and bioavailability of EPO released from HAMC at the site of injury. Thus, these studies established that EPO delivered locally with HAMC increased neuronal sparing as compared to more traditional routes of drug delivery.
In addition to providing enhanced neuroprotection, HAMC delivery may have also provided other benefits over the other two delivery strategies. Because EPO causes red blood cell production [261] thus leading to blood thickening, an intravenous route of administration might lead to more severe hemorrhaging after injury. However, a more significant impact was seen between the two localized delivery strategies of HAMC containing EPO versus intrathecal bolus of EPO. In animals that received the bolus intrathecal delivery of EPO, acute respiratory complications occurred during surgery, where deep gasping sometimes led to death. Of the 16 rats that underwent surgery in this group, 12 exhibited this behavior, 4 of which could not be resuscitated and died. Slowed breathing has been reported for EPO when it is administered in the central nervous system [262], yet these studies indicate more serious physiological implications for intrathecal bolus delivery. While this impact was not observed in animals that received intraperitoneal EPO or intrathecal HAMC alone, deep breathing was observed in 2 of 10 animals that received intrathecal HAMC delivery of EPO, both of which were successfully resuscitated. This unexpected respiratory complication may have contributed to the lack of early functional recovery after the first week in those animals that received EPO as an intrathecal bolus, where interestingly, motor function was statistically lower than those that received EPO from HAMC intrathecally. These observations demonstrate that HAMC may have an added benefit in terms of improved safety for intrathecal drug administration.

5.2 Neuroprotection Achieved with Localized Delivery

The secondary injury cascade, which leads to significant tissue damage and ultimately the neurological deficit after SCI, begins quite rapidly after the initial mechanical trauma. Limiting the early events that eventually lead to widespread cell death and neuronal destruction has been investigated with a variety of pharmacological treatments. Neuroprotection is a term describing neuronal sparing and reduced lesion volume following SCI, and attempts to achieve this largely target early secondary secondary injury events. Localized delivery with HAMC permits release of neuroprotective proteins at the site of injury early in this process; here, the tissue impacts of early delivery of EPO and FGF2 with HAMC are described.
5.2.1 Implications of EPO Crossing the BSCB

EPO delivered from HAMC provided enhanced neuronal protection, likely due to direct contact with EPO receptors on the surface of neurons [155, 159]. Cultured primary neurons treated with EPO have been protected from excitotoxic cell death [155], and experiments with EPO in stroke and traumatic brain injury models [170] have also shown a neuroprotective effect in hypoxic and ischemic environments [169]. More indirect actions of EPO suggest that it can reduce the inflammatory response following traumatic brain injury [163, 263, 264] and can generally decrease apoptosis [163, 264]. When delivered from HAMC, EPO showed greater functional benefit at 7 d post-injury over EPO delivered IT, although this impact was not retained at later times. Notably, none of the delivery methods of EPO achieved the same high level of functional recovery that was previously reported [49]; however, an attempt at replicating this work in a different lab showed that no significant functional recovery could be achieved with EPO administration [312], thus validating the lack of sustained functional recovery that we observed even with the same route of delivery as the original work.

The spatial limitations of HAMC in the intrathecal space results in a substantial hydrogel-spinal cord interface, whereby drugs are delivered in a sustained manner for several hours to the spinal cord surface. The drug delivered to the surface of the cord must diffuse through the pia mater and through spinal cord tissue to reach cell receptors. For an intrathecal bolus delivery method, this would also be the case; however, since it disperses into the CSF, drug concentrations would be significantly lower than those at the HAMC-spinal cord interface. Since EPO crosses the blood-brain barrier [172], it may in fact have a shorter diffusion distance when administered systemically, since it must only diffuse from the luminal surface of the endothelium into tissue. For highly vascularized tissue, particularly in the spinal cord where a dense capillary network exists [313], a significant surface area of endothelium could potentially allow EPO to reach the ventral motor neurons faster than EPO delivered via HAMC. This is substantiated by the fact that neuron counts were nearly equivalent for EPO delivered IP or IT. However, local delivery of EPO with HAMC still resulted in greater motor neurons counted than both of these groups, suggesting that the local dose when EPO was sustained at the site of injury for several hours was greater than the dose that could be delivered with transient delivery intrathecally or systemically.
5.2.2 Improvements in SCBF and Permeability with FGF2 Delivery

FGF2 is a molecule that does not cross the BSCB [314] and since FGF2 is mitogenic [182, 197], it was not delivered systemically, thus localized delivery of FGF2 from HAMC was compared to controls of HAMC alone and aCSF intrathecally. Immediately following an injury, several vascular changes occur at the site of injury. Vessels that are grossly injured will leak blood and fluid into the parenchyma of the tissue, leading to hemorrhage and edema. This leads to a disruption of the BSCB since cells and proteins normally absent in the CNS consequently accumulate in the tissue. Immune cells, such as leukocytes, infiltrate the tissue via hemorrhage and through the leaky vasculature, stimulating a significant immune response and emitting reactive oxygen species and other free radicals. To seal the burst blood vessels and limit the hemorrhage, vasoconstriction occurs, thus lowering blood flow to the area. However, this global vasoconstriction leads to ischemia in the surrounding tissue penumbra. Following SCI, localized delivery of FGF2 from HAMC stabilized SCBF near pre-injury values at the injury epicenter and at 2 rostral sites for the first 24 h post-injury. Also, at 7 d post-injury perfusion rates were increased beyond pre-injury measurements at all 3 sites observed. The control injections of HAMC and aCSF showed a characteristic decrease in SCBF within the first hour, and remained lower than pre-injury values for up to 24 h. At 7 d, SCBF was statistically higher than pre-injury values for FGF2 treated animals. Blood flow was also higher for controls, but not statistically higher than pre-injury values, suggesting that FGF2 had a greater impact on the tissue. In vivo angiogenesis requires persistent vasodilation; however, this impact has been difficult to link to a biochemical process [286]. Systemic FGF2 infusion has been shown to cause vasodilation in vivo by opening ATP sensitive K+ channels and enhancing release of nitric oxide [285]. Although FGF2 was only detected in the spinal cord for up to 6 h in vivo, it may have caused early vasodilation on the large vessels that are on the periphery of the cord, since it would have reached these vessels first, based on in vivo diffusion data for FGF2. In areas distal to the epicenter where localized FGF2 delivery with HAMC appeared to stabilize blood flow at 1 d post-injury, and higher perfusion rates at 7 d post-injury, several possibilities exist. One possibility is that, as described above in section 5.1.2.1, exogenously delivered FGF2 was potentially sequestered in the extracellular matrix or bound to cells, and could continue to have actions much later than the expected delivery window. Another possibility is that FGF2 diffusing into the tissue several hours after the injury would cause earlier vasodilation of smaller
vessels than controls, at a time when hemorrhage was beginning to be controlled. This early vasodilation of smaller vessels could have stimulated angiogenic pathways earlier than controls, thus increasing blood flow at later time points. Additionally, FGF2 may have directly stimulated angiogenic impacts in cells at these early times, either by inducing expression of VEGF [194] or nitric oxide [286] in endothelial cells, which are known angiogenic regulators. Based on cerebral blood flow studies, capillary density is directly correlated to cerebral blood flow [315]. If capillary density was increased due to angiogenesis, then increased blood flow would be expected. Although the extent of angiogenesis required to produce a beneficial impact on tissue sparing and functional recovery is not known, the capillary density of the uninjured spinal cord could be used as a baseline goal for a given vertebral segment. While the mechanism for improved SCBF observed here was not fully elucidated, FGF2 did provide long lasting benefits on SCBF after its localized delivery.

Disruption of the BSCB occurs quite quickly from the mechanical impact at the site of injury, although the molecular mechanisms by which disruption occurs in the rostro-caudal regions of the injury are not completely understood. The inner lumen of blood vessels in the CNS has a glycoprotein-rich, anionically charged glycocalyx coating, providing a repulsive barrier to plasma proteins of similar charge. The tight junctions between endothelial cells that line the vessels effectively block passive transport of large molecules across the BSCB [290], and these are surrounded by a basement membrane abluminally. Astrocytes surround the basement membrane to further regulate what is passed into the parenchyma. Following injury, the glycocalyx transiently loses anionic charges leading to indiscriminate extravasation of plasma proteins [52], likely also regulated by tight junction disruptions. After SCI, the BSCB remains permeable to very small molecules for at least 28 d [55], although larger molecules are permeable for closer to 14 days [52]. Permeability of the BSCB has been observed several segments away from the epicenter of injury [52, 55], suggesting a widespread biochemical mechanism. Localized delivery of FGF2 with HAMC stabilized PS at regions rostral to the epicenter of injury at 1 d post-injury. At 7 d post-injury, FGF2 lowered PS values with respect to pre-injury measurements, showing significantly lower PS values than aCSF controls at 7d in the region most rostral to the epicenter at T1/C7. Knockout mice for FGF2 showed reduced levels of tight junction proteins in the CNS [191], suggesting that FGF2 regulates their expression. In organotypic brain slices, significantly higher expression of tight junction proteins and intact
blood vessels persisted for 7 d in vivo when bathed in media containing FGF2 [190]. Additionally, the interaction of astrocytes and endothelial cells are necessary for functioning of the BSCB [23, 291]. In angiogenesis, paracrine signalling leads to changes in both astrocytes and endothelial cells [16], thus localized FGF2 may have impacted permeability due to its effects on either astrocytes or endothelial cells.

5.3 PEGylation for Improving Tissue Delivery

Since prior experiments showed that FGF2 did not significantly penetrate injured spinal cord tissue [87], PEG was conjugated to FGF2 with the goal of enhancing tissue delivery. PEGylation has previously been used to improve molecular stability [234], allowing for greater bioavailability. It also reduces immunogenicity [236] by shielding immunogenic sites of proteins, which can lead to decrease tissue clearance [237]. PEG has increased the biologic half-life of several molecules and also enhanced distribution in tissues [238]. The distribution of PEGylated proteins in tissue depends on both the physico-chemical factors of the PEG-protein conjugate and the physiological and anatomical factors of the host tissue. While PEG increases the size and molecular weight of a protein, it also can produce changes in steric hindrance, the electrostatic binding properties, hydrophobicity, and the PI of the protein. All of these changes may impact its bioactivity and action once in vivo. Additionally, the physiological factors of the tissue in which a PEGylated protein is diffusing will impact the effectiveness of PEGylation.

FGF2 is a polypeptide that retains its conformation mainly through van der Waals forces and hydrogen bonding [175]. Although it contains 4 cysteine residues, none of them participate in disulfide bonding [178]. Heparin and heparin sulfates bind to FGF2 and make them conformationally more stable and able to bind to FGFRs. This suggests that FGF2 alone does not have an extremely stable tertiary structure and may be easily rendered inactive, thus both FGF2 and PEG-FGF2 were co-delivered with heparin. When PEG was attached to FGF2, in vitro bioactivity did not decrease, suggesting that it retains its ability to attach to cell receptors. Two interesting findings came from the localized delivery of FGF2 intrathecally with HAMC: 1) FGF2 does in fact penetrate into spinal cord tissue, but previous methods were not sufficiently sensitive to detect it; and 2) PEGylation of FGF2 improves its diffusion into tissue. Assuming that PEG-FGF2 retains the ability to bind to receptors, we showed that PEGylation increased the
concentration of FGF2 in the injured spinal cord tissue by nearly twofold. Diffusivity of PEG-FGF2 was expected to decrease relative to FGF2 due to the increase in molecular size, which would cause it to take a more tortuous path through the tissue. However, PEGylation was thought to also decrease the elimination of FGF2 diffusing through the tissue. While each parameter could not separately be determined from the concentration profiles, the inverse relationship between diffusivity and elimination, given by \( \varphi = \frac{\text{diffusivity}}{\text{elimination}} \), could empirically be determined based on these data. The \( \varphi_{\text{FGF2}} / \varphi_{\text{PEG-FGF2}} \) ratio was expected to be \(<1\) if the \( \varphi \) value reflected only a change in diffusivity, assuming elimination remained constant. However, instead, the ratio \( \varphi_{\text{FGF2}} / \varphi_{\text{PEG-FGF2}} = 1.7 \), suggesting that PEGylation of FGF2 caused significantly reduced elimination of the protein. Thus, the greater tissue penetration observed for PEG-FGF2 was ascribed to reduced elimination.

5.4 Spinal Cord Blood Flow Imaging Strategies

The primary goal of this thesis was to achieve localized neuroprotective drug delivery to the injured spinal cord. However, since FGF2 mechanistically impacts vasculature in tissue, a method to functionally assess blood flow and permeability was desired to better understand its local delivery. Several methods have been utilized for imaging SCBF, including xenon [60, 61] and hydrogen [53, 62] clearance methods and autoradiographical methods [50, 64]. All of these methods are limited to either single session or terminal measurements of SCBF, leading to a large quantity of animals needed. Also, since variability between animals can be high for such measurements, several measurements of SCBF in the same animal were desirable within 1 w, which is the time frame when post-traumatic angiogenesis occurs. We first investigated ultrasound imaging paired with laser Doppler to image blood flow through spinal cord tissue. This method seemed promising, since an external probe applied to the muscle tissue over the cord could be used for measurement. Measurements from individual vessels of arterial and venous flow could be observed in the healthy spinal cord using this method (Appendix 3, Figure 0.5), but several difficulties with this method were encountered. Although the probe could be
placed on the skin surface, high frequencies were necessary to probe as far as the spinal cord, and much of the signal was lost due to the shadow of the laminae that were not removed. Other groups that do this type of imaging apply the probe directly to the spinal cord surface, with the muscle and fascia removed [278, 316], which is invasive and impractical. For this reason, we pursued CT imaging to study blood flow dynamics, a technique which also allowed us to study permeability.

CT imaging does not require a probe to be applied to the tissue; instead it provides sectional images of the whole body. When a contrast agent is injected into the bloodstream close to the heart, the increase in density as the tracer moves through the tissue slice is detected, and the perfusion rate can be derived from this first pass through the tissue. Based on the concentrations that accumulate in the tissue, the permeability can be assessed, since these tracers are generally not very permeable across the uninjured BSCB. Contrast agents used in CT are approved clinically for blood flow assessments, and most compounds are quickly filtered from the blood via the kidney, and therefore have low systemic toxicity. Dynamic CT imaging has been used in several applications in both animal and human studies. Initially the method was used to investigate renal blood flow [68, 279, 280], but further technical developments allowed measurements of tumor blood flow in cerebral tumors [67, 73], lung cancers [72, 74], as well as renal and hepatic metastases [68, 70, 71]. A recent report describes the first use of CT perfusion for SCBF in humans where different software packages for calculating SCBF and permeability were tested to investigate the reproducibility of these measurements [66]. This study showed that CT perfusion provided reasonable reliability for spinal cord blood flow of the cervical spinal cord region in humans. We tested this method in rats, where their small size requires greater sensitivity and resolution in CT. Most small animal studies dealing with SCI treatements involve histological or immunohistochemical examination of tissue, which is not practical for clinical studies. Developing a minimally invasive tool for measuring SCBF and permeability in humans and small animals would provide a unique ability to compare the impacts of SCI treatment between species. Chapter 4 describes in detail the mathematical models which underlie the calculations for SCBF and permeability measurements. The SCBF parameter is easily determined using first-pass tissue and arterial influx parameters for contrast agents that are not very permeable across the BSCB, such as the iodinated compound that was employed. This method has been used for renal blood flow calculations [68, 70], and SCBF values calculated
using this model showed similar values in rats to those calculated using the hydrogen clearance method [53]. SCBF decreased following injury as previously reported [50, 53, 316], and hyper-reperfusion was observed 1 w after injury. The modified Toft's model was identified as an appropriate method for assessing the permeability-capillary surface area product, in which the intravascular and extravascular spaces are viewed as separate compartments. These data showed an increase in permeability at 1 d post-injury, which has been similarly demonstrated in other SCI models [55, 317, 318]. These characteristic trends of blood flow and permeability over time that have been shown using other methods support the data obtained with dynamic CT imaging, suggesting that this is a valid measurement technique for these parameters. Dynamic CT imaging is an important method by which functional tissue recovery can be assessed, rather than static measurements provided by immunohistochemical and histological data. Additionally, CT imaging improves on existing methods for SCBF and permeability measurements since it can be done serially on the same animal, thus reducing the size of animal studies. This data and recent advances in human SCBF measurements with dynamic CT may also provide a unique opportunity to directly compare tissue outcomes in clinical studies with the preclinical small animal studies that precede them.

5.5 Achievement of Objectives

The hypothesis originally proposed for this research was:

Localized delivery of neuroprotective agents with hyaluronan and methylcellulose will mitigate tissue damage after spinal cord injury.

This dissertation describes the use of EPO and FGF2 as neuroprotective agents to assess the utility of HAMC for localized delivery after SCI. Below is a summary of work completed toward achieving each of the objectives defined at the beginning of this document.

1. **Characterize the in vitro and in vivo release of neuroprotective agents from HAMC**

The release mechanism of proteins and water-soluble small molecules from HAMC was determined to be diffusion mediated based on in vitro data. These results suggested that HAMC was an ideal drug delivery system for neuroprotective agents in the acute phase of secondary
injury after SCI. *In vivo* release of FGF2 from HAMC resulted in therapeutic concentrations of FGF2 penetrating into injured spinal cord tissue. PEGylation of FGF2 enhanced spinal cord tissue penetration by nearly twofold when delivered locally from HAMC.

2. **Determine the extent of *in vivo* neuroprotection achieved following localized delivery of neuroprotective agents to the injured spinal cord**

HAMC delivery of EPO, a neuroprotective that crosses the blood brain barrier, showed that a greater number of motor neurons were spared after SCI relative to intraperitoneal or intrathecal bolus injections. A reduction of cavity size was also observed, suggesting that localized delivery of EPO had a tissue sparing effect. FGF2 is a protein that does not cross the intact blood brain barrier, and its local delivery with HAMC was compared to intrathecal injections of HAMC alone and aCSF. Relative to these controls, FGF2 delivered with HAMC enhanced spinal cord blood flow and reduced the permeability-surface area product at sites rostral to the epicenter. These data suggests that better functional tissue outcomes can be achieved using HAMC as a local delivery system.

3. **Develop a live animal imaging strategy to assess spinal cord hemodynamics after delivery of neuroprotective agents with HAMC**

Both ultrasound paired with laser Doppler and dynamic CT imaging were explored as methods for assessing SCBF. Dynamic CT imaging required less invasive techniques and was utilized to investigate SCBF and the permeability-surface area product in the uninjured and injured spinal cord after localized delivery of FGF2 with HAMC.

Taken together, it is clear that HAMC is an appropriate delivery system for neuroprotective agents that affect secondary injury events within the first day of injury. Proteins that do or do not cross the blood spinal cord barrier can be delivered locally with HAMC to mitigate tissue damage following SCI, demonstrated by neuron sparing achieved with EPO delivery and improved blood flow and reduced permeability with FGF2 delivery.
5.6 Conclusions

SCI is a debilitating condition for which there is a lack of safe and effective treatment strategies. The secondary injury that occurs after the initial mechanical trauma creates a complex environment that will likely require a combination of pharmacological intervention strategies to result in a significant amount of functional recovery. Our lab previously developed a polymeric blend of HA and MC that was assessed \textit{in vitro} and \textit{in vivo} to be safe for use as a minimally invasive intrathecal drug delivery system. This dissertation describes the \textit{in vivo} potential of HAMC to locally deliver neuroprotective proteins to the injured spinal cord. Release of neuroprotective proteins EPO and FGF2 was determined \textit{in vitro} to occur within the first 16 h after injection. Intrathecal drug delivery with HAMC maintained protein concentrations on the surface of the injured spinal cord for several hours, resulting in effective protein concentrations to diffuse into tissue. Localized delivery of these proteins with HAMC led to neuroprotection and improved tissue outcomes relative to controls. In addition, dynamic CT imaging was utilized for the first time to functionally assess SCBF and permeability in live animals, a tool that could be used as a clinical comparison between animal and human studies of SCI. Taken together, these results demonstrate that the current formulation of HAMC is an effective and minimally invasive drug delivery system for delivery of neuroprotective agents to the injured spinal cord. Its use could be clinically practical for acute SCI treatment following decompressive surgery performed in the first 24 h after injury, although further work is necessary to determine the optimal combination of drugs or proteins that will be most effective at improving functional recovery.
6 Limitations and Recommendations for Future Work

6.1 Extending Delivery Window for HAMC

Experimental data shown in this dissertation clearly indicate that HAMC has significant potential as an intrathecal drug delivery system. Neuroprotective agents that can limit the early tissue impacts of the secondary injury can be effectively delivered to the injured spinal cord with HAMC. However, one limitation of HAMC is that drug release is largely diffusion-mediated, thus only short term drug delivery can be achieved for molecules that are simply mixed into the HAMC blend. Since HAMC only remains in the intrathecal space for 4-7d, conjugation of a drug or protein to the polymer chains would still generally be limited to delivery during the acute phase of secondary injury, which can last for up to a month. Additionally, a combinatorial strategy to maximize the utility of HAMC would include neuroregenerative agents that could stimulate tissue regrowth after the injury has been resolved. To deliver these molecules from HAMC would require: 1) reformulation or crosslinking of the hydrogel network to extend the useful delivery lifetime and 2) a method of controlling the release rate for at least 30 d to deliver neuroregenerative agents.

6.1.1 Define Mechanism for HAMC Elimination from CNS

To adequately develop HAMC as a drug delivery system, it is important to understand how both HA and MC are eliminated from the intrathecal space. Both components were initially thought to dissolve into the CSF and be cleared through the arachnoid granulations, where CSF normally enters the bloodstream. However, HA was eliminated from the gel more quickly than expected, suggesting that perhaps it is degraded by free radicals or enzymes that enter through the hemorrhage following injury. While HA may play a biological role as a therapeutic polymer as previously discussed, understanding both this role and the mechanism by which it is eliminated is important for further development of the gel. For example, if it does play an early biological role, then crosslinking HA to increase the \textit{in vivo} lifetime would be undesirable. Confirming that MC is not enzymatically degraded may be important for the same reasons.
6.1.2 Extend *In vivo* Lifetime of HAMC

Increasing the wt% of the polymer in the gel would likely increase the stability of the gel; however, it also reduces the injectability of the material, an important property for its clinical relevance. Both HA and MC in the formulation employed in this thesis were of relatively high molecular weight; however, both polymers are commercially available at higher molecular weights. Increasing the chain length of individual polymers that are interacting, namely MC, could increase the lifetime by limiting chain dissolution into CSF. Since each MC polymer unit possesses several hydrophobic moieties, and hydrophobic association is a dynamic process, increasing the molecular weight would allow for re-association of some hydrophobic moieties before the chain becomes disentangled from the polymer network and dissolves away [254]. Also, increasing the chain length of MC makes the path length of diffusion from the gel more tortuous, thus increasing the time for dissolution of individual polymer chains.

To slow degradation of polymers, chemical crosslinking is commonly used. This could be done by chemically crosslinking either MC or HA, or by crosslinking HA to MC. This could be done by chemically modifying the free hydroxyls or carboxyls of HA and MC with reactive moieties to induce crosslinking, either through a spontaneous mechanism or using an initiator. However, chemical crosslinking can be toxic when performed in situ. Additionally, chemical crosslinking often increases the swelling behavior of the material, since chains are no longer able to dissolve out of the gel and are bound together, producing a large mesh size where water can accumulate. This property would need to be limited for the intrathecal application so further compression of the cord does not occur upon injection.

6.1.3 Controlled Release

Extending the release of molecules from polymeric devices can be done using a variety of techniques. Utilizing strong intermolecular interactions, such as the heparin-FGF2 interaction is one strategy where release is mediated by dissociation rates of the interacting pairs [319]. Another method could be attach proteins or drugs to the polymer backbone via enzymatically cleavable side chains [320], where release could be mediated by the presence of degrading enzymes during the secondary injury of SCI. A method to significantly increase the length of
time that drugs can be delivered is by encapsulating them into solid polymer particles. Release can be mediated by surface or bulk erosion of the particles and the particles can be synthesized in varying sizes, even into the nanometer size range. Release can be extended for weeks to months with polymeric microparticles, potentially allowing for effective neuroregenerative therapies in SCI patients.

Our lab has pursued some of these avenues and further development of HAMC has shown promise in vitro for both extended stability and long term delivery [209]. Higher molecular weight HA and MC in the HAMC blend, paired with poly(lactide-co-glycolide) nanoparticles, swell similarly to the original formulation of HAMC that was pursued here, degrades in vitro beyond 56 d. Polymeric particles embedded in the hydrogel can tailor the delivery of molecules for days to weeks, suggesting that this new composite HAMC can provide sustained and localized release of therapeutic agents after SCI. In vivo data suggests that it is safe and biocompatible for this purpose [321].

6.2 Drug Choice Matrix

Several neuroprotective agents are being investigated for treatment of SCI, many of which are summarized in Table 1.3. While HAMC has proved to be a useful material for delivery of two neuroprotective proteins, significant functional recovery was not observed with either of these molecules. This could be due to many factors, including dosage delivered, rate of delivery, or timeframe of delivery. However, due to the complexity of the secondary injury, it is unlikely a single molecule will be able to mitigate damage in the spinal cord and provide satisfactory functional recovery for patients. This will likely require a combinatorial strategy, whereby several of the secondary injury events can be targeted. Although reports for many of these molecules claim to produce functional recovery, and perhaps statistically better than controls, this is a phenomenological exploration, not a systematic and mechanistic approach to delivering an appropriate dose for an adequate amount of time to achieve long term, reproducible functional recovery. In addition, functional recovery is sometimes not reproducible when the studies are repeated in a different environment [312, 322]. For this reason, a systematic approach is proposed here to determine optimal combinations of drugs based on literature support and further testing.
The events of secondary injury and molecules that mediate these events can be categorized into the following: (1) ion channel antagonists to provide protection from excitotoxicity, (2) inflammatory and immune regulators, (3) membrane stabilizers and antioxidants, (4) blood flow mediators and angiogenic agents, (5) antiapoptotic factors and cytoskeletal stabilizers, (6) neuroregenerative agents, and (7) scar reducing agents. Each drug, which would be categorized according to these secondary injury events above, could then be assessed based on a set of weighted criteria to assign a relative value of likelihood that it would be optimal for delivery with the new formulation of HAMC [209]. For example, each drug should be assessed and assigned a numerical score based on (1) its ability to cross the BSCB, (2) the extent of reported systemic side effects, (3) whether clinical trials have been reported for the molecule (in SCI or otherwise), (4) whether the timeframe of action is clinically relevant, (5) whether it has a spectrum of mechanistic actions, and (6) the strength of the literature support for its use in SCI. The score given in each category would then be multiplied by the weighting for each assessment (based on importance). The sum of these products for each molecule provides a numerical value that could then be used to rank molecules within a category. An optimal strategy for determining an effective combination of drugs would be to select drugs from a number of these categories so that the combination could have a wide range of actions following SCI. This type of assessment would require an immense amount of literature research to determine an assessment for each molecule; however, it would also provide an unbiased and systematic approach to determining a combination of factors that could provide the greatest impact after SCI.

6.3 Development of CT for SC hemodynamics

Dynamic CT imaging has been applied here to measure SCBF and permeability in rats. While this has advantages over several other techniques for these measurements, further optimization would be ideal for both practical issues and signal optimization. Dynamic CT imaging is a minimally invasive and easily applied method in humans because intravenous administration of contrast agents can be done quite easily. However, intravenous administration is more difficult in small animals due to the size of vessels that are readily available and go directly to the heart. In these studies, we attempted to use tail vein administration of contrast agents in rats, however, successful injections were not possible since the catheters were difficult to insert into the vein.
and often the catheters did not remain in the vein during the injection of the contrast agent. To avoid these issues, animals with implanted catheters were used, where contrast was delivered directly to the heart through catheters inserted into the jugular vein. This is a reproducible and accepted method of catheterizing small animals; however, it does require a surgical procedure that is not minimally invasive, and thus not ideal. Additionally, catheters would sometimes become blocked with clotted blood, at which times the animals could no longer be infused with contrast for CT imaging. These are significant issues and considerable experience is required to perform these studies in small animals.

The SCBF measurements obtained were similar to previously published values, suggesting that dynamic CT is a valid technique. However, many previous studies of SCBF have shown considerable variability, often correlated to changes in other vital signs, such as mean arterial blood pressure, hematocrit, and body temperature [53]. In the present study, variability was also observed, which may have been due to these other physiological parameters, but since we could not measure them during CT imaging, they could not be correlated. Permeability measurements had very high variability, which was more closely correlated to low signal-to-noise ratio and low sensitivity. These could potentially be improved by varying the x-ray exposure factors, such as the tube voltage and current. These parameters should be optimized to reduce noise in the signal, thus allowing more consistent analysis for permeability parameters.

6.4 Limitations for Bench to Bedside Translation

HAMC has proven a useful tool in animal studies for local administration of therapeutic agents, where relevant concentrations of proteins were achieved within the tissue, and better neuroprotection was achieved over systemic or intrathecal delivery. However, as described previously, there are several factors that make it non-ideal for clinical use as of yet. Reformulation of HAMC and its combination with particles could significantly improve the in vivo duration and drug delivery capacity; however, these remain to be tested. The formulation used in these studies would likely not deliver drugs for a sufficiently long period in humans to offer significant functional benefit, and diffusion distances in humans are also much larger than those in small animals. Large animal studies to better understand the pharmacokinetics and
tissue distribution in vivo relevant to human studies are necessary for utilization in a clinical setting.

Many small animal studies performed do not truly replicate practical timelines for clinical studies, one of the potential reasons for many clinical trials failing to show functional improvement. Animal studies are often performed with drug treatment immediately after SCI. However, patients may not be …to receive treatment until several hours after injury, thus making correlation to pre-clinical data difficult. Similarly, HAMC was applied immediately post-injury in these small animal studies, whereas current recommendations for decompressive surgery in humans is 8-24 h after injury, the time at which HAMC would optimally be applied clinically. At this stage in the secondary injury local delivery of many excitotoxic neuroprotectants, such as NBQX, would likely have little impact since excitotoxicity would have already affected neurons in a large penumbra of tissue. This suggests that HAMC may be better suited to deliver neuroprotective agents that attack secondary injury events which occur at least 24 h after the primary injury. In addition, to make animal studies involving HAMC more clinically relevant, intrathecal injection should be done 24 h after injury, rather than immediately following injury. While this is not ideal from the viewpoint of limiting damage after injury, it makes these studies more likely to indicate the true potential of localized drug delivery with HAMC.
References & Bibliography


22. M. Eddleston and L. Mucke, Molecular profile of reactive astrocytes--implications for their role in neurologic disease, Neuroscience. 54 (1993) 15-36


29. R.B. Borgens, A.R. Blight, D.J. Murphy, and L. Stewart, Transected dorsal column axons within the guinea pig spinal cord regenerate in the presence of an applied electric field, J Comp Neurol. 250 (1986) 168-80


42. E.J. Dolan and C.H. Tator, A new method for testing the force of clips for aneurysms or experimental spinal cord compression, J Neurosurg. 51 (1979) 229-33

44. J.A. Gruner, A monitored contusion model of spinal cord injury in the rat, J Neurotrauma. 9 (1992) 123-6; discussion 126-8


52. L.J. Noble and J.R. Wrathall, Distribution and time course of protein extravasation in the rat spinal cord after contusive injury, Brain Res. 482 (1989) 57-66


58. S.S. Kety, The theory and applications of the exchange of inert gas at the lungs and tissues, Pharmacol Rev. 3 (1951) 1-41

59. S.S. Kety and C.F. Schmidt, The nitrous oxide method for the quantitative determination of cerebral blood flow in man; theory, procedure and normal values, J Clin Invest. 27 (1948) 476-83

60. I.R. Griffiths, Spinal cord blood flow in dogs. 1. The 'normal' flow, J Neurol Neurosurg Psychiatry. 36 (1973) 34-41


68. K.A. Miles, Measurement of tissue perfusion by dynamic computed tomography, Br J Radiol. 64 (1991) 409-12


70. K.A. Miles, M.P. Hayball, and A.K. Dixon, Functional images of hepatic perfusion obtained with dynamic CT, Radiology. 188 (1993) 405-11


80. S.P. Hicks and C.J. D'Amato, Motor-sensory cortex-corticospinal system and developing locomotion and placing in rats, Am J Anat. 143 (1975) 1-42


118


84. D.M. Basso, M.S. Beattie, and J.C. Bresnahan, A sensitive and reliable locomotor rating scale for open field testing in rats, J Neurotrauma. 12 (1995) 1-21


91. D. Wu, B.W. Song, H.V. Vinters, and W.M. Pardridge, Pharmacokinetics and brain uptake of biotinylated basic fibroblast growth factor conjugated to a blood-brain barrier drug delivery system, J Drug Target. 10 (2002) 239-45


123. L.J. Rosenberg, Y.D. Teng, and J.R. Wrathall, Effects of the sodium channel blocker tetrodotoxin on acute white matter pathology after experimental contusive spinal cord injury, J Neurosci. 19 (1999) 6122-33


131. J.E. Wells, R.J. Hurlbert, M.G. Fehlings, and V.W. Yong, Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice, Brain. 126 (2003) 1628-37


133. J. Luo, R. Borgens, and R. Shi, Polyethylene glycol immediately repairs neuronal membranes and inhibits free radical production after acute spinal cord injury, J Neurochem. 83 (2002) 471-80


140. R. Vavrek, J. Girgis, W. Tetzlaff, G.W. Hiebert, and K. Fouad, BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats, Brain. 129 (2006) 1534-45


150. D.C. Baptiste and M.G. Fehlings, Pharmacological approaches to repair the injured spinal cord, J Neurotrauma. 23 (2006) 318-34


155. E. Morishita, S. Masuda, M. Nagao, Y. Yasuda, and R. Sasaki, Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death, Neuroscience. 76 (1997) 105-16


167. E.J. Demers, R.J. McPherson, and S.E. Juul, Erythropoietin protects dopaminergic neurons and improves neurobehavioral outcomes in juvenile rats after neonatal hypoxia-ischemia, Pediatr Res. 58 (2005) 297-301


175. F.J. Moy, A.P. Seddon, P. Bohlen, and R. Powers, High-resolution solution structure of basic fibroblast growth factor determined by multidimensional heteronuclear magnetic resonance spectroscopy, Biochemistry. 35 (1996) 13552-61


177. S.J. Prestrelski, G.M. Fox, and T. Arakawa, Binding of heparin to basic fibroblast growth factor induces a conformational change, Arch Biochem Biophys. 293 (1992) 314-9


179. D. Gospodarowicz and J. Cheng, Heparin protects basic and acidic FGF from inactivation, J Cell Physiol. 128 (1986) 475-84


183. A.L. Vescovi, B.A. Reynolds, D.D. Fraser, and S. Weiss, bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells, Neuron. 11 (1993) 951-66


186. P. Follesa, J.R. Wrathall, and I. Mocchetti, Increased basic fibroblast growth factor mRNA following contusive spinal cord injury, Brain Res Mol Brain Res. 22 (1994) 1-8

187. K. Nozaki, S.P. Finklestein, and M.F. Beal, Basic fibroblast growth factor protects against hypoxia-ischemia and NMDA neurotoxicity in neonatal rats, J Cereb Blood Flow Metab. 13 (1993) 221-8

188. T.T. Lee, B.A. Green, W.D. Dietrich, and R.P. Yezierski, Neuroprotective effects of basic fibroblast growth factor following spinal cord contusion injury in the rat, J Neurotrauma. 16 (1999) 347-56


204. R. Langer, L.G. Cima, J.A. Tamada, and E. Winternantel, Future directions in biomaterials, Biomaterials. 11 (1990) 738-45


215. Y. Katayama, Prolonged release of BDNF from PLGA Microspheres Dispersed within a PEG hydrogel, in Chemical Engineering. 2003, University of Toronto: Toronto.


232. M.C. Tate, D.A. Shear, S.W. Hoffman, D.G. Stein, and M.C. LaPlaca, Biocompatibility of methylcellulose-based constructs designed for intracerebral gelation following experimental traumatic brain injury, Biomaterials. 22 (2001) 1113-23


261. F. Stohlman, Jr., S. Ebbe, B. Morse, D. Howard, and J. Donovan, Regulation of erythropoiesis. XX. Kinetics of red cell production, Ann N Y Acad Sci. 149 (1968) 156-72


270. Q. Yun, W.C. Xing, G.G. Mal, and Z.G. Su, Preparation and characterization of mono-
PEGylated consensus interferon by a novel polyethylene glycol derivative, Journal of
Chemical Technology and Biotechnology. 81 (2006) 776-781

271. M.F. Haller and W.M. Saltzman, Localized delivery of proteins in the brain: can transport

272. C. Nicholson, Diffusion and related transport mechanisms in brain tissue, Reports on
Progress in Physics. 64 (2001) 815-884

273. M.J. Mahoney and W.M. Saltzman, Controlled release of proteins to tissue transplants for
the treatment of neurodegenerative disorders, J Pharm Sci. 85 (1996) 1276-81

274. M. Mak, L. Fung, J.F. Strasser, and W.M. Saltzman, Distribution of drugs following
controlled delivery to the brain interstitium, J Neurooncol. 26 (1995) 91-102

275. C.M. Bernards and H.F. Hill, Morphine and Alfentanil Permeability through the Spinal
Dura, Arachnoid, and Pia Mater of Dogs and Monkeys, Anesthesiology. 73 (1990) 1214-
1219

276. M.E. Young, P.A. Carroad, and R.L. Bell, Estimation of Diffusion-Coefficients of
Proteins, Biotechnology and Bioengineering. 22 (1980) 947-955

277. A.N. Sandler and C.H. Tator, Review of the measurement of normal spinal cord blood
flow, Brain Res. 118 (1976) 181-98

278. H. Toda, H. Maruyama, B. Budgell, and M. Kurosawa, Responses of dorsal spinal cord
blood flow to noxious mechanical stimulation of the skin in anesthetized rats, J Physiol
Sci. 58 (2008) 263-70

279. A.M. Peters, J. Brown, G.G. Hartnell, M.J. Myers, C. Haskell, and J.P. Lavender, Non-
invasive measurement of renal blood flow with 99mTc DTPA: comparison with
radiolabelled microspheres, Cardiovasc Res. 21 (1987) 830-4

280. A.M. Peters, R.D. Gunasekera, B.L. Henderson, J. Brown, J.P. Lavender, M. De Souza,
J.M. Ash, and D.L. Gilday, Noninvasive measurement of blood flow and extraction
fraction, Nucl Med Commun. 8 (1987) 823-37

281. R. Hebel and M.W. Stromberg, Anatomy and Embryology of the Laboratory Rat, 2nd ed.
BioMed Verlag, Worthsee, 1986. 261

effect of insulin in the control of cell death and neurologic deficit after acute spinal cord

283. M. Murakami and M. Simons, Fibroblast growth factor regulation of neovascularization,


287. D. Presti and J.E. Scott, Hyaluronan-mediated protective effect against cell damage caused by enzymatically produced hydroxyl (OH.) radicals is dependent on hyaluronan molecular mass, Cell Biochem Funct. 12 (1994) 281-8


290. T.S. Reese and M.J. Karnovsky, Fine structural localization of a blood-brain barrier to exogenous peroxidase, J Cell Biol. 34 (1967) 207-17


297. B.C. Hancock and M. Parks, What is the true solubility advantage for amorphous pharmaceuticals? Pharmaceut Res. 17 (2000) 397-404


301. T.B. Csoka, G.I. Frost, and R. Stern, Hyaluronidases in tissue invasion, Invasion Metastasis. 17 (1997) 297-311


Appendix 1: Glossary

**Angiogenesis**: a physiological process involving the growth of new vessels from existing vessels.

**Apoptosis**: programmed cell death whereby cellular debris is limited and has less impact on surrounding tissue. This is a genetically controlled process, although it can be affected by biochemical events external to the cell.

**Astrocytic scar**: see **Glial scar**

**Bioactivity**: the ability of a molecule to perform its intended biological purpose.

**Blood-spinal cord barrier**: a cellular and molecular barrier that exists between blood and spinal cord tissue; all blood vessels entering the CNS create this barrier which allows very little molecular diffusion and passage from blood to tissue is limited by active transport mechanisms.

**Cerebrospinal fluid**: a clear fluid in the intrathecal space to cushion the brain and spinal cord.

**Diffusivity**: the extent to which a molecule can move through a given space.

**Edema**: abnormal fluid accumulation in the extracellular, extravascular space.

**Elimination**: removal of proteins from active diffusion, either through degradation or cellular binding/uptake.

**Endogenous**: originating from within the organism of interest.

**Equilibrium**: A stable situation in which forces cancel one another; a state of dynamic systems in which there is no net change.

**Exogenous**: originating from outside of the organism of interest.

**Free Radical**: molecules with unpaired electron shells which readily react and cleave other molecules to fill their electron shells.

**Glial Scar**: a network of astrocytes that forms to seal healthy tissue from injury in the CNS.

**Hypoxia**: A deficiency of oxygen in biological tissues.
**Intraperitoneal injection:** injection into the body cavity (peritoneum). This is most commonly performed in animals for systemic drug administration.

**Intrathecal space:** the space between the brain/spinal cord tissue and the membranes (dura/arachnoid mater) that envelop the tissue; the space in which CSF flows.

**Ischemia:** A deficiency of oxygen due to a lack of blood flow to a tissue.

**Immunogenicity:** the ability of a molecule to induce an immune response in the body.

**Necrosis:** the premature death of cells caused by injury and infection; characterized by cellular debris build-up in the surrounding area of dying cells.

**Neuroprotection:** mechanisms which protect neurons and neural tissue from degenerating after injury or infection; characterized by sparing of neurons or axons and reduced lesion volumes.

**Parenchyma:** a general description of tissue including all cell types and tissue regions.

**Penumbra:** the area surrounding the traumatized tissue in which the secondary injury leads to significant cell death and degeneration.

**Secondary Injury:** the cascade of biochemical events that occurs after mechanical trauma in spinal cord tissue; characterized by inflammation, edema, hypoxia, ischemia, and cell death.

**Shear thinning:** a property of a material where viscosity temporarily decreases as shear rate increases.

**Systemic:** affecting the entire bodily system, usually via the circulatory system.

**Trophic:** a growth-promoting property.

**Vasoconstriction:** narrowing of blood vessels due to contraction of muscular walls around the vessels.
Appendix 2: Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BBB</td>
<td>Basso, Beattie, and Bresnahan open field locomotor scoring method</td>
</tr>
<tr>
<td>BSCB</td>
<td>Blood-spinal cord barrier</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug delivery system</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast growth factor 2 also known as basic Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate (fluorescent tag)</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GM-1</td>
<td>Monosialotetrahexosylganglioside</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>HAMC</td>
<td>Hyaluronan and methylcellulose blend</td>
</tr>
</tbody>
</table>
IP  Intraperitoneal
IT  Intrathecal
IU  International units
LFB  Luxol fast blue
MC  Methylcellulose
MP  Methylprednisolone sodium succinate
MRI  Magnetic resonance imaging
MW  Molecular weight
NMR  Nuclear magnetic resonance
NSCISC  National Spinal Cord Injury Statistical Center
PBS  Phosphate buffered saline
PEG  Poly(ethylene glycol)
PEG-FGF2  Poly(ethylene glycol) conjugated to FGF2
PEG-mal  Poly(ethylene glycol)-maleimide
PNS  Peripheral Nervous system
PS  Permeability – surface area product
SAS  Subarachnoid space
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SCBF  Spinal cord blood flow
SCI  Spinal cord injury
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfo-NHS</td>
<td>N-hydroxysulfosuccinimide sodium salt</td>
</tr>
<tr>
<td>TM</td>
<td>Tirilizad mesylate</td>
</tr>
<tr>
<td>T1/2</td>
<td>Thoracic vertebrae 1&amp;2</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Appendix 3: Additional Data

Figure 0.1: Protein release from HAMC is linearly related to $t^{0.5}$ for >60% release.

Figure 0.2: NBQX and solubilized nimodipine (with ethanol) release from HAMC. Inset shows linear relationship with $t^{0.5}$ for >60% of release.
Figure 0.3: Nimodipine release from HAMC for solid nimodipine particulates sonicated to 380nm and 900nm. Biphasic release profile was observed where fast release was observed in the first 8 h (solid line = 380nm, dashed line = 900nm) and was likely due to soluble nimodipine fraction saturating HAMC. Slow release was observed for 3 d (small dashed line = 380 nm, large dashed line = 900 nm) likely due to fraction released as surface of particles were solubilized into surrounding media.
Figure 0.4: Comparison of nimodipine solubility in MC and HA, with different nimodipine particle sizes: (◆) 100-μm nimodipine particles in 0.25 wt% HA; and (□) 100-μm, (▲) 900-nm, and (○) 380-nm nimodipine particles in 7 wt% MC. The upper shaded concentration range indicates nimodipine solubility values achieved in 7 wt% MC, while the lower shaded range indicates aqueous nimodipine solubility reported in the literature.
Figure 0.5: (A) Longitudinal echogram imaging of the spinal cord where brightness indicates density of the tissue. The bright spots show the dense bones of the vertebrae surrounding the spinal cord; yellow line indicates where the laminectomy was performed to expose the spinal cord. The spinal cord tissue can be observed between the vertebrae only below where the laminae have been removed. (B) Arterial flow from vessel in spinal cord. (C) Venous flow from vessel in spinal cord.