FABRICATION AND EVALUATION OF A TYROSINE-DERIVED POLYCARBONATE CONDUIT TO ENHANCE FUNCTIONAL RECOVERY OF A 5 MM PERIPHERAL NERVE GAP IN A MOUSE FEMORAL NERVE MODEL

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And approved by

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ABSTRACT OF THE DISSERTATION

FABRICATION AND EVALUATION OF A TYROSINE-DERIVED POLYCARBONATE CONDUIT TO ENHANCE FUNCTIONAL RECOVERY OF A 5 MM PERIPHERAL NERVE GAP IN A MOUSE FEMORAL NERVE MODEL

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Each year, over 200,000 people in the United States are treated for peripheral nerve injuries requiring surgery. Several nerve guidance conduits (NGCs) have been approved by the FDA, however, when used to repair critical size defects, regeneration results in limited functional recovery and poor quality. Therefore, a conduit fabricated from a material encouraging regeneration, specifically enhancing neurite outgrowth and functional recovery is required. Tyrosine-derived polycarbonates (TyrPCs) are biodegradable and biocompatible polymers offering a unique chemistry that allows for the optimization of their chemical, mechanical, and cellular properties for a specific application. These materials have been used in several medical devices and are effective at supporting neurite outgrowth in vitro. Additionally, peptide mimics of HNK-1 elucidated by the Schachner laboratory show significant promise when used in soluble form within conduits used to treat short defects. For critical size defects, soluble HNK-1
may not suffice; the mimic may diffuse away from the injury site. Thus, the goal of this research was twofold: 1) to develop a TyrPC NGC to treat critical size nerve defects and 2) to establish alternative methods of HNK-1 delivery. Three methods were explored: a collagen hydrogel filler grafted with HNK-1 (developed and provided by the Shreiber laboratory), the secretion of HNK-1 from genetically engineered stem cells, and slow release of HNK-1 from the NGC outer walls. TyrPC was compared to commercially available polyethylene in vitro and conduits fabricated from both materials were evaluated in the mouse femoral nerve model. In vitro results indicated greater protein adsorption and neurite outgrowth on TyrPC as compared to polyethylene. In vivo results showed improved functional recovery and quality of nerve regeneration in animals treated with TyrPC and suggested greater Schwann cell presence and fibrin matrix formation. Furthermore, in vitro results confirmed usefulness of 2 new methods for HNK-1 delivery, release from stem cells and the NGC itself. In vivo studies demonstrated that the influence of a collagen hydrogel with and without HNK-1 depends upon the TyrPC nerve conduit structure: whether or not the conduit was porous. In conclusion, conduits fabricated from TyrPC offer the potential for treatment of critical size nerve gaps.
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To my family and friends who have supported me along this journey,

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Figure 5.3: Analysis of motor neuron enriched spinal cord population viability and outgrowth in vitro. (A) 20X image of rat spinal cord neurons seeded on 2D control coverslip coated with PLL and Laminin in neurobasal media. (B) 20X image of rat spinal cord neurons seeded on 2D control coverslip coated with PLL and Laminin in 50% neurobasal media, 50% conditioned media from peptide engineered MSCs. (C) Total live neurite cell count on coverslips treated with different media (control neurobasal or conditioned media). Values represent averages ± standard deviation. (D) Neurite outgrowth distribution on coverslips treated with different media (control neurobasal or conditioned medium) as determined by β-III-tubulin. All three engineered MSC’s conditioned media (blue, green, red lines) cause a shift to the right of the neurite outgrowth distribution as compared to the control conditions (black line) indicating greater neurite outgrowth.

Figure 5.4: Retrograde labelling technique (A) Retrograde labelling of the sensory branch (red) and motor branch (yellow) is used to follow projection of neurons. A different color tracer is applied to each branch of the femoral nerve and is allowed to transport back to the spinal cord. (B) Section of a spinal cord marked with retrograde tracers in the ventral root, indicative of motoneurons correctly projecting through the nerve conduit to the motor branch of the femoral nerve. (C) 3 cell bodies are seen in the magnified image.

Figure 5.5: HNK-1 effect on E15 rat spinal cord neurons cultured for 48 hours (A) Total live neurite cell count in neurobasal media spiked with soluble HNK-1 at 3 concentrations, 0nM (Control), 9.7 nM (10 ng/mL), 97 nM (100 ng/mL), and 970 nM (1 µg/mL). Values represent averages ± standard deviation. (B) Neurite distribution in media spiked with soluble HNK-1 at 3 concentrations, 0nM (Control), 9.7 nM (10 ng/mL), 97 nM (100 ng/mL), and 970 nM (1 µg/mL). All three concentrations of HNK-1 cause a shift in the neurite distribution to the right of the control indicating greater neurite outgrowth.

Figure 5.6: HNK-1 stability in simulated body fluid. Samples were evaluated via HPLC at days 0, 1, 3, 7, and 14 in order to determine if the peptide mimic is stable in this solution over 2 weeks.

Figure 5.7: Residual amounts of HNK-1 in conduits as detected by HPLC. Three polymer compositions were evaluated: E0004(1K) (red square), E0502(1K) (green triangle), and E1001(1K) (purple star). Conduits were fabricated with an initial loading of 4.4 µg HNK/mg of polymer. Values represent averages ± standard deviation. Results show similar release kinetics for all three types of conduits fabricated.

Figure 5.8: Neurite outgrowth distribution of motor enriched spinal cord neurons cultured in media conditioned by TyrPC conduits containing HNK-1 for 7 days. All three release mediums are effective at enhancing outgrowth as indicated by the shift of the all three experimental peaks (E0004, E0502, E1001) to the right of the control peak.
Figure 5.9: Scanning electron microscope (SEM) images of conduits fabricated from TyrPC. (A and B) Outer wall and cross section of a conduit fabricated from E0004(1K), respectively. (C and D) Outer wall and cross section of a conduit fabricated from E0004(1K) with HNK-1 peptide mimic, respectively. Scale bar: 100 µm.

Figure 5.10: Metrics of functional recovery promoted by HNK-1 releasing conduits (blue), conduits filled with phosphate buffered saline (red), and conduits filled with a collagen hydrogel grafted with HNK-1 peptide mimic (green). (A) FBA for all conditions. Values represent averages ± standard error. A decrease in the FBA over time indicates functional recovery. (B) Recovery Index (RI) for FBA at week 8. Each dot represents one animal in the group. Line indicates average RI value for each condition. (C) PLR for all conditions. Values represent averages ± standard error. (D) Recovery Index for FBA at week 8. Each dot represents one animal in the group. Line indicates average RI value for each condition.
LIST OF ABBREVIATIONS

ACN – Acetonitrile

DT – Desaminotyrosyl tyrosine

DTE – Desaminotyrosyl-tyrosine ethyl ester

ECM – Extracellular matrix

FBA – Foot base angle

hMSC – Human mesenchymal stromal cell or mesenchymal stem cell

HNK-1 – Human natural killer

Ln – Laminin

MeCl₂ – Dichloromethane (DCM or methylene chloride)

MeOH – Methanol

MSC – Mesenchymal stromal cell or mesenchymal stem cell

MW – Molecular weight

NGC – Nerve guidance conduit

NP – Non-porous

P – Porous

PCR – Polymerase chain reaction

PE – Polyethylene

PEG – Poly(ethylene glycol)

PLL – Poly(L-lysine)

PLR – Protration limb ratio

PMR – Preferential motor reinnervation
PNI – Peripheral nerve injury
PNR – Peripheral nerve regeneration
PNS – Peripheral nervous system
RI – Recovery index
r-SBF – Revised simulated body fluid
SCN – Spinal cord neuron
TFA – Trifluoroacetic acid
TyrPC – Tyrosine-derived polycarbonate
CHAPTER 1

INTRODUCTION

Peripheral Nerve Function and Anatomy

The nervous system is organized into two compartments each having its own physiology and functions: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of the brain and spinal cord and is responsible for interpreting sensory signals coming from the PNS and then converting them into motor signals to send out to the muscles of the body. The PNS, the focus of this dissertation, consists of the nerves outside of the CNS that serve to function the limbs and organs of the body. These nerves are responsible for receiving stimuli from the external environment (via sensory neurons), transmitting the signals back to the CNS, and then carrying the signals (via motor neurons) back to the muscles (2). Signals are carried from the soma to the end of the axon via action potentials, fast changes in the membrane potential. Conduction of these axon potentials down the nerve is how information is transmitted from the cell body to the processes at the end of the axon extensions. (2-4)

Within the nervous system there are two cell types: neurons and neuroglia. Neurons are the basic unit in the nervous system and consist of a cell body with a long extension, or axon, which can reach up to 1 m in length. The axon is responsible for carrying the signals away from the soma. Glial cells, or neuroglia, are the non-neuronal cells present in the nervous system that maintain the neurons, providing support and protection. In the PNS the neuroglia are called Schwann cells and they are responsible
for depositing myelin around the axons. Myelin is an insulating layer that helps to increase propagation velocity of the signals being sent from the CNS down the length of the axons in the PNS to the target muscles. Action potentials occur at the gaps present between the insulated axon segments. (2, 5)

Peripheral nerves consist of bundles of sensory and motor neurons grouped together. Each group is surrounded by a series of tissue layers (Figure 1.1). First, the individual nerve fibers are surrounded by the endoneurium that provides protection and nourishment directly to the axons. On top of this layer is the perineurium, composed of fibroblasts and collagen, which provides strength to the nerve and also separates groups of nerve fibers into fascicles. The outer most layer is the epineurium which consists of fibrocollagenous tissue that encircles the fascicles altogether. (2, 6)

![Peripheral Nerve Anatomy](image)

**Figure 1.1: Peripheral Nerve Anatomy.** Individual axons are surrounded by Schwann cells depositing myelin. These axons are enclosed within the endoneurium. The perineurium groups axons together to form fascicles. The epineurium groups fascicles together to form a final nerve trunk. (7)
Degeneration and Regeneration

Peripheral nerve injuries present a serious medical concern, constituting approximately 2.8% of all trauma cases in the United States and approximately 100,000 neurosurgical procedures in the United States and Europe annually (8-11). In addition to trauma related injuries, diseases or surgeries that require the transection of the nerve in order to reach the site of surgery (for example the removal of a tumor near a nerve) can also cause significant peripheral nerve injuries. Although the PNS has the ability to regenerate, the regeneration process (Figure 1.2) is characterized by poor functional recovery as axons extend slowly and often make improper reconnections with their distal targets (12, 13). This results in a limb with no nerve function and lifelong pain for the patient.

In 1850, Augustus Waller described the degenerative processes that occur in the distal nerve following a peripheral nerve transection: this series of events soon became known as “Wallerian degeneration” (14). Following complete transection, the distal portion of a peripheral nerve breaks down due to lack of nutrients naturally supplied by the cell body. Within hours Schwann cells, macrophages, and monocytes become increasingly active and begin to invade the wound site to clear away axonal, myelin, and tissue debris via phagocytosis and to secrete supportive cytokines (2, 6, 13, 15). After the injury site is “clean” (within a few days) a fibrin cable forms across the nerve gap to act as a substrate for the migration of Schwann cells (5, 16). The Schwann cells migrate along the cable and organize themselves into longitudinally oriented microtubes (the bands of Büngner) that provide guidance and neurotrophic factors to enhance axonal regeneration (16). In addition, they produce a basal lamina consisting of extracellular
matrix (ECM) proteins capable of supporting axonal regrowth and display a variety of cell adhesion molecules on their surface supporting axonal regrowth (17). Regeneration begins from the proximal end of the nerve as axons attempt to traverse the nerve gap through the bands of Büngner and reconnect with their distal target (either muscle or skin) (18). The growth cone, at the leading front of the axons, extends outward and navigates the environment looking for the basil lamina of the Schwann cells to grow along (17, 19). Distal stumps provide chemotactic cues that help entice axons to reach their end targets. However, many axons do not enter into the appropriate endoneurial tubes at the distal end, making improper reconnections and improper target reinnervation. The axons that do become myelinated and enter the correct tubes form neuromuscular junctions with their target tissue and start to show signs of functional recovery. Some of the microtubes remain empty and any axons that do not enter a tube to cross the gap get pruned and eventually die. (2, 6)
Figure 1.2: Events associated with peripheral nerve injury. (A) Within days after transection (arrowhead), degeneration begins and axons and myelin break down. (B) Days to weeks later, Wallerian degeneration is almost complete, with macrophages phagocytosing tissue debris and Schwann cells proliferating at the injury site. Axons begin to regenerate from the proximal end of the nerve. (C) By the first few months, some axons have bridged the nerve defect and are supported by Schwann cells in the bands of Büngner. Some axons enter into the appropriate endoneurial tubes reaching through the distal nerve stump. The target muscle begins to atrophy as it has lost contact with the neuron cell body for a great period of time. (D) Some axons are able to extend through the bands of Büngner and reinnervate their target muscle. This allows for the reversal of muscle fiber atrophy. (E) Axons that are not able to traverse the injury gap result in neuroma formation. (17)
**Standard of Care**

A complete transection of the peripheral nerve, where the nerve trunk is severed and the proximal and distal stumps only remain, continues to be a major clinical challenge (20). When the distance axons must regenerate is greater than the critical size (5.0 cm in humans, 1.5 cm in rats, 5.4 ± 1.0 mm in the mouse sciatic nerve model (21)), a fibrin cable cannot traverse the long nerve gap on its own. Without this initial cable, regeneration cannot occur successfully as cells depend on it for contact guidance to bridge the nerve gap (22). Standards of care for this type of critical nerve injury include primary repair, suturing severed ends back together (only feasible when the nerve endings are adjacent and the nerve gap is small enough to prevent tension on the nerve stumps), or using an autologous nerve graft such as the sural nerve or the medial cutaneous nerve (11). However, with both of these treatment options motor and sensory function recovery is usually limited. Recovery of full motor function is observed in 25% of patients and recovery of full sensory function is observed in only 3% of patients following direct suturing of nerve stumps (23). Treatment with an autologous nerve graft holds several other disadvantages such as loss of function in the donor site, multiple surgeries for the patient, risk of the development of a neuroma, poor functional recovery, and limited availability of donor nerve in both length and diameter (2, 24-26). Therefore, there is a need for an alternative nerve graft or scaffold that can bridge the gap between the nerve stumps. The scaffold must encourage proper target reinnervation of the newly regenerated axons ultimately leading to a higher quality of functional recovery.
Nerve Guidance Conduits

Due to the aforementioned difficulties associated with the current standard of care for peripheral nerve injuries, research has focused on the development of a bioartificial nerve guidance conduit (NGC) as an alternative treatment method for severed nerves. Entubulation, the act of inserting both nerve stumps into each end of a nerve guidance conduit or tube and enclosing the nerve defect within the inner lumen of the tube, provides a way to bridge a nerve defect and physically guide regenerating axons to their distal end targets (27, 28). The NGC also acts as a physical barrier, concentrating secreted factors and supporting cells inside the lumen and preventing any external inhibitory factors such as non-supporting cells or other tissues that may cause scar tissue formation from reaching the injury site (29).
Figure 1.3: The phases of regeneration occurring in a nerve guidance conduit. (A) Within the first few hours following implantation a fluid fills the inner lumen containing neurotrophic factors as well and inflammatory cells. (B) After several days, the fibrin cable begins to form between the proximal and distal stump. (C) Between 1 and 2 weeks Schwann cells, fibroblasts, and endothelial cells enter into the conduit along the fibrin matrix. (D) Between 15-21 days axons begin to regenerate from the proximal end of the conduit. (30, 31)

Following NGC implantation, several events must occur for successful regeneration to happen (Figure 1.3). Within the first day following implantation of a NGC, a fluid phase occurs during which a clear fluid with neurotrophic activity fills the inner lumen of the conduit. Between days 2-6, the matrix phase begins during which a fibrin matrix forms providing a physical scaffold for the migration and seeding of cells. From days 7-14, the cellular phase occurs. This is when all of the non-neuronal cells enter the lumen and migrate across the fibrin matrix. These cells, including Schwann cells, fibroblasts, endothelial cells, and perineurial cells, precede the axons in order to lay down a perineurial sheath. Finally, between days 15-21 axons begin to enter the conduit and reach the midpoint. Blood vessels enter into the conduit inner lumen during this time.
**Natural Nerve Guidance Conduits**

Many naturally existing conduits can be used to bridge nerve gaps including vein, artery, muscle, or nerve segments. In addition to these natural tubular structures, additional biologic materials can be fabricated into nerve conduits such as chitosan, collagen, gelatine, hyaluronic acid, and silk. These materials are attractive because of their biocompatibility, ability to support cell attachment and growth, and degradable nature. However, because of the variability seen between isolations and the need for strict purification and characterization of these materials, reproducibility is low and properties vary greatly from one tube to another. For treating longer gaps, natural materials often result in poor recovery associated with collapse of the conduit outer wall, scar formation at the ends of the conduit, and early resorption leading to mechanical failure (32). If not treated properly, natural materials may also cause an immune response upon implantation or carry microbes from the donor tissue to the patient. (32-34)

**Synthetic Nerve Guidance Conduits**

An alternative to using natural nerve guidance conduits is to use synthetic biomaterials fabricated into the shape of a tube or conduit that may be placed around the injury site in order to try and physically assist axons to their distal targets (35). Many methods are used to fabricate synthetic NGCs, including solvent casting, extrusion, freeze-drying, and dip-coating (36).

The ideal material used to fabricate a NGC is non-cytotoxic, has a controllable degradation rate, can be fabricated into a conduit of the proper dimensions, and has sufficient mechanical properties to prevent collapsing or kinking over time. A NGC
fabricated from this material should be easy to handle and suture into place, and should be semi-permeable, allowing for the diffusion of oxygen and nutrients into and waste products out of the inner lumen. (27, 37, 38)

Synthetic polymer NGCs offer several advantages over natural materials. These advantages include altering the surface chemistry of the material, controlling its degradation rate by way of the molecular weight and/or polymer composition, and being able to fabricate the material into designs with various dimensions to fit different size defects (27, 39). More importantly, using synthetic conduits eliminates the need to sacrifice an existing nerve elsewhere in the body.

Silicone was the first synthetic material to be used as a nerve conduit due to its elastic properties and chemical stability. However, because NGCs fabricated from silicone are non-degradable and non-porous, silicone conduits can cause harmful effects such as nerve compression or fibrosis leading to a secondary surgery necessary to remove the conduit (40). To avoid this issue, degradable polymers may be preferrable as an entubulation material (3, 39). By using degradable polymers, the conduit is able to provide a protective environment to the nerve during its regeneration time, and then to degrade after nerve regenerates, with no long-term tissue response. Also, whereas silicone is an inert material, research now focuses on selecting a material that actively stimulates regeneration of the severed axons (2).

Recently, a number of new different synthetic materials have been explored for their use as nerve conduits including poly(esters), such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), and polycaprolactone
(PCL). These materials are used so often because they are readily available and U.S. Food and Drug Administration (FDA) approved for use. In addition, these materials are easy to handle and have the ability to degrade over time. (2, 39)

Currently available artificial conduits are able to match the performance of autografts in smaller sized defects, yet they do not allow for regeneration across critical size defects due to problems associated with collapse, scar formation, and early resorption (8, 32). In addition, the degradation products of some of these materials can be harmful to the body and can cause an inflammatory response that inhibits or slows down nerve regeneration (8). Therefore, a new biomaterial with improved properties is required.

Some of the most recent academic research work concerning new materials for peripheral nerve conduits is listed in Table 1.1. The table displays the various materials being used and the defect gap lengths being bridged in rat models to evaluate each conduit’s potential. In addition, some of the materials shown are enhanced with biological, chemical, or physical cues to help increase the functional outcome over long defects.

**FDA-Approved Nerve Guidance Conduits**

Studies from over the past 20 years have resulted in several clinically available nerve conduits being approved by the FDA and Conformit Europe (CE). Clinically available biodegradable NGCs approved by the FDA are shown in Table 1.2 (3, 38, 39, 41). All of these conduits are based on the simplistic design of a hollow tube (Figures 1.4
and 1.5) lacking an internal architecture or a biological enhancer and are limited in the lengths available.

Experimental and clinical results collected from studies using these FDA-approved nerve conduits show comparable and sometimes better nerve regeneration as compared to autografts when the nerve defect gaps being bridged are small. However, when used to bridge critical size nerve defects, treatment with these currently available conduits results in reduced regeneration and functional recovery (39, 42-45). Reasons for this low success rate may be due to the fact that these conduits only retain their mechanical properties for limited amounts of time, providing inadequate long-term physical support and protection to the nerve. Problems related to swelling (occluding the inner lumen), early resorption, or too slow degradation, leading to nerve compression and fragmentation of the conduit in vivo have also been reported (26, 27). In addition, some of the degradation products of these materials are highly acidic and have a low solubility. This can lead to a severe inflammatory response leading to macrophage invasion, fibrosis, and disordered axonal outgrowth (8, 31, 46). For example, a study in 2009 by Meek et al. evaluating a 1 cm rat sciatic nerve defect implanted with an empty Neurolac tube showed signs of very fast degradation of the conduit with a high degree of swelling, fragmentation of the conduit, and collapse of the inner lumen. In addition, a severe foreign body reaction occurred inhibiting regeneration (47). Due to these aforementioned reasons, the FDA-approved NGCs are most commonly used to bridge small sensory nerves (usually in the digits of the hands) and rarely used for large nerve defects or motor nerves. (11, 34, 38, 41, 48)
Table 1.1: Nerve conduits currently being investigated for peripheral nerve repair. Table shows material used with or without modifications, defect size being bridged in a rat model, diameter of the nerve conduit, and the outcome to the *in vivo* evaluation.

<table>
<thead>
<tr>
<th>Author of Study</th>
<th>Material</th>
<th>Defect Size and Tube Diameter</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chioni et al.</em> 2011 (49)</td>
<td>Poly(ester urethane)</td>
<td>18 mm length ID 1.5 mm OD 1.0 mm sciatic nerve</td>
<td>Electromyography and histological analysis show the presence of regenerating fibers in the distal stump</td>
</tr>
<tr>
<td><em>Kakinoki et al.</em> 2011 (50)</td>
<td>Poly(L-lactic acid) (PLLA) Nanofiber with Oligo(D-lactic acid) Bioactive-Peptide Conjugates</td>
<td>10 mm length ID 1.0 mm OD 2.0 mm sciatic nerve</td>
<td>Electrophysiological evaluation showed better functional reinnervation than silicone tube or unmodified PLLA nanofibrous conduit</td>
</tr>
<tr>
<td><em>Wang et al.</em> 2009 (51)</td>
<td>Photo-Crosslinked Poly(e-caprolactone fumarate)</td>
<td>10 mm length sciatic nerve</td>
<td>Proved in vivo biocompatibility and functionality of guiding axon growth Nerve cable with myelinated axons showed cell response and tissue ingrowth</td>
</tr>
<tr>
<td><em>Wang et al.</em> 2009 (52)</td>
<td>Polyurethane (PU)-collagen</td>
<td>10 mm length sciatic nerve</td>
<td>Walk track analysis, electrophysiological and histological evaluations showed better nerve repair than the controls</td>
</tr>
<tr>
<td><em>Kemp et al.</em> 2009 (53)</td>
<td>Collagen</td>
<td>5.0 and 10 mm-length ID 1.5 mm sciatic nerve</td>
<td>Displayed enhanced axonal regeneration, myelination, and vascularization</td>
</tr>
<tr>
<td><em>Hsu et al.</em> 2009 (54)</td>
<td>Asymmetric and symmetric porous poly(D,L-lactide) (PLA)</td>
<td>10 mm length ID 1.5 mm sciatic nerve</td>
<td>Maintained stable supporting structure through regeneration process. Asymmetric conduits showed greatest myelination and highest degree of functional recovery</td>
</tr>
<tr>
<td><em>Kim et al.</em> 2008 (35)</td>
<td>Aligned poly(acrylonitrile-co-methylacrylate) (PAN-MA)</td>
<td>17 mm length ID 1.5 mm tibial nerve</td>
<td>Axons regenerated, reinnervated muscles, and reformed neuromuscular junctions. Electrophysiological and behavioral analyses showed tube facilitated both sensory and motor nerve regeneration and significantly improved functional outcome</td>
</tr>
</tbody>
</table>
Table 1.2: FDA-approved nerve conduits including name of conduit, company manufacturing the conduit, material used, degradation time, length, and diameters available for clinical use. (3, 11, 38, 41)

<table>
<thead>
<tr>
<th>Name, Company</th>
<th>Material</th>
<th>Degradation time</th>
<th>Length (maximum cm)</th>
<th>Inner Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuraGen, Integra NeuroSciences</td>
<td>Type I collagen</td>
<td>4 years</td>
<td>3</td>
<td>1.5-7</td>
</tr>
<tr>
<td>NeuroMatrix and NeuroFlex, Collagen Matrix Inc.</td>
<td>Type I collagen</td>
<td>7 months</td>
<td>2.5</td>
<td>2-6</td>
</tr>
<tr>
<td>NeuroTube, Synovis</td>
<td>polyglycolic acid</td>
<td>3 months</td>
<td>4</td>
<td>2.3-8</td>
</tr>
<tr>
<td>Neurolac, Polyganics, BV</td>
<td>poly-DL-lactide-caprolactone</td>
<td>16 months</td>
<td>3</td>
<td>1.5-10</td>
</tr>
<tr>
<td>Avance, AxoGen Inc.</td>
<td>Processed human nerve graft</td>
<td>No degradation</td>
<td>1.5-5</td>
<td>1-5</td>
</tr>
<tr>
<td>SaluBridge, SaluMedica LLC.</td>
<td>Polyvinyl alcohol hydrogel</td>
<td>No degradation</td>
<td>6.35</td>
<td>2-10</td>
</tr>
</tbody>
</table>
Figure 1.4: Photographs of FDA-approved nerve conduits. (A) Left to right: Collagen type I conduit (NeuraGen; Integra LifeSciences, Plainsboro, NJ); polyglycolic acid conduit (Neurotube; Synovis, Micro Companies Alliance, Birmingham, AL); poly(DL-lactid-e-caprolactone) conduit (Neurolac; Polyganics BV, Groningen, Netherlands) (1). (B) Axogen decellularized nerve graft http://startsfl.com (C) Neuroflex Flexible Collagen Nerve Conduit, http://www.collagenmatrix.com (D) NeuroMatrix Collagen Nerve Conduit, http://www.collagenmatrix.com

Figure 1.5: Cross-section images of FDA approved conduits. (A) NeuraGen (B) Neurotube (C) Neurolac. (11)
**Tyrosine-Derived Polycarbonates**

Although treatment options for nerve injuries have advanced, there is still an unmet need for conduits that can successfully bridge together critical size defects. To achieve this goal, the development of a nerve conduit prepared from a material with improved mechanical properties, long-term biocompatibility, and a controllable degradation rate is required. Therefore, biomaterials from the family of tyrosine-derived polycarbonates (TyrPCs) are explored as a potential material for fabrication and evaluation of nerve conduits to treat critical size gaps. These polymers are based on the natural amino acid tyrosine, and are non-cytotoxic and bioresorbable, breaking down primarily via hydrolysis, into naturally occurring components. Therefore, they elicit only a mild inflammatory response as compared to the more commonly used polymers in medical applications such as poly (L-lactic acid) (55). TyrPCs, synthesized utilizing previously published procedures (55-60), offer a flexible chemistry allowing one to work with this polymer family for very specific applications within a wide range of material properties (55-60). For example, TyRx Pharma utilizes these polymers for the fabrication of a hernia repair device that was cleared for clinical use in 2006 by the US Food and Drug Administration (FDA). TyRx Pharma also works with polymers from the same polymer family for a very different application: the AIGISRx™ Anti-Bacterial Envelope, a product for the surgical repair of damaged or ruptured soft tissue that was cleared by the US FDA in 2010 (http://www.tyrx.com/index.htm).

TyrPC terpolymers with the following chemical structure are used in this work:

Poly((100-XX-YY)% DTE(desaminotyrosyl-tyrosine ethyl ester)-co-XX% DT (desaminotyrosyl tyrosine)-co-YY% PEG(polyethylene glycol)) carbonate) (Figure 1.6).
The percentages of each component can be altered in order to change the material properties of the polymer. By modifying the ratio of DTE to DT, the hydrophobicity/hydrophilicity balance of the polymer and rate of in vivo degradation will change. For example, increasing the % DT, the molar fraction of free carboxylates, will decrease the % DTE, and will increase the degradation and bioresorption rate of the polymer. The % PEG will alter polymer properties such as cell-material interactions, protein adsorption, cell growth, and the physicomechanical properties of the polymer. To simplify the naming of the TyrPC terpolymers, throughout this dissertation the notation EXXYY(1K) is used to name poly(DTE-co-XX% DT-co-YY% PEGMW carbonate) where E is the ethyl ester, XX is the mole percent of DT, YY is the mole percent of PEG and MW is the weight average molecular weight of PEG (MW is “1K” in all studies described in this dissertation, stating that all PEG blocks were of 1000 molecular weight). As an example, poly(DTE carbonate) without any DT or PEG is designated as E0000 while poly(DTE-co-10%-DTco-0.5%-PEG1K carbonate) will have a notation of E1000.5(1K). (47, 61-64)

Figure 1.6: Chemical structure of TyrPC terpolymer. 100-XX-YY is the mole percent of DTE, XX is the mole percent of DT, and YY is the mole percent of PEG. DTE: Desaminotyrosyl-tyrosine ethyl ester DT: Desaminotyrosyl tyrosine PEG: Polyethylene glycol (MW 1000Da)
**Modifications to the Single-Lumen Nerve Guidance Conduit**

In order to enhance regeneration further and increase the gap length that can be bridged with a hollow conduit, additions can be made to the simplistic design of a hollow synthetic tube (Figure 1.7). These alterations include adding an internal microarchitecture, transplanting an external supply of support cells (65-68), delivering growth factors (36, 69-72), electrical stimulation of nerve during the regeneration period (73, 74), mechanical stimulation of the target musculature during the regeneration period (75), using conductive polymers for the fabrication of the conduit (76), or making the conduit outer walls porous and therefore permeable to certain cell types and molecules (77, 78). Some of these modifications will be discussed further below. (2, 24, 74, 75, 79-81)

Adding an intrinsic framework to the conduit inner lumen, such as polymeric fibers, collagen sponges, denatured muscle grafts, or longitudinally oriented channels will help to stabilize the fibrin matrix that naturally forms across the injury gap or will mimic this matrix and the Bands of Büngner if they are not able to form on their own (32, 82-85). The additional structure will also provide contact guidance cues and a greater surface area for migrating non-neuronal cells (including Schwann cells, fibroblasts, and endothelial cells) and regenerating axons. This will improve upon their ability to attach and proliferate speeding up the regeneration process. (32, 85, 86)

The addition of support cells, especially Schwann cells, is the most commonly investigated modification made to the single-lumen NGC (80). An external supply of Schwann cells will migrate along the fibrin matrix and form Bands of Büngner along which axons can grow. In the case of a long defect when the fibrin matrix cannot grow
on its own, these Bands of Büngner will be necessary for axon regeneration. Schwann cells will also produce extracellular matrix molecules (such as laminin) and neurotrophic factors (such as nerve growth factor) to enhance recovery further. In addition to Schwann cells, stem cells and genetically modified cells are commonly added to the inner lumen of the NGC. Stem cells can be used to differentiate into neurons and glial cells and genetically modified cells are often used to deliver a continual supply of active neurotrophins (2). (87-94)

As well as providing guidance and structural support, a NGC can also function as a mode of delivery for bioactive molecules, such as growth factors, proteins, or neurotrophic molecules. These biologic factors need to be introduced locally and slowly over a prolonged period of time, delivering as little as nanogram quantities per day in order to achieve the desired therapeutic effect. In this manner, the conduit can support regeneration, but also promote and direct neurite outgrowth. Different growth factors including nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), neurotrophin-3, and fibroblast growth factor (FGF) have been added previously to NGCs (Table 1.3). Growth factors can enhance regeneration via several ways: by increasing axonal outgrowth, neuron survival, or by acting on select groups of neurons, such as motor or sensory. Most often growth factors are added in solution, are secreted by cells transplanted into the inner lumen or are secreted by cells already present within the inner lumen but that are genetically altered to secrete the growth factors (Figure 1.8). (36, 89, 95)

Finally, using conductive polymers to fabricate NGCs can enhance regeneration. Materials like polypyrrole that have an electrical activity have been shown to help
accelerate axonal elongation on their charged surfaces (96, 97). In addition, low frequency electrical stimulation to the proximal nerve stump following transection has been used to significantly accelerate axonal outgrowth (73, 74, 81). (2, 24, 80, 98)

As mentioned already above, the addition of porosity to the conduit outer walls is a common modification made to the NGC design. The pore size and the degree of porosity determine whether or not cells, nutrients, or blood vessels can pass through the conduit wall into the inner lumen (16, 39, 61). Some studies show that impermeable conduits help regenerate healthy nerve in short gaps (99), while other studies show that pores are critical for regeneration across longer gaps (62). Of these later studies, the optimal pore sizes used in conduits vary. For example, some studies show that pores large enough to allow for both cells and fluid to pass through to (5 µm in diameter) enhance regeneration more than when only fluid can pass through the outer wall (1.2 µm in diameter pores) (61-63). Despite the possible advantages of having pores in the outer wall, porous conduits have also been associated with the following disadvantages: weakening of the mechanical strength of the conduit, infiltration of the inner lumen with various cell types, physically blocking axons from regrowing, and the diffusion of bioactive growth factors out of the site of injury. (25, 29, 39)
Figure 1.7: Modifications to the single lumen nerve tube. These modifications include making the outer walls porous/permeable to allow for exchange between the external environment and the inner lumen, delivering growth factors to the regenerating nerve, transplantation of supportive cells, the addition of an internal structure to help support cell ingrowth, inclusion of channels to mimic the bands of Büngner, and use of an electrically conductive polymer. (2, 80, 100)
Table 1.3: Examples of growth factors used in peripheral nerve regeneration experiments, the targets the factors act upon, and studies in which these factors were used. Adapted from Pfister et al. (95)

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Major target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF: nerve growth factor</td>
<td>Sensory neurons, small axons</td>
<td>Chung et al. (2011) (101)</td>
</tr>
<tr>
<td>NT-3: neurotrophin 3</td>
<td>Sensory neurons, small- and medium-size axons</td>
<td>Barras et al. (2002) (69)</td>
</tr>
<tr>
<td>BDNF: brain-derived neurotrophic factor</td>
<td>Sensory neurons, large axons</td>
<td>Terris et al. (2001) (102)</td>
</tr>
<tr>
<td>GDNF: glial-derived neurotrophic factor</td>
<td>Motor neurons</td>
<td>Fine et al. (2002) (103)</td>
</tr>
<tr>
<td>FGF-1: fibroblast growth factor 1</td>
<td>Vascular endothelial cells</td>
<td>Cordeiro et al. (1989) (104)</td>
</tr>
<tr>
<td>GGF: glial growth factor</td>
<td>Schwann cells</td>
<td>Mohanna et al. (2005)(106)</td>
</tr>
<tr>
<td>PDGF: platelet-derived growth factor</td>
<td>Schwann cells</td>
<td>Wells et al. (1997)(107)</td>
</tr>
<tr>
<td>CNTF: ciliary neurotrophic factor</td>
<td>Schwann cells (injury factor)</td>
<td>Ho et al. (1998) (108)</td>
</tr>
<tr>
<td>VEGF: vascular endothelial growth factor</td>
<td>Vascular endothelial cells</td>
<td>Hobson (2002) (109)</td>
</tr>
<tr>
<td>IGF-I: insulin-like growth factor I</td>
<td>Inflammatory cells (anti-inflammatory)</td>
<td>Fansa et al. (2002)(110)</td>
</tr>
<tr>
<td>LIF: leukemia inhibitory factor</td>
<td>Neurons (injury factor)</td>
<td>McKay Hart et al. (2003) (111)</td>
</tr>
</tbody>
</table>

Figure 1.8: Representation of ways to deliver growth factors to the nerve guidance conduit. From left to right, protein delivery from the outer conduit walls that directly releases the factor into the inner lumen, transplanting cells to the inner conduit walls to synthesize and release growth factors, using gene delivery to transfect cells already present within the NGC to produce the growth factors. (95)
**HNK-1 Carbohydrate Epitope**

HNK-1, a carbohydrate epitope first discovered on the human natural killer cell (hence its name), appears to play a significant role in development of the nervous system. When this epitope, consisting of a sulfated trisaccharide with the following structure, \( \text{SO}_4^-3\text{GlcA} \beta(1-3)\text{Gal} \beta(1-4)\text{GlcNAc} \) (SO\(_4\): sulphate group, GlcA: glucuronic acid, Gal: galactose, and GlcNAc: N-acetylglucosamine – Figure 1.9), was first found on myelin-associated glycoprotein (MAG), which plays a role in severe demyelination conditions, neuropathologists became particularly interested in investigating its functional relevance further (112). It was found that many different cell adhesion molecules in the nervous system were recognized by the monoclonal antibody HNK-1 including, but not limited to, neural cell adhesion molecule (N-CAM-1), L1, P0 (myelin protein), and extracellular matrix molecules (tenascin-R and phosphacan) (113, 114). All of these molecules are functionally important during development, and therefore the presence of HNK-1 was thought to indicate its functional significance (113). HNK-1’s conservation across many species in time (both vertebrates and invertebrates) supported its functional meaning as well. It is important to note that the presence of this epitope is regulated over time and space throughout development and maturation of the nervous system, indicating there is some degree of control over its synthesis, perhaps having to do with its function. For example, during the perinatal period in the rat cerebral cortex expression of HNK-1 is high as is the degree of myelination and/or synaptogenesis (114, 115). (112, 116, 117)

To further elucidate HNK-1’s functional importance, antibodies have been used to block its activity both in cell culture and in animal models (118, 119). Results from these studies demonstrate alterations in cell-cell interactions, cell-substrate interactions, neuron
migration, and neurite outgrowth as well as changes in synaptic plasticity (120). For example, when HNK-1 found on the surface of migrating neural crest cells is presented with the HNK-1 antibody, migration of these cells is prevented (indicating that the neural crest cell-substrate interaction is being blocked) (119). When presented to the mouse hippocampus, zebrafish, or goldfish, the HNK-1 antibody causes a significant decrease in memory and the ability to learn (120-123). Removal of the enzymes responsible for HNK-1 synthesis also results in abnormal learning tasks involved in the mouse hippocampus (124, 125). Another interesting finding is that in mice the HNK-1 epitope is found in the myelin, basal lamina, and on Schwann cell surfaces associated with motoneurons only. These findings led to the hypothesis that HNK-1 may also play a role in the development of motoneurons specifically.

Figure 1.9: The chemical structure of the HNK-1 epitope. (112)
In critical size gaps specific reinnervation is rarely perfect and functional recovery is often poor due to the limited ability of neurons to navigate long gaps and reconnect with their proper distal targets. Regenerating motor axons are often misguided to skin, and sensory axons are often misguided to muscle (120). Brushart et al. showed that when given equal opportunity to regenerate towards a motor or sensory branch, significantly more motoneurons prefer to enter the motor branch as opposed to the sensory branch, leading to the phenomenon referred to as preferential motor reinnervation (PMR) (126-129). HNK-1’s functional relevance to PMR is based on the fact that even though there are neural cell proteins present in both the sensory and motor branches of the mouse femoral nerve, HNK-1 is only associated with these proteins in the motor branch and therefore may be necessary to help guide motor axons to their proper motor branch (113, 120, 126, 127, 130, 131). If able to mimic and enhance the effects of PMR during regeneration in a nerve conduit, the number of individual axons reaching their proper targets may increase, leading to increased functional recovery of the new nerve. (129) A peptide mimic (amino acid sequence FLHTRLFV) that is easier to obtain and synthesize than natural HNK-1 has been identified by phage display techniques using an L2-412 antibody for selection (112). This peptide mimic was evaluated via several assays and was found to effectively function like the natural HNK-1 carbohydrate. The mimic competed with HNK-1 glycolipids for the interaction of the HNK-1 antibody, was able to bind to both laminin-1 and motor neurons, and effectively promoted neurite outgrowth from motor neurons only in vitro (132). In vitro experiments confirmed these results and showed that substrates coated with the mimic increased neurite outgrowth from motoneurons only as compared to sensory neurons (133). When applied in soluble form
to the reconstruction of severed mouse (134) and monkey (135) femoral nerves in vivo, the HNK-1 peptide mimic increased functional recovery and PMR, helping axons reach their targets more appropriately. In addition, the mimic reduced motoneuron death, enhanced expression of endogenous HNK-1 carbohydrate in the motor nerve branch during regeneration, and reduced abnormalities in axonal myelination in the newly regenerated nerve (134).

**Cellular Therapies**

As briefly described earlier, to further enhance nerve regeneration within a NGC, an additional supply of ECM components, neurotrophic factors, and cell adhesion molecules can be introduced. One way to deliver these factors is via cellular transplantation. In particular, Schwann cells and various types of stem cells are being investigated as cell sources for transplantation to support nerve regeneration. The recent advances in molecular biology and transfection methods have further expanded the potential of cells to aid repair in the nervous system by genetically engineering the cells to express and/or secrete neurotrophic factors (2). A common way of introducing cells to the conduit is to isolate primary cells from the patient, expand them ex vivo, genetically engineering them if needed, and then transplant them back into the patient within the context of a nerve conduit (136).

Schwann cells are commonly used as a cell source for a number of reasons and have already been shown to enhance regeneration in small nerve gaps (137, 138). Following nerve injury, Schwann cells typically support axonal regeneration, by providing a substrate for which axons can elongate across, helping to lead the axons to the distal target, forming synapses, and by secreting various kinds of support factors such
as (nerve growth factor) NGF and brain derived neurotrophic factor (BDNF), cell adhesion molecules, such as L1, N-cadherin, and NCAM, extracellular molecules, such as laminin and collagen (2, 24, 138, 139). If an exogenous source of Schwann cells was added to a NGC following injury, these beneficial effects would occur faster and at a larger magnitude. However, a main disadvantage of using Schwann cells is the amount of time needed to harvest and expand enough cells from an autologous isolation. This process may take weeks and cell cultures may not be pure enough to use.

In order to bypass the obstacle of having to wait for Schwann cell isolations to expand ex-vivo, studies have shown the ability to differentiate stem cells into Schwann-like cells (2, 80, 139-141). In particular, mesenchymal stem cells (MSCs) (either bone marrow or adipose derived) have been shown to transdifferentiate into Schwann cell like cells using a cocktail of chemicals. These Schwann-like cells have the morphology of a Schwann cell and express such Schwann cell markers as p75, S-100, GFAP and O4. When evaluated in vivo in a sciatic nerve model, transdifferentiated Schwann cells increased functional regeneration and the number of myelinated nerve fibers indicating their ability to myelinate and support nerve fiber growth. (140, 142, 143)

Stem cells are more recently being used for peripheral nerve regeneration applications. MSCs (or BMSCs, bone marrow stromal cells) are of particular interest because of their ability to naturally provide cytokines that control the inflammatory and immune response, neurotrophic factors that enhance survival and outgrowth of neurites, substances that induce neoangeogenesis, and they have been shown to provide a “perineurium-like” structure that surrounds the nerve and forms a contact between the regenerating nerve and the NGC (68, 140, 141, 143-146). Also, for potential clinical use,
MSCs are a promising source of cells because they can be used for autologous transplantation, can be rapidly expanded in culture, are adult-like and therefore reduce the chance of tumor formation, survive in vivo, and integrate well with the native nerve (39, 67, 68). Additionally, when transplanted within the inner lumen of artificial conduits, MSCs improve functional recovery of regenerating peripheral nerves. (67, 68, 92, 140-143, 145-147)

Few cases in the literature show additional types of stem cells being used for peripheral nerve regeneration. For example, hair follicle stem cells (87, 93) and skin-derived stem cells (89, 91) have been used in vivo to evaluate their effects on regeneration. When located within a nerve conduit independently, both cell types enhanced the rate of regeneration and the functional recovery in a sciatic nerve injury model as well as improved electrophysiological and morphometrical parameters over the control condition. In vivo, these cells were found to transdifferentiate mostly into Schwann cells expressing high amounts of S100 and neurofilament (93). In another study, neuronal progenitor cells derived from the fetal rat hippocampus were evaluated for their potential to promote peripheral nerve regeneration (148). The cells were embedded in a collagenous gel within the inner lumen of a nerve conduit used to bridge a 15mm defect in the sciatic nerve. Following implantation, the cells were able to retain their ability to proliferate and differentiate within the gel, mostly into Schwann cell like cells. Results showed that the number and diameter of myelinated fibers were significantly increased as compared with a control conduit without the additional cells (148).
In addition to the aforementioned natural benefits, Schwann cells and stem cells have on nerve regeneration, these cells can be genetically engineered to overexpress certain neurotrophic factors and in this way serve as a delivery vehicle. These factors include the following: NGF, BDNF, NT-3, CNTF, GDNF, and bFGF, cell adhesion molecules, or extracellular matrix proteins (95). This can be done ex-vivo or in vivo by either viral or nonviral methods and allows for continual expression and/or secretion of the factors as the cell divides (2, 149, 150). For example, in one study retroviral infection was used to alter the adhesive properties of Schwann cells (151). The virus caused the cells to display the polysialylated (PSA) form of the neural cell adhesion molecule NCAM on their surface, which increased the cells’ motility in vitro. When evaluated in vivo within a collagen conduit used to bridge a gap in the rat sciatic nerve, the cells caused regeneration comparable to that of an autologous graft, enhancing the axon fiber morphology and functional recovery of the rats (151).
Organization of Thesis

The overall goal of this doctoral research was to develop a biodegradable, TyrPC nerve guidance conduit to treat critical size peripheral nerve defects and to enhance functional recovery. This dissertation describes the development of the TyrPC NGC as well as the results of several in vivo experiments evaluating a series of TyrPC conduits for nerve regeneration. In addition, results from experiments investigating new methods of delivery for the HNK-1 peptide mimics are discussed.

In this chapter, an overview of the peripheral nerve anatomy, the regeneration process following nerve transection, and currently available NGCs was given. In addition, methods for modifying the single-lumen design of a NGC were described and the TyrPC polymer chemistry was introduced as well as the therapeutic potential of HNK-1.

The remaining chapters of the dissertation present data from studies that explore the use of TyrPC as a NGC. Chapter 2 describes the fabrication and characterization techniques used to develop the NGC prior to in vivo evaluation. Chapter 3 presents in vitro work comparing TyrPC against polyethylene for use in nerve regeneration, as well as in vivo results evaluating the use of a TyrPC NGC as compared to a polyethylene NGC to bridge a critical size defect. Chapter 4 also presents in vivo results, however this chapter focuses on the ability of a modification to the inner lumen (in the form of a hydrogel with or without HNK-1) to have different effects on regeneration based on the conduit structure. Chapter 5 describes alternate methods of HNK-1 delivery to increase the lifetime of the mimic within the conduit inner lumen at the injury site. The last
chapter, Chapter 6, provides a final conclusion and discusses future directions and recommendations for this work.
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CHAPTER 2

FABRICATION AND CHARACTERIZATION OF A TYROSINE-DERIVED POLYCARBONATE NERVE GUIDANCE CONDUIT

Introduction

Repair methods for peripheral nerve injuries still remain to be a critical challenge (38). Although peripheral nerves are able to regenerate on their own following complete transection, the regeneration process can be extremely inefficient as axons extend in a random fashion across the nerve injury gap making many inaccurate reconnections. In addition, scar and/or neuroma formation is likely to occur if the injury is left untouched, further inhibiting functional recovery (152).

There are several methods of repair currently used to treat peripheral nerve injuries. If the defect is short enough, the nerve stump endings can be brought together and directly sutured. When the nerve defect is too large, end to end suturing is not possible, as it will cause too much tension on the nerve stumps. In this case an autologous nerve graft is most typically used. However, there are complications with using autologous nerve grafts: there is a limited number of donor nerves, the chance for donor site morbidity, and differences in the structure and size of the nerve tissue needed (27, 39). Furthermore, complete recovery of function following treatment with an autograft is still less than 50 %, leaving much room for improved treatment options (153).
In order to solve some of the problems associated with autografts, synthetic NGCs are being explored for use in nerve injury repair. By using conduits fabricated from synthetic materials, one has the ability to have an abundant supply of materials, to alter the conduit surface chemistry, to control the conduit’s degradation rate, and to fabricate conduits with various dimensions (27, 39). Synthetic conduits also remove the need to sacrifice an existing nerve elsewhere in the body.

Historically, non-degradable synthetic materials, such as silicone and polyethylene were commonly used to treat peripheral nerve defects. These materials are inert and therefore thought to be harmless to the body. However, long-term use of both silicone and polyethylene (Figure 2.1) for the repair of nerve defects in humans is not practical as these materials do not degrade and may become irritable to the patient over time. Silicone and polyethylene conduits can physically hinder movement, especially if used in places that exhibit a lot of bending stress, such as the ulnar nerve around the elbow joint. Their presence can lead to harmful effects such as nerve compression or fibrosis resulting in the need for secondary surgery to remove the conduit (2, 40). To avoid these concerns, degradable polymers are preferrable for use as a NGC so that once the nerve has repaired itself the conduit degrades and is removed from the body naturally (3, 39).

Ideally, a synthetic NGC has a controllable degradation rate, the proper dimensions, and promotes the formation of the initial fibrin matrix following injury (154). The fibrin matrix is particularly important for successful regeneration as it provides structural support and directs cells and regenerating axons across the nerve defect. Finally, the material used to construct a NGC must be non-cytotoxic with the cells
present in nerve tissue and must have sufficient mechanical properties to prevent collapsing of the outer walls or kinking of the inner lumen over time. (155)

Figure 2.1: Motivation for the work: Unpublished photographs provided by the Center of Molecular Neurobiology at the University of Hamburg, Germany. (A) A 2mm long commercially available polyethylene nerve tube (black arrow) used to bridge a defect in the mouse femoral nerve. The tube is filled with HNK-1 peptide mimic in soluble form. (B) 3 months following transection nerve has regenerated completely through the polyethylene tube (black arrow). Intact femoral nerve is shown (blue arrow) as reference nerve. (C) PE tube does not degrade and needs to be surgically removed following regeneration.

Porosity is another parameter considered when optimizing the design of a synthetic NGC. The use of pores in the outer conduit walls has proven to be both favorable and unfavorable for nerve regeneration depending upon the length of the nerve defect needing treatment (47, 156). It is thought that pores will allow for the influx of cells, nutrients, extracellular matrix proteins, and blood vessels into the inner lumen of the conduit to the regenerating nerve (16, 39, 61). In addition, pores may allow for the outflow of waste products away from the nerve injury. The optimal pore size utilized for the conduit outer walls varies within the literature (61-63). Despite the possible advantages of having pores in the outer wall, porous conduits have also been associated with disadvantages such as weakening of the conduit strength, infiltration of the inner lumen with various cell types and connective tissue, and the diffusion of bioactive growth factors out of the site of injury (25, 29, 39). On the other hand, impermeable, or non-porous, conduits may encourage successful regeneration as they typically have greater strength, separate the nerve injury from the external environment, preventing connective
tissue from infiltrating, and can concentrate neurotrophic factors secreted by the nerve stumps near the injured area. Despite the lack of a clear decision on whether or not pores are beneficial, studies specifically designed to investigate the differences between porous and non-porous synthetic NGC are very rare (156).

The material selected to fabricate the NGC is also a very important factor to consider. A number of synthetic materials including poly(esters), such as poly(glycolic acid) (PGA) and polycaprolactone (PCL) have been explored for this purpose. From these materials, and several others, a number of NGCs have been approved by the FDA for clinical use. When used to treat short nerve defects, these devices result in comparable and sometimes better regeneration than treatment with an autograft. However, when a critical size nerve defect is present, treatment with these conduits results in poorer regeneration and functional recovery as compared to treatment with an autograft (39, 42-45). This low success rate may be a result of several factors: the limited amount of time these materials can maintain their mechanical properties, their highly acidic degradation products, swelling, early resorption, or too slow degradation (8, 11, 31, 32, 34, 38, 41, 46, 48).

A treatment method for critical size peripheral nerve defects is still needed. A conduit fabricated from a material providing improved mechanical properties, long-term biocompatibility, and a controllable degradation rate is required. In this study tyrosine-derived polycarbonates (TyrPCs) are investigated to use for fabrication into a nerve conduit to treat critical size gaps. These materials are non-cytotoxic and biodegradable reducing the chance of an inflammatory host response to occur (55, 57).
TyrPCs offer a flexible chemistry allowing one to synthesize them into both copolymers and terpolymers (with the addition of polyethylene glycol (PEG) and/or desaminotyrosyl-tyrosine (DT)), in order to alter their mechanical and degradation properties for very specific applications (55-60, 157-160). Copolymers with PEG or DT have been shown to undergo backbone degradation at a rate much faster than poly(DTE carbonate)s (160). In the case of PEG, these copolymers are associated with an increased amount of water adsorption and a decrease in the mechanical strength of the material (59, 159, 161). In addition, the presence of PEG affects some surface properties, bulk properties, and biological properties such as the amount of protein the material adsorbs (162) and the amount of cell motility on the material, increasing with increasing molar fraction of PEG up to about 4 mol%, and then decreasing when more PEG is added (163). The use of the terpolymers (with both PEG and DT) can allow for further alteration of the rates of degradation and resorption, the bulk and surface properties, and the biological properties of the polymer (160).

For this study conduits were fabricated from two different polymer compositions with and without pores in order to determine differences in the conduit’s properties due to these variables. Characterization methods used in this study include the following: scanning electron microscopy to investigate conduit morphology, in vitro degradation studies to analyse molecular weight loss of the conduit over time, mechanical testing (tensile test and three point bend test) to determine conduit strength, and in vitro viability and neurite outgrowth assays with polymer coated coverslips to determine biocompatibility.
Materials and Methods

Polymer synthesis

Tyrosine-derived polycarbonate terpolymers were synthesized at the New Jersey Center for Biomaterials using previously published procedures (160). These terpolymers (See Figure 3.1 in Chapter 3 of chemical structure) are comprised of desaminotyrosyl tyrosine alkyl ester (DTR), desaminotyrosyl tyrosine (DT), and poly(ethylene glycol) (PEG). In order to simplify the nomenclature of the tyrosine-derived terpolymers, the notation RXXYY(MW) is used to name poly(DTR-co-XX%-DT-co-YY%-PEGMW carbonate) where R is the alkyl pendent chain, XX is the mole percent of DT, YY is the mole percent of PEG and MW is the weight average molecular weight of PEG. As an example, poly(DTE-co-00%-DT-co-04%-PEG1K carbonate) and poly(DTE-co-10%-DT-co-0.5%-PEG1K carbonate) will have the notation of E0004(1K) and E1000.5(1K), respectively, where E stands for ethyl ester pendant group.

In this study the following two polymer compositions were used: E0004(1K) and E1000.5(1K). E0004(1K) was selected because it contains no DT and was hypothesized to be the slower degrading of the two materials, remaining intact throughout the length of the study (15 weeks in vivo). In addition, the 4% presence of PEG(1K) in this composition was thought to also affect the mechanical, degradation, and biological properties of the conduit. The second polymer E1000.5(1K) was chosen as it contains 10% DT and was thought to degrade faster than the E0004(1K). However, only a very small amount of PEG(1K) (0.5%) was incorporated into this polymer, and therefore it does not adsorb as much water as the E0004(1K). We hypothesized that this difference would affect the mechanical, degradation, and biological properties as well.
**Preparation of Coverslips**

Glass coverslips were spin-coated with E0004(1K) and E1000.5(1K) for *in vitro* cell studies. Glass coverslips were cleaned by sonication in acetone for 15 minutes followed by sonication in ethanol for 15 minutes. Individual coverslips were dried under a stream of nitrogen in a lint free environment, ensuring that each coverslip was debris free. Polymer (either E0004(1K) or E1000.5(1K)) was dissolved in tetrahydrofuran (THF) to produce a 2.5 % (w/v) solution that was spin-coated on coverslips at 4000 rpm for 30 seconds. The TyrPC coverslips were placed in vacuum for 24 hours to evaporate residual THF.

**In Vitro Assessment of Conduit Material with Spinal Cord Neurons**

Motor enriched spinal cord neurons were obtained and cultured on coverslips prepared above as described in the Materials and Methods section of Chapter 3: *In vitro* Assessment of Conduit Material with Spinal Cord Neurons. Immunocytochemistry and analysis was performed as described in Chapter 3 as well. In addition to measuring outgrowth, cell viability was measured by staining with Hoechst 33258 nuclear stain (AnaSpec Inc., San Jose, CA, Cat# 83219, 20 mM solution, 1:500 dilution) for 10 minutes. Images of each coverslip were taken using a Zeiss Axiocam with a 20x objective and Axiovision imaging software. The number of neurons per field of view was counted. In each experiment, 10 random images from each coverslip were analyzed in a double-blind manner.

**Conduit Fabrication**

Hollow conduits with an inner diameter of 580 μm (inner diameter of commercially available PE conduits previously used *in vivo* (134)) were fabricated using
a dip-coating (KSV dip-coater, KSV Instruments Inc., Helsinki, Finland) technique in which a Teflon-coated mandrel is dipped at constant rate into a polymer solution. Using this technique, conduits with consistent inner diameters and wall thicknesses were routinely synthesized. A 30% weight to volume solution containing 3 grams of polymer in 10 mL of methylene chloride was used for dip-coating. Following dip-coating, the mandrels were dried in vacuum overnight to remove remaining solvent. The conduits were then pulled off the mandrels and cut to the necessary length for \textit{in vitro} characterization and \textit{in vivo} evaluation. Sucrose crystals sieved to 25-45 μm were incorporated into a E1000.5(1K) polymer solution to make porous conduits from this material. After the conduits were removed from the mandrel the sucrose was leached out in water to create a porous structure. The conduits were then dried again and cut to the proper length. Conduits fabricated without pores (impermeable or non-porous) are indicated with a “NP” following the polymer composition and conduits fabricated with pores (porous) are indicated with a “P” following the polymer composition.

\textit{Scanning Electron Microscopy}

Scanning electron microscopy (SEM) was used to examine the cross-section of the conduits, the wall thickness, and the porosity of the outer wall. The conduits were sputter-coated with gold for 2 minutes to minimize charging effects and observed with an Amray 1830I SEM at 20 kV.

\textit{In Vitro Degradation}

Conduits (5mm in length) were placed into scintillation vials containing 2 mL of pre-warmed (37 °C) simulated body fluid (r-SBF) (164). Vials were incubated at 37 °C for the duration of the study. r-SBF was changed weekly. At the predetermined time-
points, 3 samples were removed from the incubator, the supernatant was removed, and the conduits were lyophilized to remove all water. Lyophilized samples were dissolved in 1 mL of N,N-dimethylformamide : 0.1 % trifluoroacetic acid (v/v) (DMF-TFA, Sigma Aldrich) and filtered (0.45 μm syringe filters, Whatman) to determine the molecular weight distribution of the remaining polymers. Gel permeation chromatography (GPC, Waters) was performed in order to determine the molecular weight relative to polystyrene standards. The GPC system consisted of a 515 HPLC pump, 717plus autosampler, a 2414 RI detector and Empower Pro Software (Waters Corporation, Milford, MA). Two PL-gel columns (Polymer Laboratories, Amherst, MA), pore size 10³ and 10⁵ Å, were used in series. Dimethylformamide (DMF) containing 0.1 % trifluoroacetic acid (TFA) at a flow rate of 0.8 mL/min was used as the mobile phase. All analyses were done in triplicate.

**Mechanical Testing Analysis**

Mechanical properties of the NGCs were characterized by tensile and three-point bending tests using an MTS Sintech/5D Universal Testing machine equipped with a 100N load cell. The samples were hydrated in PBS (37°C) for 24 hours before testing. All tests were performed immediately after the samples were taken out of the incubator, under ambient humidity and temperature. For tensile testing, 15 mm long conduit samples were held in place by 2 split shots (Size #BB, Bullet Weights, Alda, NE, USA) with a gage length of 1.4 cm. A displacement rate of 0.5mm/min was used. The tensile structural stiffness was determined from the tangent to the stress-strain curve at the origin (0-2 % strain). The calculated stiffness and failure strength were an average of 3 measurements.
For the three-point bending test, intact 15mm long conduit samples were hydrated in PBS (37 °C) for 24 hours before testing. All tests were performed immediately after the samples were taken out from the incubator, under ambient humidity and temperature. The conduit was placed into the holder at two points 8 mm apart (i.e., L=8 mm). At a third point midway between these, an increasing load was applied on the sample from above. The bending stiffness, EI, was calculated using the following formula: $EI = \left( \frac{F}{d} \right) \left( \frac{l^3}{48} \right)$ where the best estimate of the slope $\left( \frac{F}{d} \right)$ was determined by linear regression from the initial (linear) portion of the F-d curve. $I$ is the moment of inertia about the bending axis (for a cylindrical tube: $I = 0.78(r_o^4 - r_i^4)$ where $r_o =$ outer radius and $r_i =$ inner radius).

**Results and Discussion**

**Neuron Viability and Neurite Outgrowth**

In this study TyrPC was investigated for its potential as a material for fabrication into a nerve guidance conduit. *In vitro* assessment of the TyrPC polymer with a motor enriched spinal cord neuron population showed greater numbers of cells attaching to the polymer surfaces (as compared to the control tissue culture polystyrene) (Figure 2.2A) and greater neurite outgrowth on TyrPC as indicated by a shift in both TyrPC peaks (blue and green lines) to the right of the Control (black dashed line) (Figure 2.2).

**Conduit Structure**

These polymer compositions were dip-coated to produce conduits with an inner lumen of 580 µm and an outer wall thickness (either porous or non-porous) of approximately 100 µm. Scanning electron microscope images of the conduits showed a uniform microstructure could be produced from the dip-coating technique (Figure 2.3).
Non-porous conduits displayed a smooth outer wall while porous conduits showed an outer wall with pores on the order of 25-45 μm in diameter.

Figure 2.2: *In vitro* rat spinal cord neuron viability and outgrowth assays. (A) Total live neurite cell count on polymer coated coverslips (coated with PLL and Laminin). Values represent averages ± standard deviation. (B) Neurite outgrowth distribution on polymer coated coverslips with PLL and Ln. Both polymers shift the outgrowth distribution to the right of the control substrate indicating greater neurite outgrowth.

Figure 2.3: Scanning electron microscope (SEM) images of conduits fabricated from TyrPC. (A,B,C) Outer wall, cross section, and magnification of outer surface of non-porous conduit, respectively. (D,E,F) Outer wall, cross section, and magnification of outer surface of porous conduit, respectively. Scale bars from left to right: 100 μm, 100 μm, 10 μm.
In Vitro Degradation of Conduits

The nerve conduit structure is most crucial to regeneration immediately following nerve injury. This is because there is a short time period of time for the regenerating axons to reach the distal stump before the muscle is denervated and non-functional. After this period of time, regeneration cannot increase further even if the conduit degrades. Therefore, the ideal degradation time of a conduit needs to be relatively short following nerve injury, with a half-life of approximately 2–3 weeks following implantation (64). In this study, the in vitro degradation rate of conduits fabricated from various polymer compositions was investigated. Figure 2.4A shows the molecular weight loss of porous conduits fabricated from E0004(1K), and porous (P) and non-porous (NP) conduits fabricated from E1000.5(1K) over the 15 week study. No significant difference was found between the initial molecular weight and the molecular weight following fabrication. Figure 2.4B shows the molecular weight loss of each polymer composition over a 15 week period in vitro. These results show that there is no significant difference between the degradation profiles of these polymers and that the presence of a porous outer wall does not enhance the degradation rate. All 3 types of conduits started with an initial molecular weight of approximately 250 kDa and reached a final molecular weight of approximately 100 kDa by week 15. By weeks 2-3, the conduits lost approximately 40% of their initial molecular weight. At about weeks 6-8 the conduits reached their half life of approximately 125 kDa.
Figure 2.4: Gel permeation chromatography measurements. (A) Molecular weight of the polymer before (blue bars) and after (red bars) fabrication of the conduit. Values represent averages ± standard deviation. (B) The effect of polymer composition and porosity on the in vitro degradation profiles of conduits in r-SBF at 37 °C. Values represent averages ± standard deviation.
Mechanical Testing of Conduits

Mechanical data collected are shown in Figure 2.5. From the load vs. displacement curves (Figures 2.5 A and D) conduits containing 10% DT (fabricated from E1000.5(1K)) showed greater mechanical stiffness and strength than conduits fabricated from E0004(1K). Also, the addition of pores (P vs. NP) weakened the mechanical properties of the conduits; the non-porous conduits were stiffer and stronger than the porous conduits. The tensile tests (Figures 2.5 A, B, and C) showed that the tensile structural stiffness (Figure 2.5B) of the E1000.5(NP) conduits (2.14±0.36 N/mm) and the stiffness of the E1000.5(P) (2.08±0.40 N/mm) were significantly higher than that of the E0004(1K) conduits (1.29±0.28 N/mm) (p<0.05). From the literature, commercial conduits (NeuraGen) had a structural stiffness of 2.38±0.86 N/mm which is not significantly different from the E1000.5(1K) conduits (165). The force applied to the commercial conduit at the failure point (6.89±2.6 N) is also not statistically different from the force at failure of the E1000.5(NP) conduits (6.68±1.65 N) stiffness (Figure 2.5C). However, both of these conduits have statistically higher forces at the failure point than the conduits fabricated from E1000.5(P) (3.90±1.33 N) and E0004(P) (1.55±0.84 N) (p<0.05). The data from the three-point bending study are shown in Figures 2.5 D and E. The bending stiffness of E1000.5(NP) conduits (66.42±13.20 N/mm²) was statistically higher than that of the E1000.5(P) (24.35±8.63 N/mm²) and the E0004(P) (17.41±5.70 N/mm²) (p<0.05).

The mechanical properties of the nerve conduits is important as a weak conduit may lead to fragmentation or collapse at the injury site. The structural integrity of the conduit must maintain itself during suturing and initial implantation into the nerve defect.
After surgery and during the period of regeneration, the conduits need to provide sufficient biomechanical support keeping their inner lumen open, resisting collapse or kinking of the outer walls. In particular, the conduits must resist muscular contraction, stretching, and distortion during the period of regeneration (165). In this study, TyrPC conduits were fabricated with and without DT and with varying amounts of PEG(1K). Conduits were also fabricated with and without pores in their outer walls. Conduits containing DT showed greater structural stiffness than conduits without DT and conduits without pores showed greater structural stiffness than conduits with pores in tensile and bending tests. (165)

Figure 2.5: Mechanical testing of TyrPC conduits. (A, B, C) Results from tensile testing. (A) Representative load–displacement curve of tensile testing for each type of TyrPC conduit. (B) Tensile structural stiffness of TyrPC conduits. (C) The failure strength of the conduits (maximum force obtained). (D, E) Results from three-point bending test. (D) Representative load–displacement curve of three-point bending for each type of TyrPC conduit. (E) Bending stiffness of TyrPC conduits. Values in B, C, and E represent averages ± standard deviation. (*p<0.05).
Conclusions

The overall goal of this study was to develop a biodegradable, non-cytotoxic NGC that can be used to bridge together crucial size nerve defects. Ideally, this conduit would be made from a material that supports neuron survival and outgrowth, is easily fabricated into a conduit that has the proper dimensions for in vivo evaluation in a mouse femoral nerve model, degrades in a controlled manner over the 15 weeks in vivo, and provides enough mechanical strength to prevent fragmentation and kinking. In this study we evaluated the use of 2 different TyrPCs (E1000.5(1K) and E0004(1K)) with and without pores in the outer conduit walls for nerve guidance conduits. A series of characterization techniques were used to evaluate properties of these conduits. From the results it appears promising that all three TyrPC conduits evaluated fulfil these requirements and may have potential as a NGC to treat critical size nerve defects.
References

CHAPTER 3

Preface

This chapter is in preparation for submission as:

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A Tyrosine-derived Polycarbonate Conduit to Improve Functionality and Quality of Peripheral Nerve Regeneration over a Critical Size Gap

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Abstract

Following complete nerve transection, tubulization can be employed to help guide regenerating axons to their appropriate distal targets. However, even in the presence of a conduit, regeneration often results in poor functional recovery as axons are slow to extend, reaching the target musculature long after atrophy has already occurred. Implanting a synthetic conduit made from a material with surface characteristics that help expedite the regenerative process would increase the number of distal reconnections, improving upon functional recovery. In this study, biodegradable and non-cytotoxic tyrosine-derived polycarbonate (TyrPC) terpolymers are used to fabricate conduits with either porous or non-porous outer walls, and evaluated against commercially available non-porous polyethylene (NP-PE) tubing. The two materials are characterized in vitro for differences in surface properties including the amount of protein adsorption and the ability to enhance neurite outgrowth. The conduits are then evaluated in vivo for peripheral nerve regeneration in a critical size nerve defect in the mouse femoral nerve model. Our results illustrate that the TyrPC material enhances protein adsorption and neurite outgrowth in vitro as compared to PE. In addition, in vivo the TyrPC conduits improve functional recovery significantly over NP-PE tubes, producing regenerated nerves with higher axon counts, greater percentages of myelination, and less fibrotic tissue. Early in vivo time
points support the hypothesis that TyrPC conduits allow for more Schwann cell migration and enhanced fibrin matrix formation as compared to the NP-PE tube. The use of a conduit fabricated from TyrPC without any additional biological enhancers is significantly better for the treatment of a critical size nerve defect than NP-PE tubing.

**Introduction**

Peripheral nerve injuries present a serious medical concern, constituting approximately 2.8 % of all trauma cases in the United States, and approximately 100,000 neurosurgical procedures in the United States and Europe annually (1, 2). Although peripheral nerves have the ability to regenerate on their own, their regeneration often results in poor functional recovery. As axons extend across large gaps slowly, they often make random reconnections with axonal targets and do not reach the distal stump until long after denervation has already occurred (3). This poor recovery leads to a lower quality of life for the patient, alldynia, inadequate muscle contraction and coordination, and poor reflexes (4-6).

Methods of repair for peripheral nerve defects depend upon the severity of the injury. When the defect is small enough, nerve stumps can be directly sutured together, end-to-end (7). However, when the defect is long enough to produce tension on nerve stumps if sutured together, tubulization, a process of implanting a biologic or synthetic tube or conduit around the nerve gap and bringing the nerve stumps into the conduit to direct regeneration, has become a clinically practiced method of repair. Tubulization allows factors and cells to remain in direct contact with the regenerating nerve, provides direction to axons, and protects the wound space from cells and tissues exterior to the inner lumen (8).

Many naturally existing conduits can be used to bridge nerve gaps including vein, artery, muscle, or nerve segments (9). Autologous nerves are preferred, however, limited availability
of donor nerves, donor site morbidity, and differences in the structure and size of the nerve tissue needed are complicating issues (10, 11). In addition, complete recovery of motor and sensory nerve functions following an autograft procedure is still less than 50% in treatment cases, leaving much room for improvement (12).

Synthetic conduits are commonly used as an alternative to autografts for nerve regeneration as there is no limitation on their supply, their chemical structure can be altered to adjust their properties, and they can be fabricated into designs with various dimensions to fit different size defects (10, 11). The use of synthetic conduits also eliminates the need to sacrifice an existing nerve elsewhere in the body (13). In early and some more recent studies, polyethylene and silicone have been used as synthetic materials for entubulation as they are inert, readily available, and able to facilitate repair of transected nerve gaps up to 1 cm in length when implanted empty or filled with saline (14-22). Additionally, polyethylene tubing has commonly been employed as a surrounding material in order to evaluate the effects of different intraluminal fillers on nerve regeneration (15, 17). For all of these studies described, polyethylene is sufficient as either a holding material (for the evaluation of the filler) or as an empty guide for directing nerve regeneration, however after some time, these nondegradable conduits can result in chronic host tissue response and/or nerve compression, deterring regeneration, and requiring a second surgery for removal (23-25). Many studies have addressed the concerns associated with using such nondegradable conduits such as polyethylene as a nerve conduit, however a few number of studies have actually compared alternative materials directly to polyethylene (18).

To avoid these issues, biodegradable materials are more commonly used to fabricate nerve conduits. Studies from over the past 20 years have resulted in the following clinically
available biodegradable nerve conduits being approved by U.S. Food and Drug Administration (FDA) and Conformit Europe (CE): Neurolac (poly-DL-lactide-caprolactone, Polyganics BV), Neurotube (polyglycolic acid, Synovis), NeuraGen (collagen type I, Integra NeuroSciences) and Neuro-Matrix/Neuroflex (collagen type I, Collagen Matrix Inc.) (26). However, when these conduits are used empty in experimental and clinical studies to treat injuries greater than the critical size (5.0 cm in humans, 1.5 cm in rats, 5.4 ± 1.0 mm for the mouse sciatic nerve model (27)) nerve regeneration and functional recovery are significantly impaired as compared to autografts (11, 28-31). These results have been associated to problems of swelling that occludes the inner lumen, early resorption causing premature collapse of the outer walls, and poor mechanical properties, or too slow degradation, leading to nerve compression and fragmentation of the conduit in vivo (26, 27). In addition, some of the degradation products of these materials are highly acidic and have a low solubility that together can cause a severe inflammatory response leading to macrophage invasion, fibrosis, and disordered axonal outgrowth (32-34). A recent study evaluating of the Neurolac conduit in a 1 cm rat sciatic nerve defect observed extremely rapid degradation, swelling, collapse of the inner lumen, and fragmentation (35). These events were associated with a severe foreign body reaction that inhibited regeneration (2, 9, 26, 36, 37).

Although nerve repair treatment options have progressed throughout the years, there is still an unmet need for conduits that can successfully bridge together critical size defects and provide an enhanced level of biocompatibility. To achieve this goal, the development of a nerve conduit prepared from a material with improved mechanical properties, long-term biocompatibility, and a controllable degradation rate is required. Therefore, biomaterials from the family of tyrosine-derived polycarbonates (TyrPC) are explored as a potential material for
fabrication and evaluation of nerve conduits to treat critical size gaps. TyrPCs are composed of the subunits desamino-tyrosine ethyl ester (DTE), desamino-tyrosine (DT), and poly(ethylene glycol) (PEG). DTE and PEG have counteracting influences on hydrophobicity, degradation, stiffness, strength, and protein adsorption (38). DT, which has a charged acid group instead of the ethyl ester of DTE, will increase degradability and bioresorbability (39). These materials have proven to be non-cytotoxic and biodegradable, breaking down primarily via hydrolysis into naturally occurring metabolites, reducing the chance of an inflammatory host response (40, 41).

In this study TyrPCs were evaluated for their potential to enhance nerve regeneration *in vitro* and *in vivo*. *In vitro* studies on 2D substrates indicated that protein attachment and neurite outgrowth are significantly enhanced on TyrPC as compared to polyethylene. *In vivo* experiments in a critical size defect in the mouse femoral nerve found that TyrPC conduits supported significantly greater regeneration and functional recovery than standard polyethylene conduits. Evaluation of recovery at early time points suggested that this enhancement could be correlated with enhanced Schwann cell presence and a more rapid formation of the fibrin matrix formation, both key features of peripheral nerve regeneration.

**Materials and Methods**

**Conduit Fabrication**

TyrPCs, synthesized utilizing previously published procedures (42) were used in this study with the following chemical structure: poly(DTE-co-DT-co-PEG<sub>1000</sub> carbonate) (Figure 3.1).
Figure 3.1: Chemical structure of TyrPC terpolymers consisting of DTE: Desaminotyrosyl-tyrosine ethyl ester, DT: Desaminotyrosyl tyrosine and PEG: Polyethylene glycol.

Briefly, hollow conduits with an inner diameter of 580 μm were fabricated using a dip-coating (KSV dip-coater, KSV Instruments Inc., Helsinki, Finland) technique in which a Teflon-coated mandrel was dipped at constant rate into a polymer solution. Using this technique, conduits with consistent inner diameters and wall thicknesses are routinely fabricated. For non-porous conduits, a solution containing 900 mg of polymer in 3 mL of methylene chloride was used. Following dip-coating, the mandrels were dried in vacuum overnight to remove any remaining solvent. The conduits were then pulled off the mandrels and cut to 5 mm length for in vivo evaluation. For porous conduits, a solution of 450 mg of sucrose crystals, sieved to 25-45 μm, and 450 mg of polymer dissolved in 3 mL of methylene chloride was used for dip-coating. After the porous conduits were removed from the mandrel, the sucrose was leached out in water to create the porous structure. The conduits were then dried under vacuum overnight and cut to 5 mm in length. Commercially available polyethylene tubes were also used (5 mm length, 0.58 mm inner diameter; Becton Dickinson, Franklin Lakes, New Jersey, USA).

**In Vitro Evaluation**

**In Vitro Assessment of Conduit Material with Spinal Cord Neurons**

The effect of TyrPC and PE on neurite outgrowth was determined using coverslips as prepared in the previous section. In order to help facilitate neurite survival and outgrowth, slips (both TyrPC and PE) were pre-coated with 200 μg/mL of poly-L-lysine (PLL, poly-L-lysine hydrobromide, MW 150 kDa, Sigma Aldrich, St.Louis, MO, Cat# P1399) for 1 hour at room
temperature followed by 20 µg/mL of laminin (Ln, natural, mouse, Invitrogen, Carlsbad, CA, Cat# 23017-015) overnight at 4 °C. Spinal cord neurons were isolated from 15-day-old embryonic rats and purified for a motor rich population via a series of trypsinization, trituration, and filtration steps. Each coverslip was seeded with 0.5 ml of a cell suspension containing 3.0x10⁴ cells/mL and maintained for 48 hours in a humidified chamber at 37 °C with 5 % CO₂. The cells were fixed at 48 hours after plating with 4 % paraformaldehyde for 10 minutes. Neurite outgrowth was evaluated using βIII-tubulin primary antibody (Covance, Princeton, NJ, Cat# MMS-435P, 1:1000 dilution) overnight at 4 °C followed by a secondary antibody (Alexa Fluor 488, goat-anti-mouse IgG (H+L), Invitrogen, Cat# A11001, 1:250 dilution) for 1 hour at room temperature. Nuclear staining was performed with Hoechst 33258 stain (AnaSpec Inc., San Jose, CA, Cat# 83219, 20 mM, 1:500 dilution) for 10 minutes. Images of each coverslip were taken using a Zeiss Axio.cam camera with a 40X objective and Axiovision imaging software. The length of total neurites per cell was measured by using ImageJ 1.43u software. For each coverslip 10 random images were analyzed in a double-blind manner.

**Protein Adsorption Assay**

The relative amounts of protein adsorption for three extracellular matrix (ECM) molecules (laminin (Natural, Mouse, Invitrogen, Carlsbad, CA, Cat# 23017-015), fibronectin (Bovine Plasma Fibronectin, Invitrogen, Carlsbad, CA, Cat# 33010-018), and collagen type I (PureCol, Bovine Type I (97 %) collagen, Advanced Biomatrix, San Diego, CA, Cat# 5005-B)) on TyrPC and PE films were evaluated. Briefly, TyrPC films prepared by compression molding and PE films (commercially available as described previously) were punched to the appropriate size to fit into a 96-well plate. Each extracellular matrix protein was diluted in ddH₂O to 20 µg/mL and 70 µL of protein solution was added to each polymer surface. The plate was
incubated at 37 °C for 48 hours in order to allow the proteins to adhere to the films. After the supernatant was removed, each well was rinsed 8 times with staining media (containing fetal calf serum for blocking) and rinsed 1 time with phosphate buffered saline. After rinsing, the primary antibodies to each protein were added for 1 hour at room temperature (Rabbit-anti-mouse laminin, Millipore, Cat# AB2034, Rabbit-anti-bovine fibronectin, Millipore, Cat# AB2047, Rabbit-anti-bovine collagen, Millipore, Cat# AB749P, all at a 1:100 dilution). The entire rinsing process was repeated and a secondary HRP-conjugated antibody (Goat-anti-Rabbit IgG(H+L) HRP conjugate, Millipore, Cat# AP307P, 1:200 dilution) was added to each well and incubated for 1 hour at room temperature. The rinsing process was repeated one more time and luminol was added (Reagent 1 and 2 added in equal amounts) (Novex ECL HRP Chemiluminescent Substrate Reagent Kit, Invitrogen, Cat# WP20005) to each well. After 5 minutes, the luminescence from each well was read using a Tecan plate reader with an integration time of 1000 ms and a settle time of 500 ms. Protein amounts were normalized to the control surface, tissue culture polystyrene.

**In Vivo Evaluation**

**Surgical Methods and Animal Groups**

All experiments were conducted in accordance with the Rutgers Animal Care and Facilities Committee and the Institutional Animal Care and Use Committee (IACUC). The surgical procedure was as follows: Female C57BL/6J mice were obtained at the age of 3 months. The animals were anaesthetized by intraperitoneal injections combination of xylazine:ketamine 12 mg/kg/: 80 mg/kg with a dosage of 0.1 mL/10 g body weight. The left femoral nerve was surgically exposed and a nerve transection was performed at a distance of approximately 3 mm proximal to the bifurcation of the nerve. The cut ends of the nerve were inserted into the nerve
conduit (either TyrPC or commercially available NP-PE) and fixed on each end with a 10-0 nylon suture (Fine Science Tools, Foster City, California, USA) so that a 4.5 mm gap was present between the proximal and distal stump. The inner lumen of the conduit was filled with sterile phosphate buffered saline and the skin wound was closed with size 7 mm wound clips (Fine Science Tools, Foster City, California, USA). The clips were removed at 2 weeks following surgery.

Three animal groups (8 animals each) were compared over a 15 week time period including: Porous tyrosine-derived polycarbonate conduits (P-TyrPC), non-porous tyrosine-derived polycarbonate conduits (NP-TyrPC), and commercially available non-porous polyethylene tubes (NP-PE).

**Motor Function Recovery**

Functional recovery was assessed using a single-frame motion analysis approach (43). Animals were trained to perform a classical beam walking test prior to implantation of the conduit. Following surgery, this test was performed weekly until the endpoint of the experiment (week 15). Rear view videos of the mice walking were collected using a high-speed camera (A602fc Basler, Ahrensburg, Germany) at 100 frames per second and stored on a personal computer in Audio Video Interleaved (AVI) format. The videos were used in order to observe the movements of the hind legs during the normal gait cycle. Measurements were performed on select frames of the videos in which defined phases of the walking cycle were seen using Simi Sports Player software (SIMI Reality Motion Systems, Unterschleissheim, Germany).

The foot base angle (FBA) (Figures 3.5A and B) was measured at the moment the left toes were fully extended on the beam and is defined by a line that divides the sole of the foot in half and a horizontal line across the beam. This angle evaluates the function of the quadriceps
muscle during the walking cycle (43) and is measured with respect to the medial aspect. The FBA changes significantly following injury and implantation of the conduit (Figure 3.5B) as compared to the FBA measured in intact animals (Figure 3.5A).

Another functional parameter, the protraction limb ratio (PLR) (43), was measured while the mouse performed a voluntary movement during a pencil grip test. For this test, the mouse was held upside down by its tail in front of a pencil and was allowed to grasp the pencil with its fore paws. The mouse tries to catch the pencil with its hind paws and simultaneously extends both hind limbs towards the pencil (Figure 3.5C and D). In intact animals, the relative lengths of the two hind legs (measured by lines connecting the most distal mid-point of each foot to the center of the base of the tail) is approximately equal and so the ratio of the right to left limb length (PLR) is approximately 1 (Figure 3.5C). Following injury, the limb cannot extend maximally (Figure 3.5D) and the PLR rises significantly above 1. Therefore, for this parameter, a value of 1 indicates full functional recovery. A value greater than 1 indicates poor functional recovery.

As a relative measure of functional recovery the recovery index (RI) was calculated for each animal for both the FBA and the PLR. The RI was calculated as a percentage using the following formula:

\[ RI = \frac{(X_{week \ y} - X_{week \ 1})}{(X_{week \ 0} - X_{week \ 1})} \times 100, \]

where \( X_{week \ 0} \), \( X_{week \ 1} \), and \( X_{week \ y} \) are intact values at week 0 (either FBA or PLR), values measured at week 1 after injury, and at week \( y \) (where \( y \) is the endpoint of the study, week 15), respectively. An RI value of 100 indicates complete recovery of the femoral nerve.
**Histomorphometric Analysis of Explanted Nerve**

Femoral nerves were dissected from animals fixed by perfusion with 4 % paraformaldehyde, post-fixed in osmium tetroxide, and embedded in resin according to standard protocol. Transverse 1 μm-thick sections from the mid-section of the nerve were cut and stained with 1 % toluidine blue/ 1 % borax in distilled water. Total numbers of myelinated axons per nerve cross-section were estimated with a Zeiss Axiocam using a 40X objective and Axiovision imaging software. The raw tissue area, cross-sectional area of the regenerating cable, and the % nerve regeneration were measured on the Zeiss microscope using a 10X or 20X objective and analyzed with ImageJ 1.43u software. Axonal (inside the myelin sheath) and nerve fiber (including the myelin sheath) diameters (Figure 3.8) were measured in a random sample from each section taken with a 100X oil objective on an Olympus IX80 microscope. For all myelinated axons, mean orthogonal diameters of the axon and of the nerve fiber are measured using ImageJ 1.43u software as previously described (44). The degree of myelination is estimated by the ratio of the axon to fiber diameter (g-ratio).

**Western Blot Analysis of Nerve Exudates at 1 Week In Vivo**

NP-TyrPC conduits and NP-PE conduits (n=3) were implanted into the mouse femoral nerve model using surgical methods described above. After 1 week post-implantation, the animals were sacrificed and nerve exudates within the conduits were carefully removed and run on 3–8 % SDS (PAGE) using a NuPAGE Tris-Acetate Gel (Invitrogen, Cat# LP0003) and transferred to PVDF membranes (Biorad, Cat # 162-0177). Membranes were blocked with 5 % nonfat milk for 1 hour and then probed with, S-100β (Abcam, rabbit-anti-mouse, Cat# AB868, 1:1000 dilution), GFAP (Abcam, chicken-anti-mouse, Cat# AB4674, 1:50000 dilution), GAPDH (Abcam, rabbit-anti-mouse, Cat# AB37168, 1:1000 dilution), and β-actin (Abcam, mouse
monoclonal, Cat# AB20272, 1:5000 dilution) overnight at 4 °C and detected through HPR luminescence of secondary antibodies (45).

**Morphologic Analysis of Fibrin Matrix Formation**

In order to visualize the presence of fibrin strands at 2 weeks post-implantation, NP-TyrPC and NP-PE conduits (n=3) were implanted into the mouse femoral nerve according to surgical methods described above. At 2 weeks after implantation, the animals were perfused with 4 % paraformaldehyde and the nerve explants were post-fixed in osmium tetroxide, and embedded in resin according to standard protocol. For inspection of the presence and orientation fibrin matrix, longitudinal 1 μm-thick sections of the nerve were cut and stained with 1 % toluidine blue/ 1 % borax in distilled water. Conventional light microscopy was used to visualize the presence and orientation of the fibrin matrix.

**Results**

**In Vitro Characterization**

TyrPCs are non-cytotoxic synthetic polymers that offer tremendous versatility for conduit characteristics and performance and have previously been approved for clinical use in humans (46). To evaluate TyrPCs as a potential nerve guidance conduit material, the polymer was dip-coated to produce conduits with an inner lumen of 580 μm and an outer wall thickness (either porous or non-porous) of approximately 100 μm (Figure 3.2). These conduits were compared by scanning electron microscopy (SEM) against the commercially available polyethylene conduits of similar dimensions previously used in nerve regeneration studies (14-22). SEM images of the conduits proved a uniform microstructure could be produced from the dip-coating technique and dimensions were comparable to polyethylene conduits.
Although SEM found conduits to have similar architecture, material and cell studies revealed disparate properties. Protein adsorption results showed significant differences between the two materials evaluated (Figure 3.3). The amounts of three essential extracellular matrix proteins (laminin, fibronectin, and collagen type I) adsorbing to TyrPC were significantly greater as compared to PE films. These proteins are of great importance to the nerve regeneration process as they are necessary for interactions with cell integrins and enhance cell behaviors such as adhesion, spreading, growth, and migration on the surface of the biomaterial (47).

**Figure 3.2**: Scanning electron microscope (SEM) images of conduits evaluated in this study. Cross sections of a (A, D) polyethylene conduit, (B, E) porous TyrPC conduit, and (C, F) non-porous TyrPC conduit respectively. Outer wall of a polyethylene conduit, porous TyrPC conduit, and non-porous TyrPC conduit, respectively. Scale bar in each row: 100 µm.

Following peripheral nerve injury, nerve regeneration occurs if the neuronal cell soma are intact and the axons are provided with the necessary environment to support their attachment and outgrowth (47, 48). Thus nerve guidance conduits must offer a non-cytotoxic surface that promotes neurite outgrowth. This was assessed by culturing motor neuron enriched spinal cord
neurons on TyrPC and PE 2D surfaces coated with PLL and Laminin. The results show that neurons extended axons to greater lengths on TyrPC as compared to PE (Figure 3.4), as indicated by the peak shift of TyrPC to the right of the polyethylene and control substrates’ peaks.

Figure 3.3: Relative adsorption of neurosupportive ECM proteins on 2D films of TyrPC and PE with control of TCPS. (*p<0.05, oneway analysis of variance with Tukey’s post hoc test).
In vitro rat spinal cord neurite outgrowth distribution on polymer coated coverslips pre-coated with PLL and Laminin. Peak shift to right indicates longer neurite extensions on TyrPC coated coverslips as compared to PE and Control coverslips.

**In Vivo Evaluation**

**Motor Function Recovery**

To evaluate the capacity of TyrPC conduits to enhance nerve regeneration *in vivo*, conduits were compared against polyethylene controls in a 5 mm critical size gap of the mouse femoral nerve. Functional recovery was quantified by single fame analysis of the FBA and PLR (Figure 3.6). Immediately following injury, the FBA increases from a starting value of approximately 65° to a value of approximately 110°, indicative of injury and denervation. A decrease in the FBA thereafter is indicative of nerve regeneration (15, 17, 49). By week 8, animals that received TyrPC conduits showed significant improvement in the FBA measurements as compared to mice that received NP-PE conduits. Animals with P-TyrPC or NP-TyrPC conduits showed an average FBA of 88° and 93°, respectively, while animals given
NP-PE conduits showed a higher average FBA of 107°. This trend was further accentuated at 15 weeks at which time animals that received P-TyrPC or NP-TyrPC conduits filled with saline decreased to an average FBA value of 85° while animals with NP-PE conduits filled with saline did not improve further, and demonstrated an average FBA of 108°.

To compliment the measurement of the reflexive FBA, voluntary movement which was evaluated throughout the 15 week recovery period by the PLR, as measured from the pencil grip test, confirms the improvement of motor function when a nerve injury is treated with a TyrPC conduit as compared to a PE tube (Figure 3.6C). Animals that received TyrPC conduits showed significantly improved PLRs than those that received NP-PE conduits by as early as 2 weeks following implantation. This implies that in only 14 days animals with TyrPC conduits began to regain the ability to use the injured limb for voluntary, non-load bearing movement. Results collected out to 15 weeks demonstrate that the PLR value for animals with TyrPC conduits approaches pre-surgical values at a faster rate than animals with NP-PE. Animals with TyrPC conduits significantly improve in the PLR value at week 15 (with an average PLR value of 1 as compared to animals treated with PE conduits (with an average PLR value of 1.55).

Calculation of the recovery index (RI), which normalizes the recovery of each animal at the end of the study to its pre-injury FBA or PLR, further supported that recovery promoted by TyrPCs was enhanced over polyethylene. An RI value of 100 % indicates complete recovery and RI values (for both the FBA and the PLR) approaching 100 % were evident for animals that received both porous and non-porous TyrPCs (Figures 3.6B and D). Animals that received PE conduits demonstrated an average RI value for the FBA parameter of -26 % and an average RI value of 23 % for the PLR measurement. The tight grouping of the animals within each TyrPC group indicates consistent performance in contrast with the variation in the RI values calculated...
from animals receiving polyethylene conduits. Thus, the overall functional results indicate that the use of a TyrPC conduit as compared to a NP-PE tube results in superior recovery of functional movement.

**Histomorphometric Analysis**

At the end point of the animal study the nerves were removed from the animals and transverse 1 µm sections from the center of the conduit were evaluated for histomorphometric analysis (Figure 3.7). A significantly greater number of axons were counted within regenerating nerve cables formed within TyrPC conduits as compared to a NP-PE tube, regardless of the presence of pores in the outer walls of the TyrPC conduit. Nerves within TyrPC conduits had, in addition, a smaller area of raw fibrous tissue was found in animals treated with TyrPC conduits relative to NP-PE. The cross-sectional area of the myelinated nerve fibers was significantly greater in both TyrPC conditions as compared to the NP-PE tube. Also, the percentage of myelinated nerve fibers as measured out of the total raw tissue area was significantly greater in both TyrPC conduits as compared to the regenerating nerve cables present within the NP-PE tube.
Figure 3.5: Video frames showing the functional measurements performed on mice following implantation of the conduit. The white lines drawn in the video frames show the foot base angle (A and B), and the limb lengths used for calculation of the protraction limb ratio (C and D) (A) Foot base angle (FBA) of mice pre-injury averages 50-70°.  (B) FBA of mice one week post-injury averages 90-110°.  Functional recovery is denoted by a reduction in this degree angle.  (C) The pencil grip test measures the protraction limb ratio (PLR) on a mouse pre-injury, where both limbs are similarly extended, giving a ratio of 1.  (D) PLR on a mouse one week post-injury shows the disparity in limb protraction due to injury, resulting in a PLR > 1.
Figure 3.6: Metrics of functional recovery promoted by TyrPC and polyethylene conduits in vivo. (A) FBA for 15 week period following surgical insertion of porous TyrPC conduits (P-TyrPC), non-porous TyrPC conduits (NP-TyrPC), and non-porous polyethylene conduits (NP-PE) pre-filled with saline. (B) Recovery index for FBA at week 15. Each dot represents one animal. (C) PLR for all conditions. (D) Recovery Index for PLR at week 15. (*p<0.001, oneway analysis of variance with Tukey’s post hoc test).
Figure 3.7: Histomorphometric analysis of femoral nerves regenerated in TyrPC and polyethylene conduits. (A-C) Representative cross sectional images (40X, Scale: 50 µm) of nerve sections stained with toluidine blue from the midpoint of regenerated femoral nerve after tubulization with either (A) NP-PE Saline (B) P-TyrPC Saline, or (C) NP-TyrPC Saline. (D) Axon count of myelinated axons in the regeneration cable in the mid-conduit nerve section for each conduit type. (E) Raw tissue area (F) Cross sectional area of regenerated nerve fibers. (G) % myelinated nerve fibers in regenerating nerve cable. * Asterisks indicate significant difference between group mean values from NP-PE (*p<0.05, oneway analysis of variance with Tukey’s post hoc test).
Representative 100X images of 1 µm thick cross-sections stained with toluidine blue as well as the fiber diameter distribution from each condition are shown in Figure 3.8. The NP-TyrPC and P-TyrPC conduits filled with saline generated nerve cables with a large number of axons, fascicular structures, a large range of nerve fiber diameters, and little fibrous tissue. In addition, blood vessels were present within the cross-sectional area. However, the NP-PE tubes filled with saline only contained little to no evident axons. The inner lumens were completely filled with dense, fibrous tissue and no vascularization was observed.
Figure 3.8: Representative nerve sections and fiber diameter analysis. Representative cross sectional images (100X, Scale: 20 µm) of nerve sections stained with toluidine blue from the midpoint of regenerated femoral nerve and histogram of the relative distribution of nerve fiber diameter after tubulization with either (A) NP-PE Saline, (B) P-TyrPC Saline, or (C) NP-TyrPC Saline. Histograms of fiber diameters reveals a reduced number of small axons and an increased number of larger axons in animals treated with TyrPC conduits as compared to animals treated with PE conduits.
G-ratio analysis (Figure 3.9) offers a finer look at the myelination of the individual axons present within the conduit. A high g-ratio indicates too little myelination whereas a low g-ratio is indicative of thick myelination \(^{(50)}\). Results showed that all g-ratio distributions were significantly different from one another. Animals treated with TyrPC conduits produced axons surrounded by thinner myelin sheaths as compared to animals treated with NP-PE conduits, despite the smaller number of myelinated axons present within NP-PE conduits.

**Figure 3.9: Analysis of axonal myelination in regenerated nerves.** (A) Representative 100X oil objective microscopic image of mid-conduit nerve section stained with toluidine blue. The magnified images show measurements for the mean orthogonal diameters of the axon (black arrows) and of the nerve fiber (white arrows). (B) Shown are normalized frequency distributions of g-ratios(axon/fiber diameter) from nerve samples treated with each type of conduit. Each of the distributions shown is significantly different from the other two distributions in the same panel as determined by oneway analysis of variance with Kolmogorov-Smirnov post-hoc test \((p<0.001)\).

**Early Differences in Nerve Repair between Conduit Materials**

As demonstrated by the PLR results shown above, animals treated with TyrPC conduits regained voluntary movement earlier than animals treated with NP-PE conduits. This early recovery may be due to a difference in the rate at which the regenerative nerve cable develops
and matures across the nerve defect site. The initial formation of a fibrin cable helps support axonal in-growth and Schwann cell infiltration and is crucial for determining at an early time point the final outcome of the regenerating nerve cable (51).

Schwann cell presence is an essential component of the early regenerative process, as these cells are responsible for clearing debris, delivering neurotrophic factors, and helping to establish the fibrin matrix for axons to extend across (23, 52-54). Therefore, in this study we evaluated the presence of Schwann cells within nerve conduits at 1 week post-implantation. Conduit exudate was removed and Western blot analysis was performed on these samples. Results revealed a greater presence of Schwann cell proteins, S-100β and GFAP, in nerve exudates removed from TyrPC conduits as compared to exudates removed from within PE conduits (Figure 3.10). Additionally, we visualized the formation of an initial fibrin matrix and found that fibrin polymer strands were more prevalent at 2 weeks post-implantation in TyrPC conduits as compared to PE conduits (Figure 3.11). The fibrin strands in all 3 TyrPC conduit samples evaluated appeared to display a longitudinal orientation (as indicated by the black arrow in Figure 3.11) suggesting the beginnings of the formation of a fibrin cable that forms from the proximal stump to the distal stump (55).
Figure 3.10: Western blot analysis of S-100β and GFAP collected from non-porous TyrPC and PE conduit exudates 1 week after implantation.

Figure 3.11: Representative photos of longitudinal sections of the acellular fibrin matrix within conduits at 2 weeks after implantation. (A) The natural matrix found in TyrPC conduits. (B) The matrix present in PE conduits. The polymers comprising the natural fibrin matrix in the TyrPC had a predominant longitudinal orientation (black arrow in A) whereas no fibrin strands were observed in PE conduits. White arrows mark edges of the inner lumen within each conduit type. Scale bar: 0.5 mm.
Discussion

Through this study, we have found that conduit material, rather than outer wall structure, plays a key role in the regenerative potential of a nerve guidance conduit by directly affecting the key processes associated with successful regeneration, such as protein adsorption, fibrin matrix formation, and Schwann cell infiltration. We demonstrate that conduits fabricated from TyrPC outperform conduits fabricated from PE in terms of the degrees of nerve functionally and histology that they differentially promote when evaluated in a critical size defect in the mouse femoral nerve model. Furthermore, our study provides direct evidence that the conduit material on its own (without the additional variable of outer wall structure) effectively influences the regenerative potential of a conduit in vivo.

In vitro characterization assays demonstrated the potential of the TyrPC material as the base material for nerve guidance conduits for nerve regeneration. Specifically, essential extracellular matrix proteins (laminin, fibronectin, and collagen type I) all adsorbed in significantly greater amounts to the TyrPC surfaces as compared to PE surfaces. Implanted materials are immediately coated with proteins from serum and cells and the interactions between these cell adhesion proteins and integrins within a cell are necessary to regulate cell behaviors including attachment, migration, and proliferation on the surface of the biomaterial (56, 57). For example, Schwann cells need to adhere, migrate, and proliferate, in order to organize into Bands of Büngner, through which axons are guided (58). The chemical composition and surface characteristics of the material used to fabricate the nerve conduit are critical as they will affect the amount, organization, and conformation of the adsorbed proteins presented to the cells and should therefore be optimized to favor these interactions (56). In vivo, greater amounts of protein adsorption to conduits fabricated from TyrPC as compared to
conduits fabricated from PE may lead to a more robust cellular response enhancing the rate and quality of nerve regeneration.

The immediate transection of the femoral nerve in mice leads to injury of the quadriceps muscle and prevents it from extending properly (43). This injury can be detected from analysis of individual steps of the mouse taken during the normal walking cycle (43). Functional results as early as week 6 clearly illustrate that functional recovery after injury is sensitive to the type of conduit used and that a conduit fabricated from TyrPC improves function as compared to a polyethylene tube. The addition of macropores to the outer tube wall was intended to enhance the conduit’s regenerative potential by allowing for the exchange of nutrients through the outer wall and capillary and fibrous tissue ingrowth, to potentially form a tissue sheath around the regenerating cable and prevent collapsing of the outer wall (35, 59-61). However, although others have shown the benefits of porous outer walls (62), in our study the addition of pores within the outer walls of the TyrPC did not appear to improve recovery over the use of a non-porous TyrPC conduit. Similar results have been found in studies where animals treated with semi-permeable tubes resulted in regeneration comparable to animals treated with impermeable materials (35, 61) or even resulted in worse nerve regeneration as compared to animals treated with impermeable conduits (63). Histomorphometric results confirmed that TyrPC, either porous or non-porous, significantly improve upon the quality of the regenerated nerve when used to repair a peripheral nerve injury. Axon count is significantly higher, as well as cross-sectional area of the regenerating nerve cable, and the percentage of myelination. In addition, a fiber distribution plot shows the wide range of nerve fiber diameters present within a TyrPC conduit as compared to the NP-PE conduit, which contained little to no axons.
In the literature, several studies demonstrate the advantages of using porous nerve conduits (62, 64) while others show the advantages of using nonporous conduits (35) for the treatment of peripheral nerve injuries. However, very few studies directly compare porous to nonporous nerve conduits. Of the small number of studies that do compare the two, no studies compare nonporous and porous conduits fabricated from the same material (63). While some studies show that nonporous conduits contain the fluid and cells secreted by the nerve stumps at the injury site, in direct association with the regenerating axons, other studies explain how the presence of pores allows for the exchange of the contents within the conduit with the contents extracellular to the conduit, enhancing cell migration and fibrin cable formation (64). In this study, we directly compared a nonporous conduit to a porous conduit fabricated from the same material, TyrPC, and found no statistical differences between the functional recovery of the animals treated with these conduits or in the quality of the nerve regenerated. However, when compared against a nonporous conduit fabricated from another material, PE, animals treated with TyrPC conduits (either porous or nonporous) statistically performed better than animals treated with PE conduits in terms of both parameters. By evaluating a non-porous conduit fabricated from TyrPC against a non-porous conduit fabricated from polyethylene, we were able to easily differentiate between differences in regeneration due to material interactions. Therefore, we conclude that the conduit material itself directly affects the regenerative potential of the nerve, most likely by affecting the quality of the fibrin matrix formation, the degree of Schwann cell migration into the matrix, and/or also axonal regrowth.

We believe that the differences observed in this study between the conduits fabricated from TyrPC and the conduits fabricated from PE are due to a specific interaction of the regenerating elements with the material constituting the outer conduit wall. The behavior of the
TyrPC illustrated by the results in this study is quite evident. Notably, the TyrPC conduits were able to bridge a critical size defect when filled with phosphate buffered saline only; no additional growth factors were necessary. Although it is unlikely that the fibrin matrix itself or the cell or nerve processes migrating across this matrix are in close contact with the inner walls, the inner walls of the TyrPC conduit may adsorb proteins at greater amounts than the inner walls of the PE conduit. This is important because within the first hours following nerve transection and conduit implantation, fluid, known as exudate, rich in nerve-promoting factors fills from both nerve stumps into the conduit inner lumen (55, 63, 65-68). The proteins and molecules present in this exudate, particularly fibrinogen and fibronectin, are essential as they play a significant role in fibrin matrix formation, cell migration, and axonal regrowth (63). From the longitudinal sections shown in Figure 3.11 (and additional samples not shown), it becomes apparent that the fibrin matrix precursors are present within the TyrPC conduits earlier than they appear within the PE conduits. This effect might be due to material interactions between the cells necessary to form the matrix, the extracellular molecules present within the lumen, and/or adhesion of these molecules to the inner walls. The presence of these precursors is necessary in order for the final fibrin matrix to form (69, 70). The ability for TyrPC to enhance protein adsorption may also help to keep the nerve exudate stable and effective upon entering the inner lumen leading to a faster more complete formation of a fibrin matrix resulting in an increase in functional recovery and health of the regenerated nerve (71). In the PE conduit, however, this exudate may potentially leak out of the ends of the conduit (as there is less adhesive force keeping the protein within the inner lumen) resulting in a thinner, less stable fibrin matrix (71). This matrix may ultimately collapse or degrade leading to poor cell migration and regeneration. The fibrin matrix structure is needed in order to achieve successful nerve regeneration because it acts as a physical
scaffold for non-neuronal cells, including fibroblasts, Schwann cells, and endothelial cells to migrate across preceding axonal elongation (22, 72, 73). Axons depend upon these non-neuronal cells for the bioactive factors they secrete (58) and the physical support they provide for regeneration (22, 74). As the defect size increases, the chances of the matrix forming within a saline filled conduit decrease: either it does not form or it completely degrades before cells have a chance to migrate across it (75). This leads to poor regeneration as there is no substrate for cells and axons to migrate across. Many studies have evaluated the use of empty tubes to bridge critical size gaps in a rat model (72, 76-78). Of these studies, very few samples had successful regeneration across the length of the conduit and of these few the fibrin matrices produced were very weak (79).

In addition to nerve exudate filling the conduit inner lumen upon implantation non-neuronal cells, such as Schwann cells, migrate into the conduit and begin to prepare the defect site for regeneration. Schwann cells play a critical role in regeneration as they release neurite promoting factors, such as laminin, which provide many biological signals necessary for axonal outgrowth (72, 80-83). Through biochemical analysis (Figure 3.10) we investigated the presence of proteins specific to Schwann cells within the nerve conduits at 1 week post-implantation. Results showed that these protein levels were higher in TyrPC conduits as compared to conduits fabricated from PE. For this assay GAPDH and β-actin were used as housekeeping proteins within the cells. Although equal total amounts of protein were loaded into each lane, the western blot results show that GAPDH and β-actin were lower in the PE lane as compared to the TyrPC lane. These proteins are intracellular and therefore do not take into account any extracellular matrix proteins that may have adhered to the conduit inner walls. To explain these findings, we hypothesize that extracellular matrix proteins may have adhered in greater amounts to the TyrPC
as compared to the PE and thus the total amount of protein (intra- and extracellular) present within the TyrPC conduits was greater than that present in the PE conduits.

In order to enhance the regenerative potential of nerve guidance conduits a combination type therapy is often used. This includes the addition of a biological agent, such as neurotrophic factors (84), an additional cell source, typically Schwann cells (85) or stem cells (86), an internal matrix, such as a hydrogel of extracellular matrix proteins, collagen (87), laminin, or fibronectin (88), or a combination of all of the above (89-92). In this study conduits fabricated from TyrPC filled with phosphate buffered saline only were able to repair a critical size defect in the mouse femoral nerve model. The results imply that TyrPC was able to control several nerve regeneration processes through its polymer composition and surface characteristics. These conclusions were based on results of a 15 week study. Further investigation at early stages of nerve regeneration in this model will need to be performed in order to explain the precise mechanisms involved more in depth.
References

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CHAPTER 4

Preface

This chapter is in preparation for submission as:

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The Effects of a Hydrogel Filler with and without a Biological Agent

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Porous and Non-porous Tyrosine-Derived Polycarbonate Nerve Conduits: The Effects of a Hydrogel Filler with and without a Biological Agent

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Abstract

Nerve conduits have been used clinically and experimentally for over 20 years as an alternative for nerve autografts to treat peripheral nerve injuries. The filling of the conduit inner lumen with a bioactive material has been a more recent practice used to enhance the nerve regeneration potential of the conduits. In this study, degradable nerve guidance conduits are evaluated for peripheral nerve regeneration in a critical size nerve defect in the mouse femoral nerve model. Tyrosine-derived polycarbonate terpolymers (TyrPCs) are used to fabricate conduits with either porous (permeable) or non-porous (impermeable) outer walls. Prior to implantation, the conduits are filled with a collagen type I hydrogel or a collagen type I hydrogel grafted with a biological enhancer, a peptide mimic of HNK-1. The collagen filler is used as a three-dimensional internal scaffold, providing physical support to the regenerating axons to help migrate across the nerve defect. The HNK-1 molecule is added in an effort to enhance the rate of regeneration and the survival of neurons, to increase the probability of the nerve reaching its distal target and thereby improve functional recovery. Our results show that the influence of the
hydrogel filler depends upon the structure of the nerve conduit; porous conduits filled with a hydrogel allow for all tissue types to enter into the inner lumen, occupying the physical space of the hydrogel while non-porous conduits filled with a hydrogel have a greater chance of restricting certain tissue types from entering into the inner lumen. In addition, the benefits of using a hydrogel grafted with biological agent such as HNK-1 may be dependent upon the conduit structure as well; HNK-1 enhanced regeneration in the non-porous conduits only.

**Introduction**

Once severed, peripheral nerves have the ability to regenerate on their own, however, functional recovery is often poor due to the slow rate of axonal extension, leading to atrophy of the end target organ, and the limited ability of neurons to navigate long gaps and reconnect with their proper distal targets (28, 39, 214). Over the past 20 years, several implants have been approved by the U.S. Food and Drug Administration (FDA) to address these problems and repair peripheral nerve injuries (48). However, current treatment options often result in nerve regeneration with limited functional recovery and poor quality when used to repair a critical size defect (a length that will not heal on its own during the lifetime of the animal) (39). This inadequate functional recovery following peripheral nerve injury still remains to be a serious clinical challenge (48).

The conduit composition has been found to affect the outcome of nerve regeneration. Specifically, porous conduits have shown enhanced nerve regeneration as compared to non-porous conduits, producing nerve cables containing more nerve fibers and better myelination, and causing less atrophy of the target muscle (61-63, 156). These
favorable results may be due to a better exchange of nutrients and waste across the conduit outer wall, diffusion of external growth-promoting and/or neurotrophic factors into the inner lumen, retention of growth-promoting and/or neurotrophic factors secreted by the nerve stumps, or a combination of all of the above (215).

In an attempt to mimic the natural fibrin matrix scaffold that forms following nerve injury, many have tried to use various materials to act as a structural support for the infiltration of non-neuronal cells, such as Schwann cells and fibroblasts, and the regeneration of axons. Three-dimensional gel matrices comprised of various macromolecules (including fibrin, laminin, alginate, heparin, heparin sulfate, and collagen) known to promote peripheral nerve regeneration have been pre-filled in the chamber prior to implantation (95, 106, 199, 202, 216-218). The introduction of the luminal filler is meant to enhance the efficiency of the conduit at short nerve defects but also to allow for the ability to bridge greater nerve defects. The physical attachment of a growth factor to the filler matrix can enhance regeneration further by allowing for the localized delivery of the factor over a period of days to weeks. This method of attaching a growth-promoting factor to an internal filler has been experimented with many factors including but not limited to BDNF (219), platelet-derived growth factor (PDGF) (107), and glial growth factor (GGF) (106, 216). Nerve conduits filled with a growth factor containing matrix generally resulted in better nerve regeneration than nerve conduits filled with a growth-factor-free version of the matrix (95).

HNK-1, a carbohydrate epitope first discovered on the human natural killer cell (hence its name), appears to play a significant role in development of the nervous system specifically in cell-cell interactions, cell-substrate interactions, neuron migration, and
neurite outgrowth (120). The HNK-1 also plays a major role in the development of motoneurons, guiding them to reach their proper end targets, ultimately leading to increased functional recovery of the nerve (129). A peptide mimic (amino acid sequence FLHTRLFV) that is easier to obtain and synthesize than natural HNK-1 and as effective as the natural HNK-1 carbohydrate was shown in vitro to increase neurite outgrowth from motoneurons only as compared to sensory neurons (133). When applied in soluble form to the reconstruction of the severed mouse (134) and monkey (135) femoral nerves in vivo, the HNK-1 peptide mimic increased functional recovery, helping axons reach their targets more appropriately. In addition, the mimic reduced motoneuron death, enhanced expression of endogenous HNK-1 carbohydrate in the motor nerve branch during regeneration, and reduced abnormalities in axonal myelination in the newly regenerated nerve (134).

In this study, porous (P) and non-porous (NP) tyrosine-derived polycarbonate (TyrPC) conduits were evaluated in a critical size nerve gap in the mouse femoral nerve model for their ability to promote peripheral nerve regeneration. A collagen hydrogel filler was investigated for use as a 3D matrix encouraging nerve regeneration. The peptide mimic of HNK-1 was also evaluated as a therapeutic agent for the target-directed guidance of motor axons. The conduits in this study were pre-filled with a collagen hydrogel or a collagen hydrogel grafted with a peptide mimic of HNK-1. Functional recovery and histomorphometric analyses were performed in order to evaluate the conduits’ performance. Four animal groups (8 animals each) were compared: Porous tyrosine-derived polycarbonate conduits filled with collagen (P-TyrPC Collagen), porous tyrosine-derived polycarbonate conduits filled with collagen grafted with HNK-1 (P-
TyrPC HNK), non-porous tyrosine-derived polycarbonate conduits filled with collagen (NP-TyrPC Collagen), and non-porous tyrosine-derived polycarbonate conduits filled with collagen grafted with HNK-1 (NP-TyrPC HNK).

**Materials and Methods**

**Conduit Fabrication**

TyrPCs, synthesized utilizing previously published procedures (160) were used in this study with the following chemical structure: poly(DTE-co-DT-co-PEG\textsubscript{1000} carbonate). A dip-coater (KSV dip-coater, KSV Instruments Inc., Helsinki, Finland) was used to fabricate conduits with an inner diameter of 580 μm (Figure 4.1). A polymer solution (30 % w/v) was made in methylene chloride and a Teflon-coated mandrel was dipped at constant rate into this solution. The non-porous conduits were fabricated from a solution with polymer only. The porous conduits were made in a polymer solution containing a 1:1 ratio of polymer to sucrose particles sieved to 25-45μm in methylene chloride. After dip-coating, the mandrels were dried in vacuum overnight to remove remaining solvent and the conduits were removed and cut to a length of 5mm length for in vivo evaluation. The porous conduits were put into water in order to remove the sucrose particles and to generate the porous microstructure.

**Grafting HNK-1 to Collagen**

The following work is performed and provided by Dr. David Shreiber’s laboratory (Biomedical Engineering, Rutgers University): The HNK-1 peptide mimic (sequence FLHTRLFV) is chemically synthesized and purchased from Genscript (Piscataway, NJ) and is grafted to collagen (Collagen Type I, Calf Skin Lyophilized, Elastin Products Company Inc., Owensville, Missouri, USA) using previously published
procedures (220). Briefly, the carboxy terminal of the peptide is activated with EDC (1-ethyl-3-(3 dimethyl aminopropyl) carbodiimide). This peptide-EDC mixture is then added to the collagen solution in which the activated peptide can bind to the collagen via free amine groups. Caution is taken to prevent the self-assembly of collagen fibers by using a low pH buffer. The peptide-EDC-collagen mixture is purified to remove any free peptides and remaining EDC, lyophilized, and then reconstituted in acetic acid at the appropriate concentration (3 mg/mL). The concentration of HNK-1 in the hydrogel is approximately 120 µg/mL. (220)

**Surgical Methods and Animal Groups**

All experiments were conducted in accordance with the Rutgers Animal Care and Facilities Committee and the Institutional Animal Care and Use Committee (IACUC). TyrPC conduits were prefilled with collagen or collagen grafted with HNK-1 and incubated at 37 °C for 1 hour to allow for gelation. Three month old female C57BL/6J were anaesthetized by intraperitoneal injections combination of xylazine/ketamine 12 mg/kg/80 mg/kg with a dosage of 0.1ml/10g body weight. A transection is performed approximately 3mm prior to the bifurcation of the left femoral nerve into the motor and sensory branch. The proximal and distal stumps are placed inside the nerve conduit ~5mm apart from one another and fixed with a 10-0 nylon suture (Fine Science Tools, Foster City, California, USA). 7mm wound clips (Fine Science Tools, Foster City, California, USA) were used to close the skin and were removed 2 weeks post-surgery.

Four animal groups (8 animals each) were compared over a 15 week time period including: Porous tyrosine-derived polycarbonate conduits (P-TyrPC) filled with collagen, porous tyrosine-derived polycarbonate conduits (P-TyrPC) filled with collagen
with HNK-1, non-porous tyrosine-derived polycarbonate conduits (NP-TyrPC) filled with collagen, and non-porous tyrosine-derived polycarbonate conduits (NP-TyrPC) filled with collagen with HNK-1.

**Motor Function Recovery**

Functional recovery was assessed using a single-frame motion analysis approach on videos collected of animals performing two different functional tests post-surgery (184). The first test was a classical beam walking in which differences (due to conduit and filler treatment) of the gait cycle of the mouse was observed. This test was performed prior to surgery and weekly post-surgery until the endpoint of the experiment (Week 15). Rear view videos of the mice walking along the length of the beam were collected and stored in Audio Video Interleaved (AVI) format using a high-speed camera (A602fc Basler, Ahrensburg, Germany) at 100 frames per second. Single video frames were observed for the movements of the hind legs during the normal gait cycle. Measurements were taken at specific points of the walking cycle using Simi Sports Player software (SIMI Reality Motion Systems, Unterschleissheim, Germany).

At the moment the left toes were fully extended on the beam, the foot-base angle (FBA) (Figures 4.4 A and B) was measured. This angle evaluates the quadriceps muscle’s ability to keep the knee fully extended during single-support phases necessary for the swing of the opposite leg during the walking cycle and is defined by a line that divides the sole of the foot in half and a horizontal line across the beam (184). Following conduit implantation, the FBA changes significantly (Figure 4.4 B) as compared to the intact FBA, measured prior to transection (Figure 4.4 A).
In order to observe changes in supraspinal control and evaluate movements needing precision, a test is performed to assess the ability of the mice to perform voluntary movements following treatment. A pencil grip test is performed in which the mouse is held upside down by its tail in front of a pencil and is able to grasp the pencil with its fore paws. At the same time, the mouse attempts to catch the pencil with its hind paws, which alternate from flexing to extending (Figure 4.4C and D). The protraction limb ratio (PLR) is measured while the mouse performs this test as the ratio of the relative length of the intact to the lesioned limb. In intact animal both hind legs reach equal distances towards the pencil and therefore the lines connecting the most distal midpoint of each foot to the center of the base of the tail are equal. In this case, the PLR value of intact mice is approximately 1 (Figure 4.4C). Following injury and conduit implantation the left limb cannot extend maximally (Figure 4.4D) and the PLR rises significantly above 1. A PLR value of 1 indicates full functional recovery while a PLR value greater than 1 indicates poor functional recovery.

The values of the two parameters, FBA and PLR, may vary amongst animals (due to natural causes such as differences in body weight, gender, age, etc.) and therefore, they would not be comparable to values reported from other studies. For this reason a recovery index (RI) is calculated for both parameters as a relative indication of how individual animals recovered. The RI is calculated as a percentage using the following formula:

\[ RI = \left( \frac{X_{day_y} - X_{day_7}}{X_{day_0} - X_{day_7}} \right) \times 100, \]
where $X_{day 0}$, $X_{day 7}$, and $X_{day y}$ are intact values on day 0 (either FBA or PLR), values measured on day 7 after injury, and at day y (where y is the final end time-point - 105 days), respectively.

**Histomorphometric Analysis of Explanted Nerve**

Femoral nerves are dissected from animals fixed by perfusion with 4% paraformaldehyde, post-fixed in osmium tetroxides, dehydrated, and embedded in resin according to standard protocols.

The mid-section of the conduit is transversely sectioned into 1 μm-thick sections and stained with 1% toluidine blue/ 1% borax in distilled water. From these sections the total numbers of myelinated axons per nerve cross-section are estimated under 40x observation using a Zeiss Axiocam and Axiovision imaging software. The raw tissue area, cross-sectional area of the regenerating cable, and the % nerve regeneration are measured with the same camera using a 10 X or 20 X objective and ImageJ 1.43u software. Using a 100 X oil objective and an Olympus IX80 microscope, random samples are measured in ImageJ 1.43u for axonal (inside the myelin sheath) and nerve fiber (including the myelin sheath) mean orthogonal diameters. The mean orthogonal diameter is calculated as the mean of the line connecting the two most distal points of the axon profile (longest axis) and the line perpendicular to this line passing through its middle (185). The degree of myelination is estimated by the ratio of the axon to fiber diameter (g-ratio).

**Results**

TyrPC conduits filled with a collagen hydrogel with and without HNK-1 peptide mimic were investigated for their potential as nerve guidance conduits. Conduits were
fabricated via dip-coating to produce conduits with an inner lumen of 580 µm, an outer wall thickness of approximately 100 µm, and either a porous or non-porous outer wall. Figure 4.1 shows an example of the outside surface morphology of porous (Figure 1A) and non-porous (Figure 1B) TyrPC conduits.

Figure 4.1: Scanning electron microscope (SEM) images of conduits evaluated *in vivo* (A) Outer wall of porous tyrosine-derived polycarbonate conduit and (B) non-porous tyrosine-derived polycarbonate conduit. Scale bar: 100 µm.

**Functional Recovery**

In this study, we evaluated the function of the quadriceps muscle as a method to compare treatment options. A decrease in the FBA towards its original starting value and the PLR to a value of 1 is indicative of healing and functional recovery. Results indicate that animals treated with NP-TyrPC conduits filled with HNK-1 grafted to collagen regained functional recovery more than animals in any other treatment group (Figure 4.3). Animals treated with NP-TyrPC HNK significantly improved the RI for FBA (Figure 4.3B) as compared to animals receiving P-TyrPC HNK or NP-TyrPC collagen conduits. In addition, the RI values for the PLR of animals treated with NP-TyrPC HNK are higher than the values for all other conditions. PLR values for animals treated with NP-TyrPC HNK were significantly different from all other groups by as early as 6 weeks post-implantation. It is important to note that the RI values for both the FBA and the PLR are more consistent among animals in the NP-TyrPC HNK1 group than any other
group with the RI for FBA reaching nearly 40% and the PLR reaching almost 100% (full recovery) at the final endpoint of 15 weeks.

Figure 4.2: Video frames showing the functional measurements performed on mice following implantation of the conduit. The white lines drawn in the video frames show the foot-base angle (top frames), and the limb lengths used for calculation of the protraction limb ratio (bottom frames). (A top) FBA of mice pre-injury averages 50-70°. (A bottom) The pencil grip test measures the PLR on a mouse pre-injury, where both limbs are similarly extended, giving a ratio of 1. (B top) FBA of mice one week post-injury averages 90-110°. (B bottom) PLR on a mouse one week post-injury shows the disparity in limb protraction due to injury, resulting in a PLR > 1. (C top) A FBA of mice at 15 weeks approaches an intact angle of 50-70° indicates healing and good functional recovery. (C bottom) A PLR value of mice at 15 weeks approaches a ratio of 1, indicating healing and good functional recovery.
Figure 4.3: Time course of motor function recovery following conduit implantation. Conditions listed as conduit porosity (porous (P) or non-porous (NP)), material (TyrPC: tyrosine-derived polycarbonate), and filler material – native collagen (Collagen) or HNK-1 grafted to collagen (HNK). (A) Foot Base Angle (FBA). Values represent averages ± standard error. There were no statistically significant differences between group means as determined by one-way ANOVA. (B) Recovery Index (RI) for FBA at week 15. Each circle represents one animal in the group. The line indicates the average RI for the group. Asterisks indicate significant differences between average RI value for NP-TyrPC HNK and NP-TyrPC Collagen, and NP-TyrPC HNK and P-TyrPC HNK. \( p<0.05 \), one-way ANOVA with Fisher’s LSD post hoc test) (C) Protraction Limb Ratio (PLR). Values represent averages ± standard error. Asterisks indicate significant differences between average RI values \( p<0.0001 \), one-way ANOVA with Tukey post hoc test). (D) RI for PLR at week 15. Each circle represents one animal in the group. The line indicates the average RI for the group. There were no statistically significant differences between group means as determined by one-way ANOVA.
**Histomorphometric Analysis**

Histomorphometric analysis was performed on transverse 1 µm sections from the middle of the conduit showing the regenerative cable (Figure 4.4). A significantly greater number of axons (Figure 4.5A) and larger cross-sectional area (Figure 4.5D) were observed in regenerating nerve cables formed within a NP-TyrPC HNK filled conduit as compared to the cables formed in the P-TyrPC HNK and NP-TyrPC collagen conduits. The percentage of myelinated nerve fibers as measured out of the total raw tissue area was significantly greater in NP-TyrPC HNK conduits as compared to all other conditions.

![Figure 4.4](image_url)

*Figure 4.4: Representative cross sectional images (40X and 100X) of nerve sections stained with toluidine blue from the midpoint of regenerated femoral nerve after tubulization with either TyrPC conduit filled with collagen or HNK-1 grafted to collagen. (A) P-TyrPC Collagen (B) P-TyrPC HNK (C) NP-TyrPC Collagen (D) NP-TyrPC HNK. Scale bars: 100 µm and 20 µm respectively.*
Figure 4.5: Histomorphometric analysis of regenerating nerve cable following conduit implantation. Asterisks indicate significant differences. Values represented as averages ± standard error. (A) Axon count. Statistics: One-way analysis of variance (ANOVA) was performed with Fisher’s LSD post hoc test ($p<0.05$) NP-TyrPC HNK significantly different from P-TyrPC HNK and NP-TyrPC Collagen. (B) Raw Tissue Area. (C) % Myelinated nerve fibers in regenerating nerve cable. Statistics: One-way analysis of variance (ANOVA) was performed ($p<0.1$) with Fisher’s LSD post hoc test ($p<0.05$) NP-TyrPC HNK significantly different from all other conditions. (D) Cross sectional area of regenerated nerve fibers. Statistics: One-way analysis of variance (ANOVA) was performed with Fisher’s LSD post hoc test ($p<0.05$) NP-TyrPC HNK significantly different from P-TyrPC HNK and NP-TyrPC Collagen.
Figure 4.6: Representative cross sectional images of nerve sections stained with toluidine blue from the midpoint of regenerated femoral nerve after tubulization. Images show various histological appearances including examples of acellular regions, dense fibrotic tissue, and distinct regenerative cables. Animals were treated with the following types of conduits: (A) P-TyrPC Collagen (B) P-TyrPC HNK (C) NP-TyrPC Collagen or (D) NP-TyrPC HNK. Large black circles are adipocytes common to nerve repair, acting as a soft "cushioning" around the nerve to protect it from mechanical trauma. Images show examples of obstruction to axonal regeneration due to remnants of the collagen gel filler (producing acellular regions within the regenerating nerve cable indicated by white arrows in A and B) and over deposition of fibrous tissue (indicated by white arrows in C), leading to dense areas through which axons cannot navigate. Image D illustrates the distinct regenerative cable found in an animal treated with NP-TyrPC HNK. Little to no fibrous tissue deposition occurs in these conduits. Scale bar: 100 µm.

Histological differences between treatment options were evident from sections of the mid-conduit regenerative nerve cable (Figure 4.6). In the porous conduits, distinct regenerative cables did not always form. Rather, the inner lumen was often filled with dense fibrous tissue (images not shown) or large acellular regions where the collagen gel
had not been digested (Figures 4.6A and B). In the NP-TyrPC conduits with collagen alone dense fibrous tissue was present, but in a more organized manner. The tissue commonly displayed a circular encasement of the regenerative cable (Figure 4.6C). All animals treated with NP-TyrPC HNK conduits exhibited distinct regenerative cables with little to know fibrous tissue surrounding them (Figure 4.6D).

Figure 4.7: Analysis of axonal myelination in regenerated nerves. (A) Representative 100X oil objective microscopic image of mid-conduit nerve section stained with toluidine blue. (B) A magnified image of (A) shows measurements for the mean orthogonal diameters of the axon (black arrows) and of the nerve fiber (white arrows). The degree of myelination was calculated as the ratio of the axon to fiber diameter. (C) Shown are normalized frequency distributions of g-ratios from nerve samples treated with each type of conduit. Each of the distributions shown is significantly different from all other distributions. Statistics: One-way analysis of variance (ANOVA) was performed with Tukey post hoc test ($p<0.05$).
Figure 4.8: Distribution of nerve fiber diameters. Results are acquired from 3 random 100X images of each nerve at the mid-conduit level. NP-TyrPC Collagen and NP-TyrPC HNK are significantly different from P-TyrPC HNK. Statistics: One-way analysis of variance (ANOVA) was performed with Tukey post hoc test ($p<0.05$).

The type of conduit structure appears to play a role in the degree of myelination and the fiber diameter. The overall degree of myelination, estimated by the axon-to-fiber diameter ratio (g-ratio) (Figure 4.7), was significantly reduced in animals treated with NP-TyrPC conduits, as indicated by the shift in the frequency distribution histogram toward larger values compared with nerves present in P-TyrPC conduits. In addition, animals treated with NP-TyrPC conduits with either a collagen or a collagen grafted with HNK-1 filler produced regenerative cables with larger nerve fibers as indicated by the curve shifts to the right as compared to the P-TyrPC conduits (Figure 4.8).
Discussion

The addition of biological factors to the inner lumen of a nerve conduit has proven to be effective at enhancing nerve regeneration by promoting neuronal survival and axonal outgrowth (80). In particular, an inner lumen gel matrix can be used to overcome conduit gap length limitations by providing a 3D environment favorable to axon regeneration, enticing non-neuronal cellular ingrowth, and increasing the surface area available for regeneration (174, 218, 221, 222). In spite of these positive results, a gel matrix may also impede axonal outgrowth, slowing down the progress of regeneration and reducing the size of the regenerating cable, resulting in regeneration comparable to that of an autograft or to a saline-filled conduit (197, 223-226). The functional and morphological results of this study show that the success of nerve regeneration after tubulization with a neurite-promoting gel matrix is dependent upon the conduit structure. More specifically, the presence or lack thereof of pores in the outer conduit walls will affect the gel’s effect on nerve regeneration.

Achieving functional recovery following nerve repair is still a critical challenge; only 25% of patients recover full motor function following median nerve repair at the wrist (23). Recovery of the motor function depends upon enough motor axons reaching the target muscle before it atrophies and becomes resistant to any form of reinnervation (227). Our findings show that functional recovery is improved in animals treated with a NP-TyrPC conduit filled with collagen gel grafted with HNK-1 as compared to all other test conditions. Through functional analysis, we found a decrease in the FBA parameter over time, indicative of good healing and proper reinnervation of the target quadriceps muscle. Based on the RI value for the FBA at week 15, the animals treated with NP-
TyrPC HNK conduit showed significant recovery as compared to the animals treated with P-TyrPC HNK and NP-TyrPC Collagen. In addition, PLR values measured from animals performing the pencil grip test were significantly better in animals treated with NP-TyrPC HNK conduits as compared to all other test conditions by as early as 6 weeks post-implantation. These values continued to remain significantly different until the final endpoint of the study at where they almost reached complete recovery of the initial PLR value of 1. It is important to note that Figures 4.3B and D demonstrate an overall consistency found amongst the RI values measured for animals treated with NP-TyrPC HNK conduits – reconfirming the positive results observed with this treatment method. Animals in all other test conditions displayed a wide range of RI values indicating an inconsistent response to the other treatment methods.

The overall degree of myelination, estimated by the axon-to-fiber diameter ratio (g-ratio), was reduced in regenerated nerves in the non-porous conduits, as indicated by the shift in the frequency distribution toward larger g-ratio values compared with nerves treated with porous conduits (134). From these results, we gather that porosity does indeed affect the g-ratio. The thicker myelin sheaths present on axons generated in mice treated with porous conduits may be due to a greater population of Schwann cells infiltrating the porous conduits, as Schwann cells tend to invade collagen type I gels quite vigorously (228), therefore accumulating greater amounts of myelin around the regenerating axons.

The concentration of the hydrogel filler within the conduit plays a critical role in the success of nerve regeneration (40, 229, 230). Even if the inner lumen gel contains neurite-promoting agents it may impair regeneration within permeable conduits if it is too
concentrated, physically impeding the diffusion of neurotrophic factors from the nerve stumps or external to the conduit, the migration of non-neuronal cells, or the elongation of axons (226, 230). In addition, a higher concentration gel will occupy space needed for the generation of the initial fibrin matrix bridging the nerve stumps (98). It has been found that reinnervation begins earlier and reaches slightly higher levels with collagen gels of lower concentrations (1.28 mg/mL) than with higher concentrated gels (1.92 and 2.56 mg/mL) (230). In this study, we used a collagen gel with a concentration of 3 mg/mL which may have been too high to be beneficial to regeneration and instead it became obstructive to repair. It could be that as the concentration of the gel increases, the average pore size of the filler matrix decreases becoming even more obstructive to regenerating axons and therefore reducing the size of the regenerating cable (224, 229).

Degradation of the filler gel by proteolysis is necessary for neurite extension as it allows for pathways to be formed within the 3D gel for neurites to extend through (231). If proteolysis does not occur, the strands of the collagen filler will remain tightly packed within the inner lumen and hinder regeneration, preventing axonal elongation, even if the filler gel includes growth-promoting substances (226, 230, 232). Acellular areas within the conduit lumen hypothesized to be remnants of the original filler gel have been reported in the central core of regenerated nerves even after 12 weeks following implantation (202, 226). As the concentration of the gel increases, so does the amount of residual gel (226). Such acellular regions were found in the regenerating nerves of animals treated with porous conduits in this study (Figures 4.6A and B) and potentially contributed to the end result of poor regeneration. Furthermore, as the concentration of
the gel used in this study was relatively high, the amount of gel remnants at 15 weeks may have been relatively high as well.

Pores within the conduit walls allow for the influx of nutrients and growth factors from outside the conduit across the outer walls into the inner lumen (215). The negative effects observed when the collagen gel filler was used within porous conduits could be due to the gel adhering to the inner lumen walls and physically blocking the pores, negating their original intent (226). In addition, the large pores present in the P-TyrPC conduits allow for connective tissue from outside the conduit to enter into the lumen (61-63). The addition of a 3D collagen gel filler increases this migration of any non-neuronal cells from outside the conduit into the matrix, as the collagen matrix provides a favorable substrate for cell adhesion and growth (233). This ingrowth can be extremely dense and compact, however, the depth of cellular infiltration into the collagen gel may be relatively short (233). Axonal ingrowth is dependent upon the distance reached by the non-neuronal cells as they secrete trophic factors and provide physical scaffolds necessary for axons to attach and grow along (194). As a consequence, if the non-neuronal cells do not migrate far enough along the fibrin matrix, axons will not be able to extend further either.

Fibroblasts, essential to regeneration, comprise a large percentage of the non-neuronal cells entering the collagen matrix (233) and are able to deposit a dense fibrous tissue that may physically occupy the space within the inner lumen, inhibiting initial fluid accumulation, fibrin matrix formation, cell-matrix interactions, and ultimately neurite extension (22, 40, 61, 177, 191, 221, 234-237). It is hypothesized that competitive interactions between myofibroblasts and axon growth at the nerve stump influence nerve repair. If myofibroblasts encapsulate the nerve stumps and deposit too much fibrous
tissue, a neuroma may form prior to axonal regrowth (beginning at approximately day 7 after injury). In accordance with the “pressure cuff theory” (238), these contractile cells may exert circumferential forces on the regenerating nerve cable, constricting any already regenerating axons by pressing against them during their enlargement and may completely prevent axons from extending across the injury gap and reconnecting with their distal targets (32, 239). An example of where this may have occurred in the present study is shown in Figure 4.6C. Dense fibrous tissue is present in the regenerating cable of animals treated with TyrPC conduits filled with collagen.

With the addition of a neurite-promoting factor such as HNK-1 to the collagen hydrogel, the inner lumen becomes more attractive to neurites, perhaps enticing their outgrowth faster and with greater accuracy than the ingrowth of non-neuronal cells. Figure 4.6D shows the regenerating cable of an animal treated with a NP-TyrPC conduit filled with collagen gel grafted with HNK-1. The image clearly demonstrates the ability of the HNK-1 to enhance neurite ingrowth and extension, prior to allowing non-neuronal cells to enter into the matrix and deposit fibrous tissue. If neurites are able to extend from nerve stumps faster than fibrous tissue can be deposited, they have a greater chance of reaching the distal end of the injury gap, making reconnections, and promoting functional recovery.

Our results are supported by a study by Mohanna et al. who found the presence of an alginate gel within a polymer conduit inner lumen that inhibits nerve regeneration when compared to an empty conduit. Further investigation showed that regenerating Schwann cells and axons were growing along the outer surface of the alginate hydrogel and did not enter directly into it, which clearly indicates that the alginate filler physically
obstructs axonal regeneration. In the same study, *Mohanna et al.* found that the addition of a biological substance, glial growth factor (GGF), to the alginate gel matrix enhanced regeneration within the conduits, encouraging the growth of both nerve fibres and Schwann cells. (106, 216)

The disparate results found in this study between the porous and non-porous conduits filled with a 3D gel matrix are accounted for by the following hypothesis: In the case of porous conduits, pores are large enough to allow excessive infiltration of tissues along the entire length of the construct, giving them a greater chance to enter into the lumen, lay down tissue, and obstruct neurite outgrowth (47). Whether or not HNK-1 is present on the collagen gel, tissues are able and will quickly enter all along the length of the conduit and inhibit axonal regeneration. However, in the case of non-porous conduits, the inner lumen of the conduit is only exposed to the end nerve stumps. When collagen gel is present within the inner lumen, non-neuronal cells still enter from both ends and lay down fibrous tissue, reducing the space and ability for axons to regrow. However, when collagen with HNK-1 is added to the inner lumen, this gel (only exposed at the end of the conduits, in greatest contact with regenerating nerve endings) gives neuronal cells preference (over non-neuronal cells) to enter into the lumen. HNK-1 entices axons to extend out into the inner lumen of the conduit, possibly competing with the myofibroblast capsule formation and any dense tissue formation down the length of the defect and prevents sealing of the nerve stump. (32, 218, 240, 241)

Our results show that a three-dimensional gel matrix added to the conduit inner lumen may have different effects depending upon the conduit structure. A permeable, porous conduit filled with a hydrogel can retard regeneration as opposed to an
impermeable, non-porous conduit filled with the same hydrogel (226). The matrix may enhance nerve regeneration provided that its density is not obstructive and more importantly, it contains neurite-promoting factors that increase the rate at which axonal regeneration occurs. The drawbacks of using a collagen hydrogel within TyrPC conduits may be compensated for by the action of HNK-1; when collagen grafted with HNK-1 is utilized, we observe a significant enhancement in the degree of functional recovery and the quality of axonal regeneration.
References

CHAPTER 5

SUSTAINED DELIVERY OF HNK-1 PEPTIDE MIMIC WITHIN A NERVE GUIDANCE CONDUIT

Abstract

In critical size gaps specific reinnervation is rarely perfect and functional recovery is often poor due to the limited ability of neurons to navigate long gaps and reconnect with their proper distal targets. Regenerating motor axons are often misguided to skin, and sensory axons are often misguided to muscle (1). Brushart et al. showed that when given equal opportunity to regenerate towards a motor or sensory branch, significantly more motoneurons prefer to enter the motor branch as opposed to the sensory branch, leading to the phenomenon referred to as preferential motor reinnervation (PMR) (2-5). In this study, HNK-1, a carbohydrate epitope first discovered on the human natural killer cell, is explored for its use to enhance functional recovery following nerve repair. HNK-1’s functional relevance to PMR is based on the fact that even though there are neural cell proteins present in both the sensory and motor branches of the mouse femoral nerve, HNK-1 is only associated with these proteins in the motor branch and, therefore, may be necessary to help guide motor axons to their proper motor branch (1-3, 6-8). If it is possible to mimic and enhance the effects of PMR during regeneration in a nerve conduit, the number of individual axons reaching their proper targets may increase, leading to increased functional recovery of the regenerated nerve (5). A peptide mimic (amino acid sequence FLHTRLFV) that is easier to obtain and synthesize than natural
HNK-1 has been identified by phage display techniques using an L2-412 antibody for selection (9). This peptide mimic effectively functions like the natural HNK-1 carbohydrate and was found to promote neurite outgrowth from motor neurons only in vitro (10). When applied in soluble form to the reconstruction of severed mouse and monkey femoral nerves in vivo, the HNK-1 peptide mimic increased functional recovery and PMR, helping axons reach their targets more appropriately (11, 12). In addition, the mimic reduced motoneuron death, enhanced expression of endogenous HNK-1 carbohydrate in the motor nerve branch during regeneration, and reduced abnormalities in axonal myelination in the newly regenerated nerve (11).

It is hypothesized that the presence of the HNK-1 in addition to the biodegradable NGC will accelerate and stabilize preferential motor reinnervation leading to enhanced functional recovery as well as the overall health of the nerve. However, when presented in soluble form, the HNK-1 is free to diffuse away from the injury site, out of the NGC, and therefore its effectiveness can be greatly reduced, especially if used within a conduit used to treat a critical size gap. In this chapter, we describe two different methods of delivery to increase the duration during which HNK-1 peptide mimic is available to present itself to the regenerating nerve (Figure 5.1): First, cellular delivery of HNK-1 by genetically engineered mesenchymal stem cells (MSCs) (Figure 5.1A) and second, release of HNK-1 from the polymeric conduit outer walls as the polymer degrades (Figure 5.1B).
Figure 5.1: Schematic representation of different ways to deliver the HNK-1 peptide mimic from the nerve conduit. (A) Transplantation of MSCs engineered to synthesize and secrete HNK-1 within the inner lumen of the conduit. (B) Conduit is fabricated with HNK-1 in its outer walls. HNK-1 is slowly released into the lumen as the polymer degrades over time.
**Method 1: Cellular Delivery of HNK-1 by Genetically Engineered Cells**

**Introduction**

Soluble HNK-1 delivery has proven to be effective at enhancing nerve regeneration when filled within a nerve conduit and used to bridge a nerve transection in vivo (11, 12). However, when applied in soluble fashion, the life time of the mimic in vivo is relatively short. For long nerve defects, the actions of the HNK-1 mimic may be needed for a longer period of time. Unfortunately, continuous injection of HNK-1 is not clinically feasible. Therefore, in order to enhance the lifetime of the mimic and maintain its bioactivity for a longer amount of time, an alternative approach to deliver HNK-1 is necessary. To this effort, we investigate the use of genetically engineered cells to continually secrete HNK-1 peptide mimics.

Recent advances in molecular biology and transfection have allowed for the genetic manipulation of cells to express and/or secrete neurotrophic factors such as NGF, BDNF, NT-3, CNTF, GDNF, and bFGF, cell adhesion molecules, or extracellular matrix proteins (13). This can be done ex-vivo or in vivo by either viral or nonviral methods and allows for continual expression and/or secretion of the factors as the cell divides (14-16). A common way of introducing cells to the conduit is to isolate primary cells from the patient, expand them ex vivo, genetically engineer them, and then transplant them back into the patient within the context of a nerve conduit (17). For example, Weiner et al. used a retroviral vector to infect fibroblasts with a vector that caused the secretion a proteolipid protein for treatment of multiple sclerosis (18). This study proved that the constitutive secretion of the peptide by the cells reduced (and in some cases eliminated) inflammatory cell infiltration into the central nervous system and demyelination (18).
Although many cell types can be genetically altered, Schwann cells and stem cells are most commonly selected for use in peripheral nerve regeneration. For example, Gravvanis et al. utilized a retrovirus to genetically alter the adhesive properties of Schwann cells. This modification increased Schwann cell’s motility, which resulted in enhanced myelination of the regenerating nerves in vivo, and provided a more favorable environment for axons to regrow (19). In addition, the altered Schwann cells enhanced recruitment of endogenous Schwann cells to the injury site. When evaluated in vivo within a collagen conduit used to bridge a gap in the rat sciatic nerve, the cells caused regeneration comparable to that of an autologous graft, enhancing the axon fiber morphology and functional recovery of the rats (19). In another study Li et al. used a retroviral vector containing the glial cell line-derived neurotrophic factor (GDNF) gene to transfect Schwann cells (20). This work showed that in vitro more motoneurons survived when they were co-cultured in GDNF-Schwann cell-conditioned medium than when they were cultured in normal Schwann cell-conditioned medium (20). These cells were then implanted into a conduit to bridge a rat sciatic nerve defect. Animals treated with conduits filled with GDNF-transfected Schwann cells showed enhanced nerve regeneration as compared to a control conduit without the transfected cells (20).

MSCs or bone marrow stromal cells (BMSCs), are commonly used for numerous peripheral nerve regeneration applications because of their inherent ability to promote regeneration. MSCs are known to secrete cytokines that control the inflammatory and immune response, neurotrophic factors that improve survival and outgrowth of neurites, and substances that induce neoangeogenesis. In addition, it has been shown that MSCs can provide a “perineurium-like” structure that surrounds a regenerating nerve and
creates a sheet of contact between the regenerating nerve and the NGC when transplanted
in vivo (21-27). Additionally, MSCs improve functional recovery of regenerating
peripheral nerves when implanted within a NGC (26, 28, 29). MSCs can be used for
autologous transplantation, can be rapidly expanded in culture, are adult-like (and
therefore reduce the chance of tumor formation), survive in vivo, and integrate well with
the native nerve (24, 30, 31). In addition to all of their natural nerve promoting effects,
mesenchymal stem cells can be genetically engineered to express exogenous genes and
serve as a delivery vehicle. A study by Deng et al. demonstrated the ability to infect
MSCs with an adenoviral vector to secrete bioactive calcitonin gene–related peptide for
treatment of cardiovascular diseases (32). Supernatant collected from infected cells in
culture has proven to be effective at increasing intracellular cyclic AMP levels in
pulmonary artery smooth muscle cells in vitro (33). (21-24, 26, 27, 29, 30, 34, 35).

In the current work, we attempted to produce genetically engineered stem cells
(via lentiviral infection) that supply the HNK-1 peptide mimic to the injury site. This
would allow for a longer, more continuous delivery method rather than using a fixed
amount of mimic in soluble form. Lentiviral infection was chosen due to its efficient and
stable gene insertion ability. In addition, lentiviruses are easy to use for cloning, well-
characterized, and successful at infecting both dividing and non-dividing cells. More
importantly, the lentiviruses do not cause an inflammatory response when introduced in
vivo. (36-38)

MSCs were employed as vehicles to deliver three different peptide mimics of
HNK-1. MSCs were infected with one of three sequences coding for an HNK-1 peptide
mimic. The amino acid sequences for these three mimics are the following:
TFKLSITTLEYY referred to as “K” (the best for stimulation of mouse motoneurons), TFQLSTRTLPFS referred to as “Q” (the best for stimulation of human motoneurons) and FLHTRLFV referred to as “F” (used in vivo and was the 2nd best for stimulation of mouse and human motoneurons) (9, 11). All three sequences were investigated because of the high variability of peptide secretion from sequence to sequence.

**Materials and Methods**

**Virus Construction and Production**

The plasmid backbone pLKO.1 neo (Figure 5.2A) was obtained (a gift from Dr. Rick Cohen from the W.M. Keck Center for Collaborative NeuroScience, Rutgers University). Plasmids were cleaved using two restriction enzymes AsiSI and MluI and the size of the fragments were confirmed using gel electrophoresis. Single stranded oligonucleotides coding for each of the three HNK-1 mimics were generated through polymerase cycling assembly (PCA), and designed so that as they annealed, the overhangs were left which corresponded to the restriction sequence “sticky ends”. The primers used for the three separate HNK-1 sequences were synthesized by Integrated DNA Technologies, Coralville, Iowa. The sequences for these six primers are as follows: GCCACCATGTTCCTGCACACCCCGCTGTTCGTGTAA as the “F” forward primer and CGCGTTAGCAGCCGAGCTGACGAGGTGGTGTGATGGCAT as the “F” reverse primer. GCCACCATGCCTTCAAGCTGAGCGAGACCACCCTGGGTACG TACTAA as the “K” forward primer and CGCGTTAGTAGTACTCCA GGGTGGTCTCGTCAGCTTTGAACTGTATGGGTGCAT as the “K” reverse primer. GCCACCATGCCTTCAAGCTGAGCGAGACCACCCTGGGTACGTACTAA as the “Q” forward primer and CGCGTTAGTAGTACTCCA GGGTGGTCTCGTCAGCTTTGAACTGTATGGGTGCAT as the “Q” reverse primer.
TGGAAGGTCATGGTGCGCAT as the “Q” reverse primer. These PCR products were analysed by gel electrophoresis, extracted, and purified. Each sequence was then inserted between the AsiSI and MluI sites of the pLKO.1 neo vector in the presence of T4 DNA ligase (Fermentas, Glen Burnie, MD) at a molar ratio of 3:1 at 22 °C for 5 minutes. After ligation, the vectors containing the cloned peptide sequences were amplified, analysed, and sequenced (GENEWIZ, Inc., South Plainfield, NJ) to confirm their correct insertion.

To synthesize lentivirus, each construct was packaged with a transfection reagent TransIT®-LT1 Transfection Reagent (Mirus Bio, Madison, Wisconsin), an envelope plasmid (pMD2.G), and the psPAX2 packaging plasmid to be transfected into Human Embryonic Kidney (HEK) 293FT cells. The packaging procedure was performed in Dulbecco’s Modified Eagle Medium (DMEM) + 10 % fetal bovine serum (FBS) at 37 °C in an incubator with 5 % CO₂. Media (containing virus) was collected at 48 hours and 72 hours and subsequently filtered through a 0.45 µm low protein binding filter with a syringe. The lentivirus was diluted in MSCGM™ (Mesenchymal Stem Cell Growth Medium) (Lonza, Walkersville, MD) (1:3 ratio) and put on the cells at 37 °C in an incubator with 5 % CO₂. The cells remained in culture for 3 days in order to allow expression to reach a maximum before selection and then they were exposed to 300 μg/mL of G418 for 48 hours to remove any non-expressing MSCs.

**In Vitro Assessment of HNK-1 Secreted by MSCs**

The bioactivity of the HNK-1 being secreted by the MSCs was assessed by evaluating its effect on motoneuron survival and outgrowth *in vitro* as previously described in the Materials and Methods section of Chapter 3: *In vitro* Assessment of Conduit Material with Spinal Cord Neurons. Neurons were cultured on glass coverslips
in fresh control media, and in “conditioned” media, collected from a monolayer of stem cells (cultured for 7 days) secreting each of the HNK-1 peptide mimic, “F”, “K”, and “Q” hMSCs.

**Results and Discussion**

For treatment of peripheral nerve injuries, it is likely that an advanced clinical approach will be needed, incorporating a combination of therapies, such as biodegradable NGCs and bioactive molecules. HNK-1 peptide mimics hold much promise for enhancing the regenerative capacity of a NGC as they have shown beneficial effects both *in vitro* (39) and *in vivo* when applied in soluble form (11, 12). However, the short lifetime of soluble HNK-1 within a NGC does not allow for maximal enhancement of regeneration by this molecule. Supplying a more continuous exogenous amount of HNK-1 to the nerve injury site therefore may help stimulate neuronal survival and axonal regeneration for longer periods of time.

MSCs are an ideal candidate for cell-based delivery of HNK-1 for the PNS for several reasons: 1) they can be easily isolated and expanded *in vitro*, 2) they are potentially non-immunogenic when transplanted allogeneically, and 3) there are no ethical concerns associated with their use in a clinical setting (16). MSCs can act as vehicles to deliver growth factors to the nerve injury site and thereby enhance the regenerative capacity of the PNS. Gene therapy is an effective means to manipulate stem cells to deliver trophic molecules specific to a particular type of injury. In the literature, conduits including different cell types, including Schwann cells and stem cells have
proven to result in better regeneration with greater functional recovery across long peripheral nerve gaps than hollow conduits (40-45).

**Confirmation of Virus Production**

In this study, mesenchymal stem cells were infected with a lentiviral vector encoding for the secretion of HNK-1 peptide mimics, a candidate molecule for peripheral nerve regeneration. Results demonstrate the ability to successfully clone DNA fragments coding for three different HNK-1 peptide mimic sequences into the lentivirus vector and to synthesize high titer lentivirus (> 85-90%) as found using G418 selection (Figure 5.2B) (14); MSCs infected with 1 of 3 vectors carrying a sequence for an HNK-1 peptide mimic (either “F”, “K”, or “Q”) and the neomycin resistance gene did not die in the presence of G418, indicating that these MSCs were successfully infected.

**Motoneuron Viability and Outgrowth**

In addition, the bioactivity of the HNK-1 released from the MSCS was evaluated using a motor enriched spinal cord neuron population. Figure 5.3 shows results (both total neurite count and neurite outgrowth distribution) in which spinal cord neurons were seeded in Neurobasal media conditioned by infected MSCs (control MSCs, “F” MSCs, “K” MSCs, and “Q” MSCs). Motor enriched spinal cord neurons extend greater lengths when cultured in “conditioned” media (Figure 5.3B) as compared to neurons cultured in fresh media (Figure 5.3A). This is indicated by the peak shift to the right of the conditioned medias (blue, red, and green lines) as compared to the polyethylene and control substrates’ peaks (black lines). In addition, the viability of the neurons is not compromised by the “conditioned” media indicating its biocompatibility.
Newly developed methods of genetic engineering allow for the manipulation of cells in order to express and/or secrete neurotrophic factors to add in peripheral nerve regeneration treatments (13). The aim of the present study was to improve the effects of a NGC by incorporating MSCS that secrete HNK-1 peptide mimics within the lumen of the device, therefore increasing the neuron survival and outgrowth, supporting successful regeneration and functional recovery. Ideally, cells would be isolated, infected ex vivo, and introduced into the injury site. In this study, lentiviral vectors were designed coding for one of three HNK-1 peptide mimics referred to as “F”, “K”, and “Q”, each known to have beneficial effects on motoneurons. MSCs that secrete bioactive amounts of the peptide would be transplanted into the nerve injury site by way of the NGC. A bioreactor can be used to culture cells on the inner lumen walls prior to implantation between nerve stumps. Each vector was used to infect a population of MSCs. Following culture in lentivirus-containing media, supernatants collected from these MSCs was assessed for its ability to enhance motoneuron outgrowth, indicative of HNK-1 presence and bioactivity. This system could be implanted with NGCs to support cellular and tissue regeneration following nerve transection. Combining the physical support of the NGC with HNK-1 peptide mimic will enhance regeneration in nerve injury. In conclusion, the present study is the first report that HNK-1-secreting modified cells enhance neurite outgrowth in vitro, demonstrating the clinical potential of marrow stromal cell-based HNK-1 gene therapy for the treatment of peripheral nerve injuries.
Figure 5.2: Lentiviral infection of MSCs. (A) pLKO.1 neo construct map after cleavage with restriction enzymes AsiSI and MluI around the multiple cloning site. (B) Resistance to G418 is conferred by the neo gene present in the lentiviral vector used. MSC controls not infected with the vector die in the presence of G418 within 48 hours. MSCs infected with a vector (either “F”, “K”, or “Q”) incorporating a sequence for HNK-1 are able to survive in the presence of G418 for 48 hours.
Figure 5.3: Analysis of motor neuron enriched spinal cord population viability and outgrowth in vitro. (A) 20X image of rat spinal cord neurons seeded on 2D control coverslip coated with PLL and Laminin in neurobasal media. (B) 20X image of rat spinal cord neurons seeded on 2D control coverslip coated with PLL and Laminin in 50% neurobasal media, 50% conditioned media from peptide engineered MSCs. (C) Total live neurite cell count on coverslips treated with different medias (control neurobasal or conditioned medias). Values represent averages ± standard deviation. (D) Neurite outgrowth distribution on coverslips treated with different mediums (control neurobasal or conditioned medium) as determined by β-III-tubulin. All three engineered MSC’s conditioned media (blue, green, red lines) cause a shift to the right of the neurite outgrowth distribution as compared to the control conditions (black line) indicating greater neurite outgrowth.
Method 2: Release of HNK-1 from the Nerve Guidance Conduit

Introduction

NGCs are often used to successfully bridge together short nerve defects following peripheral nerve injury. However, when employed in critical size defects, conduits on their own are incapable of successfully achieving functional recovery. In order to increase the gap length that can be bridged by a nerve conduit, modifications may be made to improve upon the single lumen conduit design. To this end, in addition to providing structural support, nerve conduits can also function as a vehicle for localized delivery of neurotrophic factors (46-51). As the conduit material degrades in vivo breaking down into smaller fragments, the neurotrophic factor is released into the injury site. The ability to combine neurotrophic factor delivery with a conduit can provide additional biological support to promote and direct neurite outgrowth (48, 52). Some growth factors that have been used in peripheral nerve regeneration, including their delivery method, are illustrated in Table 5.1. Several other methods of delivery are mentioned including the use of an osmotic pump to deliver neurotrophic factors, or the use of a filler gel containing neurotrophic factors (as was evaluated in Chapter 4).
Table 5.1: Growth factors and their delivery methods commonly used in peripheral nerve regeneration with some references of work where they have been used. (43)

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Delivery Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF: nerve growth factor</td>
<td>Micro-injection ports</td>
<td>Kemp et al. (53)</td>
</tr>
<tr>
<td></td>
<td>Silk fibroin matrices</td>
<td>Uebersax et al. (54)</td>
</tr>
<tr>
<td>NT-3: neurotrophin 3</td>
<td>Collagen matrix</td>
<td>Midha et al. (52)</td>
</tr>
<tr>
<td>BDNF: brain-derived neurotrophic factor</td>
<td>Osmotic mini-pump</td>
<td>Boyd et al. (55)</td>
</tr>
<tr>
<td></td>
<td>Calcium alginate spheres</td>
<td>Vogelin et al. (56)</td>
</tr>
<tr>
<td>GDNF: glial-derived neurotrophic factor</td>
<td>EVA polymer tubes</td>
<td>Barras et al. (46)</td>
</tr>
<tr>
<td>FGF-1: fibroblast growth factor1</td>
<td>Collagen Matrix</td>
<td>Midha et al. (52)</td>
</tr>
<tr>
<td>FGF-2: fibroblast growth factor 2</td>
<td>Heparin/alginate gel</td>
<td>Ohta et al. (57)</td>
</tr>
<tr>
<td>GGF: glial growth factor</td>
<td>PHB conduits</td>
<td>Mohanna et al. (58)</td>
</tr>
<tr>
<td>PDGF: platelet-derived growth factor</td>
<td>Collagen tubules</td>
<td>Ho et al. (59)</td>
</tr>
<tr>
<td>CNTF: ciliary neurotrophic factor</td>
<td>Collagen tubules</td>
<td>Ho et al. (59)</td>
</tr>
<tr>
<td>VEGF: vascular endothelial growth factor</td>
<td>Silicone chambers</td>
<td>Hobson et al. (60)</td>
</tr>
<tr>
<td>IGF-I: insulin-like growth factor I</td>
<td>Osmotic pumps</td>
<td>Fansa et al. (61)</td>
</tr>
<tr>
<td>LIF: leukemia inhibitory factor</td>
<td>PHB conduits</td>
<td>McKay Hart et al. (62)</td>
</tr>
</tbody>
</table>

Although all of the methods used in Table 5.1 have proven success, we chose to evaluate delivery of HNK-1 directly from the polymeric NGC outer walls. As the polymer degrades in vivo, the mimic is slowly released into the inner lumen to the regenerating nerve. This method, as opposed to others shown in Table 5.1, allows for the NGC lumen to remain open for axonal growth which may result in faster reinnervation of the distal nerve end with better functional recovery. For example, the method used in Chapter 4, collagen grafted with HNK-1, physically fills the NGC lumen, occupying the space needed for regenerating axons (13).
In this study, non-porous tyrosine-derived polycarbonate (NP-TyrPC) conduits were fabricated with HNK-1 in their outer walls. Following fabrication, release studies were performed in order to determine the release profile of the HNK-1 over 14 days. In addition, the bioactivity of the released HNK-1 was evaluated using a motor enriched spinal cord population. Finally, these conduits were implanted into a critical size nerve gap in the mouse femoral nerve model and will be evaluated for their ability to promote peripheral nerve regeneration.

**Methods and Materials**

**Conduit Fabrication**

TyrPCs, synthesized utilizing previously published procedures (63) with the following chemical structure were used in this study: Poly((100-XX-YY)%DTE(desaminotyrosyl-tyrosine ethyl ester)-co-XX%DT(desaminotyrosyl tyrosine)-co-YY%PEG(polyethylene glycol)) carbonate). To simplify the naming of the TyrPC terpolymers the notation EXXY(1K) as described in Chapter 1: Tyrosine-derived polycarbonates will be used. For this study, the following polymers were used to fabricate conduits: E1001(1K), E0502(1K), and E0004(1K). HNK-1 peptide mimic (4.5 mg, amino acid sequence FLHTRLFV custom synthesized by Genscript, Piscataway, NJ) was first solubilized in 666 μL of dimethylformamide (DMF) due to its strong hydrophobic nature. Then, 900 mg of polymer were dissolved in 3.34 mL methylene chloride. To this, all 666 μL of the HNK-1/DMF solution was added. The final solution was parafilmed and vortexed over night before dip-coating.

The following day, a dip-coater (KSV dip-coater, KSV Instruments Inc., Helsinki, Finland) was used to fabricate conduits with an inner diameter of 580 μm. The conduits
were made by dipping Teflon coating mandrels at a constant rate into the polymer/HNK-1 solution. After dip-coating, the mandrels were dried in vacuum overnight to remove the remaining solvent. The conduits were removed from the mandrels and cut to a length of 5 mm length for \textit{in vitro} and \textit{in vivo} evaluation. Conduits were also fabricated without HNK-1. These conduits were prepared from solutions containing 900 mg of polymer solubilized in 4 mL methylene chloride only.

It is important to note that there are no toxicity concerns associated with the HNK-1 peptide mimic and, therefore, greater amounts than necessary for effectiveness can be used without concern.

\textit{Establishing Minimum Effective Dose}

The minimum effective dose of HNK-1 was established in order to be sure that the conduits would release enough HNK-1 to be effective \textit{in vivo}. From the literature, a vast range of concentrations have been used, depending upon the delivery technique used. This includes as 0.75 µg/mL and 100 µg/mL of the HNK-1 mimic on a spin-coated coverslip (9, 11), 120 µg/mL used \textit{in vivo} (collagen hydrogel grafted with HNK-1 in Chapter 4), and as much as 200 µg/mL used \textit{in vivo} in soluble form (11). \textit{In vitro} motoneuron survival and outgrowth assays (as previously described in the Materials and Methods section of Chapter 3: \textit{In vitro Assessment of Conduit Material with Spinal Cord Neurons}) were performed in order to evaluate the minimum amount of HNK-1 needed in soluble form to enhance neurite outgrowth and viability. Three concentrations of soluble HNK-1 were evaluated: 10 ng/mL (9.7 nM), 100 ng/mL (97 nM), and 1µg/mL (970 nM).
**HNK-1 Stability in Simulated Body Fluid**

Prior to release studies in simulated body fluid, the stability of the peptide mimic was evaluated. Briefly, 5 individual samples of 10 µg of fresh HNK-1 powder were incubated in separate vials of 1 mL of r-SBF (64) at 37 ℃ shaking at 80 RPM. At select time-points, days 0, 1, 3, 7, and 14, one sample was analysed via HPLC methods described below in order to quantify the amount of HNK-1 detected at that time.

**Release Studies of HNK-1 from Conduits**

_In vitro_ release studies will be performed in order to determine the release profile of the mimic over time for various polymer compositions before evaluation _in vivo_. A reversed phase HPLC method was developed in order to detect down to 1 µg/mL of the HNK-1 peptide mimic remaining in the polymer conduit. The HPLC system used consisted of a Waters Alliance 2695 Module, Waters 2487 Dual 1 Absorbance Ultraviolet (UV) Detector and Empower Pro Software (Waters Corporation, Milford, MA). The column was a Perkin-Elmer Pecosphere C18 (33 mm x4.6 mm; 3 µm particle size; Cat # 0258-1064, Waltham, MA). The established HPLC method utilized two buffers (Buffer A is 0.1 % TFA in deionized water and buffer B is 0.1 % TFA in ACN) in order to detect HNK-1 concentrations in conduits over time. Samples were dissolved in MeCl₂, ACN, and MeOH at a ratio of 1:3:1. The separation was monitored by UV at 214 nm. The column temperature was 25℃. A linear gradient method was used as follows: 0 min: 70 % A and 30 % B; 7 min: 60 % A and 40 % B; 8 min: 70 % and 30 % B. Standard solutions for the HPLC experiments were prepared from a 1 mg/mL stock solution of HNK-1 in 20 % acetic acid. Standard solutions were obtained by diluting this solution
with the blank buffer (MeCl₂, ACN, and MeOH at a ratio of 1:3:1) to give final concentrations of 1, 2.5, 5, 10, and 20 µg/mL for preparation of the standard curve.

Release studies were carried out in r-SBF (64) at 37 °C shaking at 80 RPM. On days 1, 3, 7, and 14 one conduit of each material was removed from solution, rinsed with PBS two times, and prepared for HPLC as described above in order to determine residual amounts of HNK-1. All analyses were done in triplicate.

**Bioactivity Assay of Released HNK-1**

Conduits (5 mm in length) fabricated with HNK-1 were placed into a scintillation vial and incubated in 2 mL neurobasal media at 37 °C shaking at 80 RPM for 7 days. At this time, the release media was collected from each vial and used to culture rat spinal cord neurons (as described in the Materials and Methods section of Chapter 3: *In vitro* Assessment of Conduit Material with Spinal Cord Neurons). Control experiments included neurons cultured in fresh neurobasal media, and neurons cultured in neurobasal media spiked with various concentrations of soluble HNK-1 (< 10 ng/mL), and release media collected from conduits not containing HNK-1. All experiments were performed in triplicate with replication.

**In Vivo Evaluation Using Mouse Femoral Nerve Model**

The conduits containing HNK-1 fabricated from E0004(1K) (which was previously evaluated *in vivo* in the mouse femoral nerve model in Chapter 3) were selected for *in vivo* evaluation in this study. Three animal groups (5 animals each) were compared over a 15 week time period including: NP-TyrPC conduits filled with saline, NP-TyrPC conduits filled with collagen grafted with HNK-1 (as evaluated in Chapter 4), and NP-TyrPC fabricated with HNK-1 mimic in the outer walls.
Surgical Methods

Surgical methods were performed as described in Chapter 3: Materials and Methods.

Motor Function Recovery

Functional recovery was based on both the foot base angle (FBA) and the protraction limb ratio (PLR) before surgery and weekly after surgery until the endpoint of the study at week 15. Methods for these measurements were performed as described in Chapter 3: Motor Function Recovery. The functional recovery is currently ongoing and will be complete during the week of August 1, 2011.

Histomorphometric Analysis of Explanted Nerve

From the mid-section of the explanted nerve samples, the number of myelinated axons, raw tissue area, cross-sectional area of the regenerating cable, percent nerve regeneration, fiber diameter, and g-ratio were measured. Methods for these measurements were performed as described in Chapter 3: Histomorphometric Analysis of Explanted Nerve. The study is currently ongoing and therefore the histomorphometric analysis has not been performed yet.

Electrophysiology Assessment

Electromyography (EMG) is a technique used in order to evaluate and record the electrical activity produced by muscles. At week 15, when the animals are anesthetized for retrograde labelling (described below) motor reinnervation will be assessed by means of nerve conduction tests. For this assessment, the left femoral nerve is stimulated with EMG electrodes to measure nerve conduction velocities and muscle action potentials. Transcutaneous or intramuscular recordings can be made of latency to the muscle action
potential and amplitude of the signal in the quadriceps muscle since this is the muscle the femoral nerve innervates. After the EMG measurements are taken, retrograde labelling will be performed (described below). (65)

**Retrograde Labelling**

A primary hypothesis is that the peptide mimic of HNK-1 will spur targeted regeneration of motor axons to the appropriate nerve branch. In order to evaluate whether more motor neurons are entering the motor or sensory branch, retrograde labeling of the nerve will be performed. The animals will be perfusion fixed and the nerves and spinal cords harvested for immunohistochemistry and analysis.

After functional assessment at 15 weeks, neurons will be retrogradely labelled and spinal cords will be removed for analyses. The animals are anaesthetized by intraperitoneal injections with a combination of 80 mg ketamine:12 mg xylazine with a dosage of 0.1 mL/10 g body weight and the left femoral nerve is exposed. A piece of Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) is placed under each nerve trunk and the two nerve branches are transected ~5 mm distal to the bifurcation and powdered fluorescence retrograde tracers are applied to the motor branch (Alexa Fluor 546, Dextran, Invitrogen, Carlsbad, California) and sensory branch (Alexa Fluor 488 Dextran, Invitrogen, Carlsbad, California) (Figure 5.4A) of the cut nerve for 30 minutes after which the wound is clipped shut. Seven days later, after which the tracers are able to transport back to the spinal cord, the mice are transcardially perfused and fixed with 4% formaldehyde. The lumbar spinal cord is removed, post-fixed overnight and cryoprotected in 20% sucrose solution in phosphate buffered solution. The cord is then transversely cut (serial section of 50 µm thickness) on a cryostat (Leica CM 1850, Leica
Microsys
ts Inc., Bannockburn, IL USA). Ten serial sets are collected from each
spinal cord. From this set, two slides that are evenly spaced apart (i.e. serial slides 1 and
6) are analysed under a 40X objective for fluorescently labelled cell profiles (Figures
5.4B and 5.4C). Therefore, each measured section is spaced 250µm apart, eliminating
the worry of double counting. Evaluation is performed by focusing through the entire
depth of each section starting from the top. The cell profiles labelled with the two tracers
are all located within the lumbar part of the spinal cord, and occupy between 35 and 45
cross-sections. Each section usually contains 2-5 labelled cell profiles. The number of
correctly and incorrectly projecting neurons will be measured.

Figure 5.4: Retrograde labelling technique (A) Retrograde labelling of the sensory
branch (red) and motor branch (yellow) is used to follow projection of neurons. A
different color tracer is applied to each branch of the femoral nerve and is allowed
to transport back to the spinal cord. (B) Section of a spinal cord marked with
retrograde tracers in the ventral root, indicative of motoneurons correctly
projecting through the nerve conduit to the motor branch of the femoral nerve. (C)
3 cell bodies are seen in the magnified image.
**Wet Muscle Mass Ratios**

At the time of sacrifice (16 week end-point) the quadriceps muscles will be isolated, removed, and then weighed on an analytical balance. The wet muscle mass ratio between the experimental (left) and control (right) quadriceps will be measured. The ratio is then used as an indicator of muscle mass recovery.

**Results and Discussion**

**Motoneuron Viability and Outgrowth**

The HNK-1 mimic has been used previously in *in vitro* experiments coated onto substrates and was shown to enhance neurite outgrowth from motoneurons (39) and to maintain motoneuron survival as compared to control substrates without the mimic. In this study, we aimed to determine how effective the HNK-1 mimic was by evaluating the effects of varying amounts of soluble HNK-1 on motoneuron viability and outgrowth. Figure 5.5 shows results (both total neurite count and neurite outgrowth distribution) of neurons cultured in media containing different amounts of soluble HNK-1. All three evaluated concentrations had similar effects on the outgrowth and viability of spinal cord neurons indicating that as little as 9.7 nM (10 ng/mL) HNK-1 solution is effective at enhancing outgrowth. (Data for other controls confirm these results but is not shown.)
Figure 5.5: HNK-1 effect on E15 rat spinal cord neurons cultured for 48 hours (A) Total live neurite cell count in neurobasal media spiked with soluble HNK-1 at 3 concentrations, 0nM (Control), 9.7 nM (10 ng/mL), 97 nM (100 ng/mL), and 970 nM (1 µg/mL). Values represent averages ± standard deviation. (B) Neurite distribution in media spiked with soluble HNK-1 at 3 concentrations, 0 nM (Control), 9.7 nM (10 ng/mL), 97 nM (100 ng/mL), and 970 nM (1 µg/mL). All three concentrations of HNK-1 cause a shift in the neurite distribution to the right of the control indicating greater neurite outgrowth.

**HNK-1 Stability in Simulated Body Fluid**

In an attempt to determine the release kinetics of the HNK-1 from the different polymer compositions fabricated into nerve conduits, an HPLC method was established. However, prior to performing these release studies, the stability of HNK-1 in r-SBF had to be confirmed. Results from the stability study are shown in Figure 5.6. Results show that the peptide mimic remains stable at 10 µg/mL in r-SBF for at least 14 days and is detectable by the HPLC method developed.
Figure 5.6: HNK-1 stability in simulated body fluid. Samples were evaluated via HPLC at days 0, 1, 3, 7, and 14 in order to determine if the peptide mimic is stable in this solution over 2 weeks.

**HNK-1 Release Studies**

We hypothesize that a sustained release of the HNK-1 peptide mimic over the first 2 weeks of regeneration will have an effect on the overall functional recovery of a critical size defect in the mouse femoral nerve model. To test this hypothesis, release studies were performed with conduits fabricated from various polymer compositions including E0004(1K), E0502(1K), and E1001(1K). Figure 5.7 shows the residual amount of HNK-1/mg of polymer over a 2 week period as measured in r-SBF via HPLC methods described above. Table 5.2 reports the amount of HNK-1 released over the 14 day period calculated from the HPLC data in Figure 5.7. The Table shows values calculated from Days 0-1, 1-3, 3-7, and 7-14. In addition, cumulative amount released over the total 14 days (Day 0-14) is shown.
Figure 5.7: Residual amounts of HNK-1 in conduits as detected by HPLC. Three polymer compositions were evaluated: E0004(1K) (red square), E0502(1K) (green triangle), and E1001(1K) (purple star). Conduits were fabricated with an initial loading of 4.4 µg HNK/mg of polymer. Values represent averages ± standard deviation. Results show similar release kinetics for all three types of conduits fabricated.
Table 5.2: HNK-1 Release from polymer conduits over a 14 day incubation period in r-SBF. Values represent an average amount of HNK-1 (µg/mg of polymer). Values are calculated from HPLC data quantifying the residual amount of HNK-1 remaining in conduits at each time-point. Values shown are calculated from Days 0-1, 1-3, 3-7, and 7-14. In addition, cumulative amount released over the total of 14 days (Day 0-14) is shown.

<table>
<thead>
<tr>
<th>HNK-1 Released (µg/mg polymer)</th>
<th>Day 0-1</th>
<th>Day 1-3</th>
<th>Day 3-7</th>
<th>Day 7-14</th>
<th>Day 0 – 14 Total Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>E0004(1K)</td>
<td>0.56</td>
<td>0.33</td>
<td>0</td>
<td>2.04</td>
<td>2.85</td>
</tr>
<tr>
<td>E0502(1K)</td>
<td>0.90</td>
<td>0.53</td>
<td>0</td>
<td>2.04</td>
<td>3.00</td>
</tr>
<tr>
<td>E1001(1K)</td>
<td>0.62</td>
<td>0</td>
<td>0.9</td>
<td>1.18</td>
<td>1.80</td>
</tr>
</tbody>
</table>

*Bioactivity of Released HNK-1*

The bioactivity of the HNK-1 released by each type of conduit was assessed by evaluating its effects on motoneuron outgrowth *in vitro*. HNK-1 released from the conduits over a 7 day period was found to be bioactive and stimulated neurite outgrowth (Figure 5.8) as demonstrated by the peak shift in % incidence to the right of all three conduits fabricated with HNK-1 (blue, green, and red lines) as compared to the control value (black line). Furthermore, there was no statistical difference between polymer compositions used: each conduit released equal amounts of bioactive HNK-1 over this time period.
Figure 5.8: Neurite outgrowth distribution of motor enriched spinal cord neurons cultured in media conditioned by TyrPC conduits containing HNK-1 for 7 days. All three release mediums are effective at enhancing outgrowth as indicated by the shift of the all three experimental peaks (E0004, E0502, E1001) to the right of the control peak.

Conduit Structure

TyrPCs are non-cytotoxic degradable polymers that can be fabricated into conduits for bridging critical size defects in the mouse femoral nerve model. In order to enhance the potential of this conduit further, HNK-1 peptide mimic was incorporated into the fabrication technique in order to enhance motoneuron survival, neurite outgrowth, and overall functional recovery of the nerve. A dip-coating technique was used to produce conduits with an inner lumen of 580 µm and a non-porous outer wall thickness of approximately 100 µm (Figure 5.9). Control conduits were fabricated from the same polymer, however, HNK-1 was not incorporated into the solution used for dip-coating. These conduits were compared against one another by scanning electron microscopy.
(SEM) in order to ensure that the physical structure was not compromised once HNK-1 was included. SEM images of the conduits showed a uniform microstructure could be obtained from the dip-coating technique and dimensions were comparable whether or not HNK-1 was included.

Figure 5.9 shows the example structure of conduits fabricated from E0004(1K) with (C and D) and without (A and B) HNK-1 in the outer conduit walls. From these SEM images, we can see that the conduits have smooth outer walls and cross sections of approximately 580 µm. The outer wall thicknesses for both samples are approximately 100 µm.

Figure 5.9: Scanning electron microscope (SEM) images of conduits fabricated from TyrPC. (A and B) Outer wall and cross section of a conduit fabricated from E0004(1K), respectively. (C and D) Outer wall and cross section of a conduit fabricated from E0004(1K) with HNK-1 peptide mimic, respectively. Scale bar: 100 µm.
**Motor Function Recovery**

In order to evaluate the ability of the HNK-1 releasing TyrPC conduits to enhance nerve regeneration *in vivo*, conduits were compared against control conduits (without HNK-1) and conduits prefilled with collagen grafted with HNK-1 in a 5 mm critical size gap of the mouse femoral nerve. A single frame motion analysis approach was used to determine functional recovery of the mice over a 15 week time period. In particular, the FBA and PLR were measured as mice performed a beam walking experiment and a pencil grip test, respectively (Figure 5.10).

A complete transection of the femoral nerve compromises the motor branch of the nerve innervating the quadriceps muscle. Therefore, the FBA is measured to indicate the trend of recovery of the ability of the quadriceps muscle to extend the knee and support body weight during the contralateral swing phases of the gait cycle (66). An FBA decreasing towards its initial starting value is indicative of healing whereas an FBA increasing over time indicates worsening of the nerve function. From the functional data collected so far, conduits releasing HNK-1 (blue line, Figure 5.10A) appear to enhance recover more than conduits prefilled with saline (red) or collagen grafted with HNK-1 (green).

In order to evaluate the ability of the mouse to perform voluntary movements without any body weight support following femoral nerve transection and conduit implantation, the pencil grip test is performed. This enables one to measure how far the injured limb can extend as compared to the non-injured limb. Results at 8 weeks demonstrated by the protraction limb ratio (PLR) confirm the improvement of motor function when a nerve injury is treated with a HNK-1 releasing conduit (blue line, Figure
5.10C) as compared to a conduit filled with saline (red line) or collagen grafted with HNK-1 (green line).

Given the description of the parameters described above, it becomes apparent that the absolute values of the FBA and PLR may vary between mice, as each mouse behaves differently at week 0 and recovers from the initial surgery at a different rate. Therefore, in order to make the results comparable to one another, we calculate a recovery index in which animals are normalized on an individual basis to their own FBA or PLR values at week 0 and at week 1. A recovery index of 100 % indicates that the mouse has regained motor function completely whereas a recovery index below 40 % indicates no functional recovery. At week 8 the RI value for the FBA parameter appears to be higher on average for the animals treated with HNK-1 releasing conduits as compared to the other conditions. The animals treated with collagen-HNK-1 filled conduits show a higher average RI value than animals treated with saline-filled conduits. For the RI value of the PLR parameter we observe that the animals treated with collagen-HNK-1 filled conduits had the highest average, although this value is not much higher than the average RI for animals treated with HNK-1 releasing conduits. Animals treated with conduits filled with saline have the lowest average RI value. Results at 15 weeks will be more accurate in determining which condition was best at achieving functional recovery.
Figure 5.10: Metrics of functional recovery promoted by HNK-1 releasing conduits (blue), conduits filled with phosphate buffered saline (red), and conduits filled with a collagen hydrogel grafted with HNK-1 peptide mimic (green). (A) FBA for all conditions. Values represent averages ± standard error. A decrease in the FBA over time indicates functional recovery. (B) Recovery Index (RI) for FBA at week 8. Each dot represents one animal in the group. Line indicates average RI value for each condition. (C) PLR for all conditions. Values represent averages ± standard error. (D) Recovery Index for FBA at week 8. Each dot represents one animal in the group. Line indicates average RI value for each condition.
Conclusions

Functional recovery following unassisted peripheral nerve injury is limited despite the ability for peripheral nerves to regenerate. Several causes of this poor recovery are the slow regeneration speed of axons, large nerve gaps, and chronic denervation of the distal nerve. Because experimental work on nerve regeneration has revealed functional improvement with the application of an HNK-1 peptide mimic in soluble form over a 2 mm gap in vivo (11) we aimed for an alternative approach to present the mimic in a localized and sustained manner by releasing it from a nerve guidance conduit over time. In this study, biodegradable TyrPC conduits were fabricated with HNK-1 incorporated into their outer walls. A sustained release of the peptide mimic was observed for at least 14 days. Released HNK-1 retained its bioactivity, as demonstrated by its ability to stimulate neurite outgrowth from motor enriched spinal cord neurons. Conduits were then implanted in vivo for a 15 week study bridging a critical size defect in the mouse femoral nerve. Functional and histomorphometric results are currently being evaluated through the end point of the study. Results thus far show that animals treated with conduits releasing HNK-1 hold promise in terms of promoting functional recovery. Electrophysiology assessment, retrograde labeling, and wet muscle mass ratios at the end of the study will confirm our findings further. We hypothesize that conduits fabricated with HNK-1 and with controllable peptide release may enhance nerve regeneration by guiding and stimulating neurite outgrowth. (46, 48, 50)
References

CHAPTER 6

CONCLUSIONS

Summary

The goal of this doctoral research was to develop a biodegradable, tyrosine-derived polycarbonate (TyrPC) nerve guidance conduit to use in the repair of critical size defects in the mouse femoral nerve model. We aimed to achieve greater functional recovery and quality of regeneration by using a conduit made from this material. This dissertation has described the methods and findings for the fabrication, characterization, and evaluation of the TyrPC conduit. In addition, new methods for sustained delivery of a biologic enhancer, HNK-1, were discussed as well as some in vivo results evaluating HNK-1 grafted to a collagen hydrogel filler.

In Chapter 2, materials were evaluated for use as nerve guidance conduits for treatment of peripheral nerve injuries. An in vitro assessment of the ability for various TyrPCs to enhance neuron viability and outgrowth was performed. In addition, conduits were fabricated from the TyrPCs, with either a porous or non-porous structure, and were analyzed via scanning electron microscopy. The degradation rate and mechanical properties of the conduits were also determined prior to in vivo analysis. Results show that by using TyrPC, we were able to fabricate porous and non-porous conduits of proper dimensions for in vivo implantation. Additionally, TyrPC was found to enhance neuron viability and outgrowth in vitro and demonstrated ideal degradation and mechanical properties for in vivo application.
Chapter 3 presents data from \textit{in vitro} and \textit{in vivo} studies directly comparing TyrPC to PE, either with 2D substrates or conduits fabricated from each material. The introduction of pores in the outer conduit walls (producing permeable conduits) was explored in order to determine whether or not they had an effect on nerve regeneration in critical size gaps. The \textit{in vivo} results indicate that the femoral nerve model is able to clearly distinguish between two different materials and that the TyrPC supports nerve regeneration over conduits fabricated from PE. Functional and histomorphometric analysis both confirmed these findings. Moreover, the addition of pores to the outer conduit wall did not appear to affect the ability of TyrPC to enhance nerve regeneration.

The focus of Chapter 4 was to enhance the regenerative potential of the TyrPC conduits further by introducing a three-dimensional filled matrix as well as a biological enhancer, the peptide mimic HNK-1. Porous and non-porous conduits were implanted into the femoral nerve model with either a collagen only filler or a collagen filler grafted with the HNK-1 peptide mimic. Functional results demonstrated that animals treated with NP-TyrPC filled with collagen grafted with HNK-1 outperformed animals treated with all other conduits. \textit{In vivo} histomorphometric results showed animals treated with conduits filled with collagen only regenerated nerves with large acellular regions and regions of dense fibrotic tissue. Little to no axons were present within these conduits. Animals treated with porous conduits with collagen grafted with HNK-1 also showed nerve sections with little axons and dense fibrotic tissue. Conversely, animals treated with non-porous conduits filled with collagen grafted with HNK-1 showed little to no dense fibrotic tissue and the greatest axonal growth with organized fascicles. We hypothesized that there was a competition between the infiltration of fibrous tissue and axons under
these conditions. A three-dimensional collagen gel filler matrix has different effects depending upon the conduit structure. The drawbacks of using a collagen hydrogel within TyrPC conduits may be compensated for by the action of HNK-1; when collagen grafted with HNK-1 is used, we see a significant enhancement in degree of functional recovery and the quality of axonal regeneration in NP-TyrPC conduits.

Chapter 5 describes alternative methods to presenting the HNK-1 peptide mimic in a sustained and localized manner in conjunction with a TyrPC conduit. The two methods described include 1) cellular delivery of HNK-1 by genetically engineered mesenchymal stem cells and 2) release of HNK-1 from the polymeric conduit outer walls. *In vitro* results from the first method show that cell culture media containing MSCs infected with lentivirus encoding for 3 different HNK-1 peptide mimics effectively enhances neuron viability and outgrowth as compared to neurons cultured in fresh MSC media. *In vitro* results from method 2 show the ability to fabricate TyrPC conduits containing HNK-1 that release bioactive amounts of HNK-1 over a 2 week period. Preliminary results from the *in vivo* evaluation show promise that HNK-1 releasing conduits improve functional recovery by 8 weeks as compared to saline-filled conduits. Results at 15 weeks will provide more meaningful findings. In conclusion, nerve guidance conduits fabricated from TyrPC offer the potential for improved functional recovery and quality of nerve regeneration in critical size defects.
**Recommendations and Future Work**

1) TyrPC conduits filled with saline alone appear to support nerve regeneration over a critical size gap in the femoral nerve model (Chapter 3). The material itself plays a significant role in the process of regeneration. For larger nerve gaps, the dip-coating technique may not provide enough mechanical strength to support regeneration completely; the conduit walls may kink or collapse, physically occluding the inner lumen. Future work includes investigating alternative fabrication techniques (including extrusion, injection molding, and braiding) offering better mechanical properties and flexibility that would allow for implantation into larger animal models (i.e. rat sciatic nerve). These experiments are currently in progress and are necessary in order to confirm the *in vivo* potential of TyrPC in critical size defects following peripheral nerve injury.

2) As collagen grafted with HNK-1 did not support nerve regeneration in porous TyrPC conduits (Chapter 4), it is recommended to investigate alternative filler materials including three-dimensional hydrogel matrices such as laminin or aligned-collagen hydrogels which have shown promise in regeneration (1, 2). Also, it is recommended to investigate collagen hydrogels with lower concentrations in order to find an optimized level of gel porosity and density to support regeneration as concentration has been found to play a crucial role in regenerative potential of filler materials (3-5).

3) The neuroregeneration process follows a predictable and reproducible series of events following peripheral nerve injury (6) as shown in Chapter 1. These processes include a cooperative effort from non-neuronal cells, extracellular matrix proteins, and axons as
well. In order to fully understand the effects TyrPC have on nerve regeneration, it is highly recommended to investigate early in vivo time-points further to follow the progression of regeneration. Weekly time-points used for both immunohistochemistry and histomorphometric analysis through week 6 following implantation may provide further insight into how TyrPC specifically affects cell infiltration, fibrin matrix formation, protein deposition, axonal outgrowth, etc. as compared to PE. (6-9)

4) Although data is not shown, a retrograde labelling technique was attempted in this work in order to track the fate of individual axons following nerve regeneration. Unfortunately, the technique that successfully works in a 2 mm gap (10) (crystal application) did not prove successful in our 5 mm femoral nerve gap. Therefore, it is highly recommended to investigate alternative methods for performing retrograde labelling including using a conduit reservoir, intramuscular injection, and pressure injection (11). Results from retrograde labelling can vary in staining intensity, neuron size, and background staining. Therefore, a new technique to count the stained neurons should be investigated. Various tracers area available for the retrograde labelling technique including the following: fast blue, fluoro-gold, fluoro-ruby (dextran conjugate), and diamino-yellow. It is recommended to investigate the use of various tracers in order to optimize the outcome of the retrograde labelling technique. (11)

5) Future work includes continuing the use of genetically engineered MSCs to express and/or secrete HNK-1 peptide mimics for a prolonged amount of time (Chapter 5). This work still needs to be performed in order to quantify the secretion of the HNK-1 mimics
– either by Western blot analysis or enzyme linked immunoassay – and to determine the specificity of the secreted peptide to enhance nerve regeneration using an antibody to bind and sequester HNK-1 peptides in solution (either Zn-12 antibody (mouse anti-Zn12, Developmental Studies Hybridoma Bank, University of Iowa), L2-412 antibody, or HNK-1 antibody). Future work also includes determining whether or not infection of the MSCs with lentivirus alters the morphology, proliferation, or viability of the cells. Ultimately, if found to secrete effective levels of HNK-1 mimics, these cells will be coated within nerve guidance conduits and evaluated in vivo to bridge a critical size defect. (12, 13)

6) HNK-1 releasing conduits are currently being evaluated in vivo in the femoral nerve model (Chapter 5). Future work includes collecting functional data through the end point of the study (15 weeks) and performing histomorphometric analysis at that time. In addition, future work aims to identify if the HNK-1 peptide mimic influenced preferential motor reinnervation. For this, retrograde labelling will be performed at week 15 to track the fate of individual axons regenerating through the conduit. Electrophysiological analysis (bare-nerve stimulation) will be performed as well as changes in quadriceps muscle mass at the final time-point in order to further corroborate functional and histomorphometric results.
References

Curriculum Vitae

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